

Neurofilaments: neurobiological foundations for biomarker applications

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Abstract word count:

Main text word count: 9,592

Summary box word count: 188

Number of figures: 6

Number of tables: 2

Number of boxes: 1

Keywords

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.

IN CONFIDENCE. PRE-PUBLICATION

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 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 saccin, 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 saccin (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 Charveloix-

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*-acetylglucosamine)(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-M^{S→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 $\mu\text{m/s}$ 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 aetiologically distinct sub-type 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 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 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 claspings, 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 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^{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(Beitner-Johnson *et al.*, 1992; Berhow *et al.*, 1996), not unlike the effects of chronic exposure to drugs of abuse in humans(Beitner-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 saccin (*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).

Saccin 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 saccin in regulating mitochondrial dynamics; loss of saccin 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 sacsine 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 sacsine 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 IF. 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(Furukawa *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*

al., 2008; Ganay *et al.*, 2011). 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 (Scrivero *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 $[Ca^{2+}]$ (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 (A β) 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 A β from the ageing brain in the genesis of Alzheimer's disease (e.g., reflected by the accumulation of A β 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 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 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 (A β), 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. These are then 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 A β (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(Malmstrom *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(Theelin *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 NfL 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

PB's laboratory was funded for NF biology by INSERM (ATIP-Avenir program) and the Association Française contre les Myopathies (AFM). RW and RC's laboratory was funded by Alzheimer's Research UK (ARUK) and Biogen. The RAN laboratory has been supported by National Institute on Aging (R01AG0560; P01AG017617). HZ is a Wallenberg Academy Fellow supported by grants from the Swedish Research Council (#2018-02532), the European Research Council (#681712), Swedish State Support for Clinical Research (#ALFGBG-720931), the Swedish Brain Foundation (FO2019-0228) and the UK Dementia Research Institute at UCL. PMM has been in receipt of generous personal and research support from the Edmond J Safra Foundation and Lily Safra, the Imperial College Healthcare Trust – National Institute for Health Research (NIHR) Biomedical Research Centre, an NIHR Senior Investigator's Award, the Medical Research Council and the UK Dementia Research Institute, which is supported by the Medical Research Council, The Alzheimer's Society and Alzheimer's Research UK.

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

Neurofilament pathology	Proteins affected	Associated Diseases
Primary neurofilament gene mutations	<ul style="list-style-type: none"> • NfH, Peripherin • NfL, NfH 	<ul style="list-style-type: none"> • Amyotrophic Lateral Sclerosis • Charcot-Marie Tooth Disease
Mutations in genes involved in NF assembly, turnover and degradation	<ul style="list-style-type: none"> • Sacsin • TRIM2 • Gigaxonin 	<ul style="list-style-type: none"> • Autosomal Recessive Spastic Ataxia of Charveloix • Charcot-Marie Tooth Disease Type 2R • Giant Axonal Neuropathy

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

IN CONFIDENCE. PRE-PUBLICATION

Clinical condition	Key references
<ul style="list-style-type: none"> ● Peripheral neuropathies <ul style="list-style-type: none"> ○ Charcot-Marie-Tooth Disease ○ Guillain-Barré syndrome ○ Chronic Inflammatory Demyelinating Polyneuropathy ● Ageing ● Multiple Sclerosis ● Amyotrophic lateral sclerosis ● Dementia <ul style="list-style-type: none"> ○ Pre-clinical Alzheimer's Disease ○ Alzheimer's Disease ○ Frontotemporal dementia ○ Atypical Parkinsonian disorders (e.g., progressive supranuclear palsy) ● Stroke <ul style="list-style-type: none"> ○ Subarachnoid haemorrhage ○ Ischaemic stroke ● Traumatic brain injury ● Huntingdon's Disease ● Neuropsychiatric conditions <ul style="list-style-type: none"> ○ Bipolar disorder ● Spinal muscular Atrophy 	<p>(Sandelius <i>et al.</i>, 2018)</p> <p>(Petzold <i>et al.</i>, 2006)</p> <p>(van Lieverloo <i>et al.</i>, 2019)</p> <p>(Disanto <i>et al.</i>, 2017; Mattsson <i>et al.</i>, 2017; Barro <i>et al.</i>, 2018)</p> <p>(Lycke <i>et al.</i>, 1998; Malmstrom <i>et al.</i>, 2003; Norgren <i>et al.</i>, 2004; Teunissen <i>et al.</i>, 2009; Gunnarsson <i>et al.</i>, 2011; Kuhle <i>et al.</i>, 2016b; Kuhle <i>et al.</i>, 2017)</p> <p>(Lu <i>et al.</i>, 2015; Steinacker <i>et al.</i>, 2016)</p> <p>(Weston <i>et al.</i>, 2017; Preische <i>et al.</i>, 2019; Weston <i>et al.</i>, 2019)</p> <p>(Mattsson <i>et al.</i>, 2017)</p> <p>(Meeter <i>et al.</i>, 2016)</p> <p>(Rojas <i>et al.</i>, 2016; Donker Kaat <i>et al.</i>, 2018)</p> <p>(Nylen <i>et al.</i>, 2006; Zanier <i>et al.</i>, 2011)</p> <p>(Gattringer <i>et al.</i>, 2017)</p> <p>(Shahim <i>et al.</i>, 2016; Shahim <i>et al.</i>, 2017)</p> <p>(Byrne <i>et al.</i>, 2017; Johnson <i>et al.</i>, 2018)</p> <p>(Jakobsson <i>et al.</i>, 2014)</p> <p>(Darras <i>et al.</i>, 2019)</p>

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 o-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 ($A\beta$) 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 $A\beta$ is draining out of the brain. The IPAD pathway for $A\beta$ 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-Glial 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.

References

- Ocrelizumab for treating primary progressive multiple sclerosis.
- Abe A, Numakura C, Saito K, Koide H, Oka N, Honma A, *et al.* Neurofilament light chain polypeptide gene mutations in Charcot-Marie-Tooth disease: nonsense mutation probably causes a recessive phenotype. *J Hum Genet* 2009; 54(2): 94-7.
- Ackerley S, Grierson AJ, Brownlees J, Thornhill P, Anderton BH, Leigh PN, *et al.* Glutamate slows axonal transport of neurofilaments in transfected neurons. *J Cell Biol* 2000; 150(1): 165-76.
- Adebola AA, Di Castri T, He CZ, Salvatierra LA, Zhao J, Brown K, *et al.* Neurofilament light polypeptide gene N98S mutation in mice leads to neurofilament network abnormalities and a Charcot-Marie-Tooth Type 2E phenotype. *Human molecular genetics* 2015; 24(8): 2163-74.
- Albargothy NJ, Johnston DA, MacGregor-Sharp M, Weller RO, Verma A, Hawkes CA, *et al.* Convective influx/glymphatic system: tracers injected into the CSF enter and leave the brain along separate periarterial basement membrane pathways. *Acta neuropathologica* 2018; 136(1): 139-52.
- Aldea R, Weller RO, Wilcock DM, Carare RO, Richardson G. Cerebrovascular Smooth Muscle Cells as the Drivers of Intramural Periarterial Drainage of the Brain. *Frontiers in aging neuroscience* 2019; 11: 1.
- Amor S, van der Star BJ, Bosca I, Raffel J, Gnanapavan S, Watchorn J, *et al.* Neurofilament light antibodies in serum reflect response to natalizumab treatment in multiple sclerosis. *Multiple sclerosis* 2014; 20(10): 1355-62.
- Asbury AK, Gale MK, Cox SC, Baringer JR, Berg BO. Giant axonal neuropathy--a unique case with segmental neurofilamentous masses. *Acta neuropathologica* 1972; 20(3): 237-47.
- Aspelund A, Antila S, Proulx ST, Karlsen TV, Karaman S, Detmar M, *et al.* A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *The Journal of experimental medicine* 2015; 212(7): 991-9.
- Badner JA, Gershon ES. Meta-analysis of whole-genome linkage scans of bipolar disorder and schizophrenia. *Mol Psychiatry* 2002; 7(4): 405-11.
- Balastik M, Ferraguti E, Pires-da Silva A, Lee TH, Alvarez-Bolado G, Lu KP, *et al.* Deficiency in ubiquitin ligase TRIM2 causes accumulation of neurofilament light chain and neurodegeneration. *Proceedings of the National Academy of Sciences of the United States of America* 2008; 105(33): 12016-21.
- Barro C, Benkert P, Disanto G, Tsagkas C, Amann M, Naegelin Y, *et al.* Serum neurofilament as a predictor of disease worsening and brain and spinal cord atrophy in multiple sclerosis. *Brain : a journal of neurology* 2018.
- Basak JM, Kim J, Pyatkovskyy Y, Wildsmith KR, Jiang H, Parsadanian M, *et al.* Measurement of apolipoprotein E and amyloid beta clearance rates in the mouse brain using bolus stable isotope labeling. *Mol Neurodegener* 2012; 7: 14.
- Bateman RJ, Munsell LY, Morris JC, Swarm R, Yarasheski KE, Holtzman DM. Human amyloid-beta synthesis and clearance rates as measured in cerebrospinal fluid in vivo. *Nature medicine* 2006; 12(7): 856-61.
- Beaulieu JM, Julien JP. Peripherin-mediated death of motor neurons rescued by overexpression of neurofilament NF-H proteins. *Journal of neurochemistry* 2003; 85(1): 248-56.
- Beaulieu JM, Nguyen MD, Julien JP. Late onset of motor neurons in mice overexpressing wild-type peripherin. *J Cell Biol* 1999; 147(3): 531-44.

Beck R, Deek J, Safinya CR. Structures and interactions in 'bottlebrush' neurofilaments: the role of charged disordered proteins in forming hydrogel networks. *Biochem Soc Trans* 2012; 40(5): 1027-31.

Beirowski B, Nogradi A, Babetto E, Garcia-Alias G, Coleman MP. Mechanisms of axonal spheroid formation in central nervous system Wallerian degeneration. *Journal of neuropathology and experimental neurology* 2010; 69(5): 455-72.

Beitner-Johnson D, Guitart X, Nestler EJ. Neurofilament proteins and the mesolimbic dopamine system: common regulation by chronic morphine and chronic cocaine in the rat ventral tegmental area. *J Neurosci* 1992; 12(6): 2165-76.

Berger T, Stuve O. Neurofilament light chain: An important step toward a disease biomarker in multiple sclerosis. *Neurology* 2019; 92(10): 451-2.

Bergman J, Dring A, Zetterberg H, Blennow K, Norgren N, Gilthorpe J, *et al.* Neurofilament light in CSF and serum is a sensitive marker for axonal white matter injury in MS. *Neurol Neuroimmunol Neuroinflamm* 2016; 3(5): e271.

Berhow MT, Hiroi N, Nestler EJ. Regulation of ERK (extracellular signal regulated kinase), part of the neurotrophin signal transduction cascade, in the rat mesolimbic dopamine system by chronic exposure to morphine or cocaine. *J Neurosci* 1996; 16(15): 4707-15.

Bocquet A, Berges R, Frank R, Robert P, Peterson AC, Eyer J. Neurofilaments bind tubulin and modulate its polymerization. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2009; 29(35): 11043-54.

Boizot A, Talmat-Amar Y, Morrogh D, Kuntz NL, Halbert C, Chabrol B, *et al.* The instability of the BTB-KELCH protein Gigaxonin causes Giant Axonal Neuropathy and constitutes a new penetrant and specific diagnostic test. *Acta Neuropathol Commun* 2014; 2: 47.

Bomont P. Degradation of the Intermediate Filament Family by Gigaxonin. *Methods in enzymology* 2016; 569: 215-31.

Bomont P, Cavalier L, Blondeau F, Ben Hamida C, Belal S, Tazir M, *et al.* The gene encoding gigaxonin, a new member of the cytoskeletal BTB/kelch repeat family, is mutated in giant axonal neuropathy. *Nat Genet* 2000; 26(3): 370-4.

Bomont P, Koenig M. Intermediate filament aggregation in fibroblasts of giant axonal neuropathy patients is aggravated in non dividing cells and by microtubule destabilization. *Human molecular genetics* 2003; 12(8): 813-22.

Bouchard JP, Richter A, Mathieu J, Brunet D, Hudson TJ, Morgan K, *et al.* Autosomal recessive spastic ataxia of Charlevoix-Saguenay. *Neuromuscul Disord* 1998; 8(7): 474-9.

Bradshaw TY, Romano LE, Duncan EJ, Nethisinghe S, Abeti R, Michael GJ, *et al.* A reduction in Drp1-mediated fission compromises mitochondrial health in autosomal recessive spastic ataxia of Charlevoix Saguenay. *Human molecular genetics* 2016; 25(15): 3232-44.

Bragina L, Conti F. Expression of neurofilament subunits at neocortical glutamatergic and GABAergic synapses. *Frontiers in Neuroanatomy* 2018 In Press.

Byrne LM, Rodrigues FB, Blennow K, Durr A, Leavitt BR, Roos RAC, *et al.* Neurofilament light protein in blood as a potential biomarker of neurodegeneration in Huntington's disease: a retrospective cohort analysis. *The Lancet Neurology* 2017; 16(8): 601-9.

Campos-Melo D, Droppelmann CA, He Z, Volkening K, Strong MJ. Altered microRNA expression profile in Amyotrophic Lateral Sclerosis: a role in the regulation of NFL mRNA levels. *Mol Brain* 2013; 6: 26.

Carare RO, Bernardes-Silva M, Newman TA, Page AM, Nicoll JA, Perry VH, *et al.* Solutes, but not cells, drain from the brain parenchyma along basement membranes of capillaries and

arteries: significance for cerebral amyloid angiopathy and neuroimmunology. *Neuropathology and applied neurobiology* 2008; 34(2): 131-44.

Cavalier L, BenHamida C, Amouri R, Belal S, Bomont P, Lagarde N, *et al.* Giant axonal neuropathy locus refinement to a < 590 kb critical interval. *Eur J Hum Genet* 2000; 8(7): 527-34.

Chen JX, Sun YJ, Wang P, Long DX, Li W, Li L, *et al.* Induction of autophagy by TOCP in differentiated human neuroblastoma cells lead to degradation of cytoskeletal components and inhibition of neurite outgrowth. *Toxicology* 2013; 310: 92-7.

Chou SM, Wang HS, Taniguchi A, Bucala R. Advanced glycation endproducts in neurofilament conglomeration of motoneurons in familial and sporadic amyotrophic lateral sclerosis. *Mol Med* 1998; 4(5): 324-32.

Coleman M. Axon degeneration mechanisms: commonality amid diversity. *Nature reviews Neuroscience* 2005; 6(11): 889-98.

Corbo M, Hays AP. Peripherin and neurofilament protein coexist in spinal spheroids of motor neuron disease. *Journal of neuropathology and experimental neurology* 1992; 51(5): 531-7.

Cote F, Collard JF, Julien JP. Progressive neuronopathy in transgenic mice expressing the human neurofilament heavy gene: a mouse model of amyotrophic lateral sclerosis. *Cell* 1993; 73(1): 35-46.

Crisp MJ, Mawuenyega KG, Patterson BW, Reddy NC, Chott R, Self WK, *et al.* In vivo kinetic approach reveals slow SOD1 turnover in the CNS. *The Journal of clinical investigation* 2015; 125(7): 2772-80.

Darras BT, Crawford TO, Finkel RS, Mercuri E, De Vivo DC, Oskoui M, *et al.* Neurofilament as a potential biomarker for spinal muscular atrophy. *Ann Clin Transl Neurol* 2019; 6(5): 932-44.

de la Fuente-Sandoval C, Leon-Ortiz P, Favila R, Stephano S, Mamo D, Ramirez-Bermudez J, *et al.* Higher levels of glutamate in the associative-striatum of subjects with prodromal symptoms of schizophrenia and patients with first-episode psychosis. *Neuropsychopharmacology* : official publication of the American College of Neuropsychopharmacology 2011; 36(9): 1781-91.

de Leon MJ, Li Y, Okamura N, Tsui WH, Saint-Louis LA, Glodzik L, *et al.* Cerebrospinal Fluid Clearance in Alzheimer Disease Measured with Dynamic PET. *Journal of nuclear medicine* : official publication, Society of Nuclear Medicine 2017; 58(9): 1471-6.

De Stefano N, Filippi M, Miller D, Pouwels PJ, Rovira A, Gass A, *et al.* Guidelines for using proton MR spectroscopy in multicenter clinical MS studies. *Neurology* 2007; 69(20): 1942-52.

Dequen F, Bomont P, Gowing G, Cleveland DW, Julien JP. Modest loss of peripheral axons, muscle atrophy and formation of brain inclusions in mice with targeted deletion of gigaxonin exon 1. *Journal of neurochemistry* 2008; 107(1): 253-64.

Dequen F, Filali M, Lariviere RC, Perrot R, Hisanaga S, Julien JP. Reversal of neuropathy phenotypes in conditional mouse model of Charcot-Marie-Tooth disease type 2E. *Human molecular genetics* 2010; 19(13): 2616-29.

Disanto G, Barro C, Benkert P, Naegelin Y, Schadelin S, Giardiello A, *et al.* Serum Neurofilament light: A biomarker of neuronal damage in multiple sclerosis. *Annals of neurology* 2017; 81(6): 857-70.

Donker Kaat L, Meeter LH, Chiu WZ, Melhem S, Boon AJW, Blennow K, *et al.* Serum neurofilament light chain in progressive supranuclear palsy. *Parkinsonism Relat Disord* 2018; 56: 98-101.

Duncan EJ, Lariviere R, Bradshaw TY, Longo F, Sgarioto N, Hayes MJ, *et al.* Altered organization of the intermediate filament cytoskeleton and relocalization of proteostasis modulators in cells lacking the ataxia protein sacs1. *Human molecular genetics* 2017; 26(16): 3130-43.

Dyakin VV, Chen Y, Branch CA, Veeranna, Yuan A, Rao M, *et al.* The contributions of myelin and axonal caliber to transverse relaxation time in shiverer and neurofilament-deficient mouse models. *Neuroimage* 2010; 51(3): 1098-105.

Ehlers MD, Fung ET, O'Brien RJ, Huganir RL. Splice variant-specific interaction of the NMDA receptor subunit NR1 with neuronal intermediate filaments. *J Neurosci* 1998; 18(2): 720-30.

Engelhardt B, Vajkoczy P, Weller RO. The movers and shapers in immune privilege of the CNS. *Nature immunology* 2017; 18(2): 123-31.

Engert JC, Berube P, Mercier J, Dore C, Lepage P, Ge B, *et al.* ARSACS, a spastic ataxia common in northeastern Quebec, is caused by mutations in a new gene encoding an 11.5-kb ORF. *Nat Genet* 2000; 24(2): 120-5.

Fabrizi GM, Cavallaro T, Angiari C, Bertolasi L, Cabrini I, Ferrarini M, *et al.* Giant axon and neurofilament accumulation in Charcot-Marie-Tooth disease type 2E. *Neurology* 2004; 62(8): 1429-31.

Faure J, Lachenal G, Court M, Hirrlinger J, Chatellard-Causse C, Blot B, *et al.* Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci* 2006; 31(4): 642-8.

Figlewicz DA, Krizus A, Martinoli MG, Meininger V, Dib M, Rouleau GA, *et al.* Variants of the heavy neurofilament subunit are associated with the development of amyotrophic lateral sclerosis. *Human molecular genetics* 1994; 3(10): 1757-61.

Furukawa M, He YJ, Borchers C, Xiong Y. Targeting of protein ubiquitination by BTB-Cullin 3-Roc1 ubiquitin ligases. *Nat Cell Biol* 2003; 5(11): 1001-7.

Gaetani L, Høglund K, Parnetti L, Pujol-Calderon F, Becker B, Eusebi P, *et al.* A new enzyme-linked immunosorbent assay for neurofilament light in cerebrospinal fluid: analytical validation and clinical evaluation. *Alzheimers Res Ther* 2018; 10(1): 8.

Gafson AR, Savva C, Thorne T, David M, Gomez-Romero M, Lewis MR, *et al.* Breaking the cycle: Reversal of flux in the tricarboxylic acid cycle by dimethyl fumarate. *Neurol Neuroimmunol Neuroinflamm* 2019; 6(3): e562.

Gaiottino J, Norgren N, Dobson R, Topping J, Nissim A, Malaspina A, *et al.* Increased neurofilament light chain blood levels in neurodegenerative neurological diseases. *PLoS one* 2013; 8(9): e75091.

Gallien S, Duriez E, Crone C, Kellmann M, Moehring T, Domon B. Targeted proteomic quantification on quadrupole-orbitrap mass spectrometer. *Mol Cell Proteomics* 2012; 11(12): 1709-23.

Ganay T, Boizot A, Burrer R, Chauvin JP, Bomont P. Sensory-motor deficits and neurofilament disorganization in gigaxonin-null mice. *Mol Neurodegener* 2011; 6: 25.

Garcia ML, Rao MV, Fujimoto J, Garcia VB, Shah SB, Crum J, *et al.* Phosphorylation of highly conserved neurofilament medium KSP repeats is not required for myelin-dependent radial axonal growth. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2009; 29(5): 1277-84.

Gasperini C, Prosperini L, Tintore M, Sormani MP, Filippi M, Rio J, *et al.* Unraveling treatment response in multiple sclerosis: A clinical and MRI challenge. *Neurology* 2019; 92(4): 180-92.

Gattringer T, Pinter D, Enzinger C, Seifert-Held T, Kneihsl M, Fandler S, *et al.* Serum neurofilament light is sensitive to active cerebral small vessel disease. *Neurology* 2017; 89(20): 2108-14.

Gentil BJ, Lai GT, Menade M, Lariviere R, Minotti S, Gehring K, *et al.* Sacsin, mutated in the ataxia ARSACS, regulates intermediate filament assembly and dynamics. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2019; 33(2): 2982-94.

Gillette MA, Carr SA. Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry. *Nature methods* 2013; 10(1): 28-34.

Girard M, Lariviere R, Parfitt DA, Deane EC, Gaudet R, Nossova N, *et al.* Mitochondrial dysfunction and Purkinje cell loss in autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS). *Proceedings of the National Academy of Sciences of the United States of America* 2012; 109(5): 1661-6.

Girault JA, Greengard P. The neurobiology of dopamine signaling. *Arch Neurol* 2004; 61(5): 641-4.

Gisslen M, Price RW, Andreasson U, Norgren N, Nilsson S, Hagberg L, *et al.* Plasma Concentration of the Neurofilament Light Protein (NFL) is a Biomarker of CNS Injury in HIV Infection: A Cross-Sectional Study. *EBioMedicine* 2016; 3: 135-40.

Gros-Louis F, Lariviere R, Gowing G, Laurent S, Camu W, Bouchard JP, *et al.* A frameshift deletion in peripherin gene associated with amyotrophic lateral sclerosis. *J Biol Chem* 2004; 279(44): 45951-6.

Gunnarsson M, Malmstrom C, Axelsson M, Sundstrom P, Dahle C, Vrethem M, *et al.* Axonal damage in relapsing multiple sclerosis is markedly reduced by natalizumab. *Annals of neurology* 2011; 69(1): 83-9.

Hakansson I, Tisell A, Cassel P, Blennow K, Zetterberg H, Lundberg P, *et al.* Neurofilament levels, disease activity and brain volume during follow-up in multiple sclerosis. *Journal of neuroinflammation* 2018; 15(1): 209.

Hashimoto R, Nakamura Y, Komai S, Kashiwagi Y, Tamura K, Goto T, *et al.* Site-specific phosphorylation of neurofilament-L is mediated by calcium/calmodulin-dependent protein kinase II in the apical dendrites during long-term potentiation. *J Neurochem* 2000; 75(1): 373-82.

Hawkes CA, Hartig W, Kacza J, Schliebs R, Weller RO, Nicoll JA, *et al.* Perivascular drainage of solutes is impaired in the ageing mouse brain and in the presence of cerebral amyloid angiopathy. *Acta neuropathologica* 2011; 121(4): 431-43.

Heo S, Diering GH, Na CH, Nirujogi RS, Bachman JL, Pandey A, *et al.* Identification of long-lived synaptic proteins by proteomic analysis of synaptosome protein turnover. *Proc Natl Acad Sci U S A* 2018; 115(16): E3827-E36.

Hoffman PN, Lasek RJ. The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian neurons. *J Cell Biol* 1975; 66(2): 351-66.

Homayoun H, Moghaddam B. NMDA receptor hypofunction produces opposite effects on prefrontal cortex interneurons and pyramidal neurons. *J Neurosci* 2007; 27(43): 11496-500.

Hsieh ST, Kidd GJ, Crawford TO, Xu Z, Lin WM, Trapp BD, *et al.* Regional modulation of neurofilament organization by myelination in normal axons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1994; 14(11 Pt 1): 6392-401.

Huntley GW, Vickers JC, Janssen W, Brose N, Heinemann SF, Morrison JH. Distribution and synaptic localization of immunocytochemically identified NMDA receptor subunit proteins in sensory-motor and visual cortices of monkey and human. *J Neurosci* 1994; 14(6): 3603-19.

Iloff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, *et al.* A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. *Sci Transl Med* 2012; 4(147): 147ra11.

Israeli E, Dryanovski DI, Schumacker PT, Chandel NS, Singer JD, Julien JP, *et al.* Intermediate filament aggregates cause mitochondrial dysmotility and increase energy demands in giant axonal neuropathy. *Human molecular genetics* 2016; 25(11): 2143-57.

Jacomy H, Zhu Q, Couillard-Despres S, Beaulieu JM, Julien JP. Disruption of type IV intermediate filament network in mice lacking the neurofilament medium and heavy subunits. *Journal of neurochemistry* 1999; 73(3): 972-84.

Jakobsson J, Bjerke M, Ekman CJ, Sellgren C, Johansson AG, Zetterberg H, *et al.* Elevated concentrations of neurofilament light chain in the cerebrospinal fluid of bipolar disorder patients. *Neuropsychopharmacology* 2014; 39(10): 2349-56.

Johnson EB, Byrne LM, Gregory S, Rodrigues FB, Blennow K, Durr A, *et al.* Neurofilament light protein in blood predicts regional atrophy in Huntington disease. *Neurology* 2018; 90(8): e717-e23.

Johnson-Kerner BL, Ahmad FS, Diaz AG, Greene JP, Gray SJ, Samulski RJ, *et al.* Intermediate filament protein accumulation in motor neurons derived from giant axonal neuropathy iPSCs rescued by restoration of gigaxonin. *Human molecular genetics* 2015a; 24(5): 1420-31.

Johnson-Kerner BL, Garcia Diaz A, Ekins S, Wichterle H. Kelch Domain of Gigaxonin Interacts with Intermediate Filament Proteins Affected in Giant Axonal Neuropathy. *PLoS one* 2015b; 10(10): e0140157.

Johnson-Kerner BL, Roth L, Greene JP, Wichterle H, Sproule DM. Giant axonal neuropathy: An updated perspective on its pathology and pathogenesis. *Muscle Nerve* 2014; 50(4): 467-76.

Jordanova A, De Jonghe P, Boerkoel CF, Takashima H, De Vriendt E, Ceuterick C, *et al.* Mutations in the neurofilament light chain gene (NEFL) cause early onset severe Charcot-Marie-Tooth disease. *Brain : a journal of neurology* 2003; 126(Pt 3): 590-7.

Julien JP, Mushynski WE. Multiple phosphorylation sites in mammalian neurofilament polypeptides. *J Biol Chem* 1982; 257(17): 10467-70.

Julien JP, Mushynski WE. The distribution of phosphorylation sites among identified proteolytic fragments of mammalian neurofilaments. *J Biol Chem* 1983; 258(6): 4019-25.

Julien JP, Tretjakoff I, Beaudet L, Peterson A. Expression and assembly of a human neurofilament protein in transgenic mice provide a novel neuronal marking system. *Genes Dev* 1987; 1(10): 1085-95.

Kalra S. Magnetic Resonance Spectroscopy in ALS. *Frontiers in neurology* 2019; 10: 482.

Kato A, Rouach N, Nicoll RA, Brecht DS. Activity-dependent NMDA receptor degradation mediated by retrotranslocation and ubiquitination. *Proc Natl Acad Sci U S A* 2005; 102(15): 5600-5.

Keable A, Fenna K, Yuen HM, Johnston DA, Smyth NR, Smith C, *et al.* Deposition of amyloid beta in the walls of human leptomeningeal arteries in relation to perivascular drainage

pathways in cerebral amyloid angiopathy. *Biochimica et biophysica acta* 2016; 1862(5): 1037-46.

Kern S, Syrjanen JA, Blennow K, Zetterberg H, Skoog I, Waern M, *et al.* Association of Cerebrospinal Fluid Neurofilament Light Protein With Risk of Mild Cognitive Impairment Among Individuals Without Cognitive Impairment. *JAMA neurology* 2018.

Kesavapany S, Patel V, Zheng YL, Pareek TK, Bjelogrlc M, Albers W, *et al.* Inhibition of Pin1 reduces glutamate-induced perikaryal accumulation of phosphorylated neurofilament-H in neurons. *Mol Biol Cell* 2007; 18(9): 3645-55.

Khalil M, Teunissen CE, Otto M, Piehl F, Sormani MP, Gatringer T, *et al.* Neurofilaments as biomarkers in neurological disorders. *Nature reviews Neurology* 2018.

Kida S, Pantazis A, Weller RO. CSF drains directly from the subarachnoid space into nasal lymphatics in the rat. Anatomy, histology and immunological significance. *Neuropathol Appl Neurobiol* 1993; 19(6): 480-8.

Kioumourtzoglou MA, Rotem RS, Seals RM, Gredal O, Hansen J, Weisskopf MG. Diabetes Mellitus, Obesity, and Diagnosis of Amyotrophic Lateral Sclerosis: A Population-Based Study. *JAMA neurology* 2015; 72(8): 905-11.

Kleijmeer M, Ramm G, Schuurhuis D, Griffith J, Rescigno M, Ricciardi-Castagnoli P, *et al.* Reorganization of multivesicular bodies regulates MHC class II antigen presentation by dendritic cells. *J Cell Biol* 2001; 155(1): 53-63.

Kong J, Tung VW, Aghajanian J, Xu Z. Antagonistic roles of neurofilament subunits NF-H and NF-M against NF-L in shaping dendritic arborization in spinal motor neurons. *J Cell Biol* 1998; 140(5): 1167-76.

Kristiansen LV, Beneyto M, Haroutunian V, Meador-Woodruff JH. Changes in NMDA receptor subunits and interacting PSD proteins in dorsolateral prefrontal and anterior cingulate cortex indicate abnormal regional expression in schizophrenia. *Mol Psychiatry* 2006; 11(8): 737-47, 05.

Kriz J, Zhu Q, Julien JP, Padjen AL. Electrophysiological properties of axons in mice lacking neurofilament subunit genes: disparity between conduction velocity and axon diameter in absence of NF-H. *Brain Res* 2000; 885(1): 32-44.

Kuhle J, Barro C, Andreasson U, Derfuss T, Lindberg R, Sandelius A, *et al.* Comparison of three analytical platforms for quantification of the neurofilament light chain in blood samples: ELISA, electrochemiluminescence immunoassay and Simoa. *Clin Chem Lab Med* 2016a; 54(10): 1655-61.

Kuhle J, Barro C, Disanto G, Mathias A, Sonesson C, Bonnier G, *et al.* Serum neurofilament light chain in early relapsing remitting MS is increased and correlates with CSF levels and with MRI measures of disease severity. *Multiple sclerosis* 2016b; 22(12): 1550-9.

Kuhle J, Kropshofer H, Haering DA, Kundu U, Meinert R, Barro C, *et al.* Blood neurofilament light chain as a biomarker of MS disease activity and treatment response. *Neurology* 2019; 92(10): e1007-e15.

Kuhle J, Leppert D, Petzold A, Regeniter A, Schindler C, Mehling M, *et al.* Neurofilament heavy chain in CSF correlates with relapses and disability in multiple sclerosis. *Neurology* 2011; 76(14): 1206-13.

Kuhle J, Malmstrom C, Axelsson M, Plattner K, Yaldizli O, Derfuss T, *et al.* Neurofilament light and heavy subunits compared as therapeutic biomarkers in multiple sclerosis. *Acta neurologica Scandinavica* 2013; 128(6): e33-6.

Kuhle J, Nourbakhsh B, Grant D, Morant S, Barro C, Yaldizli O, *et al.* Serum neurofilament is associated with progression of brain atrophy and disability in early MS. *Neurology* 2017; 88(9): 826-31.

Lachenal G, Pernet-Gallay K, Chivet M, Hemming FJ, Belly A, Bodon G, *et al.* Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. *Mol Cell Neurosci* 2011; 46(2): 409-18.

Lariviere R, Gaudet R, Gentil BJ, Girard M, Conte TC, Minotti S, *et al.* Sacs knockout mice present pathophysiological defects underlying autosomal recessive spastic ataxia of Charlevoix-Saguenay. *Human molecular genetics* 2015; 24(3): 727-39.

Lee MK, Marszalek JR, Cleveland DW. A mutant neurofilament subunit causes massive, selective motor neuron death: implications for the pathogenesis of human motor neuron disease. *Neuron* 1994; 13(4): 975-88.

Lee S, Pant HC, Shea TB. Divergent and convergent roles for kinases and phosphatases in neurofilament dynamics. *J Cell Sci* 2014; 127(Pt 18): 4064-77.

Lee YL, Burke B. LINC complexes and nuclear positioning. *Semin Cell Dev Biol* 2018; 82: 67-76.

Leung CL, He CZ, Kaufmann P, Chin SS, Naini A, Liem RK, *et al.* A pathogenic peripherin gene mutation in a patient with amyotrophic lateral sclerosis. *Brain Pathol* 2004; 14(3): 290-6.

Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I, *et al.* Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. *Am J Hum Genet* 2003; 73(1): 34-48.

Lewis SE, Nixon RA. Multiple phosphorylated variants of the high molecular mass subunit of neurofilaments in axons of retinal cell neurons: characterization and evidence for their differential association with stationary and moving neurofilaments. *J Cell Biol* 1988; 107(6 Pt 2): 2689-701.

Li F, Tsien JZ. Memory and the NMDA receptors. *The New England journal of medicine* 2009; 361(3): 302-3.

Li Y, Jung P, Brown A. Axonal transport of neurofilaments: a single population of intermittently moving polymers. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2012; 32(2): 746-58.

Lin CH, Huang YJ, Lin CJ, Lane HY, Tsai GE. NMDA neurotransmission dysfunction in mild cognitive impairment and Alzheimer's disease. *Curr Pharm Des* 2014; 20(32): 5169-79.

Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, *et al.* Structural and functional features of central nervous system lymphatic vessels. *Nature* 2015; 523(7560): 337-41.

Lu CH, Macdonald-Wallis C, Gray E, Pearce N, Petzold A, Norgren N, *et al.* Neurofilament light chain: A prognostic biomarker in amyotrophic lateral sclerosis. *Neurology* 2015; 84(22): 2247-57.

Lycke JN, Karlsson JE, Andersen O, Rosengren LE. Neurofilament protein in cerebrospinal fluid: a potential marker of activity in multiple sclerosis. *Journal of neurology, neurosurgery, and psychiatry* 1998; 64(3): 402-4.

Mahammad S, Murthy SN, Didonna A, Grin B, Israeli E, Perrot R, *et al.* Giant axonal neuropathy-associated gigaxonin mutations impair intermediate filament protein degradation. *The Journal of clinical investigation* 2013; 123(5): 1964-75.

Malmstrom C, Haghighi S, Rosengren L, Andersen O, Lycke J. Neurofilament light protein and glial fibrillary acidic protein as biological markers in MS. *Neurology* 2003; 61(12): 1720-5.

Manek R, Moghieb A, Yang Z, Kumar D, Kobeissy F, Sarkis GA, *et al.* Correction to: Protein Biomarkers and Neuroproteomics Characterization of Microvesicles/Exosomes from Human Cerebrospinal Fluid Following Traumatic Brain Injury. *Molecular neurobiology* 2018; 55(7): 6129.

Manser C, Stevenson A, Banner S, Davies J, Tudor EL, Ono Y, *et al.* Deregulation of PKN1 activity disrupts neurofilament organisation and axonal transport. *FEBS Lett* 2008; 582(15): 2303-8.

Mariosa D, Kamel F, Bellocco R, Ye W, Fang F. Association between diabetes and amyotrophic lateral sclerosis in Sweden. *European journal of neurology* 2015; 22(11): 1436-42.

Marrie RA. Comorbidity in Multiple Sclerosis: Some Answers, More Questions. *Int J MS Care* 2016; 18(6): 271-2.

Mattsson N, Andreasson U, Zetterberg H, Blennow K, Alzheimer's Disease Neuroimaging I. Association of Plasma Neurofilament Light With Neurodegeneration in Patients With Alzheimer Disease. *JAMA neurology* 2017; 74(5): 557-66.

Mattsson N, Insel PS, Palmqvist S, Portelius E, Zetterberg H, Weiner M, *et al.* Cerebrospinal fluid tau, neurogranin, and neurofilament light in Alzheimer's disease. *EMBO molecular medicine* 2016; 8(10): 1184-96.

Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, *et al.* Decreased clearance of CNS beta-amyloid in Alzheimer's disease. *Science (New York, NY)* 2010; 330(6012): 1774.

McIntee FL, Giannoni P, Blais S, Sommer G, Neubert TA, Rostagno A, *et al.* In vivo Differential Brain Clearance and Catabolism of Monomeric and Oligomeric Alzheimer's Abeta protein. *Frontiers in aging neuroscience* 2016; 8: 223.

Meeter LH, Dopfer EG, Jiskoot LC, Sanchez-Valle R, Graff C, Benussi L, *et al.* Neurofilament light chain: a biomarker for genetic frontotemporal dementia. *Ann Clin Transl Neurol* 2016; 3(8): 623-36.

Merritt K, Egerton A, Kempton MJ, Taylor MJ, McGuire PK. Nature of Glutamate Alterations in Schizophrenia: A Meta-analysis of Proton Magnetic Resonance Spectroscopy Studies. *JAMA psychiatry* 2016; 73(7): 665-74.

Mersiyanova IV, Perepelov AV, Polyakov AV, Sitnikov VF, Dadali EL, Oparin RB, *et al.* A new variant of Charcot-Marie-Tooth disease type 2 is probably the result of a mutation in the neurofilament-light gene. *Am J Hum Genet* 2000; 67(1): 37-46.

Millecamps S, Gowing G, Corti O, Mallet J, Julien JP. Conditional NF-L transgene expression in mice for in vivo analysis of turnover and transport rate of neurofilaments. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2007; 27(18): 4947-56.

Miyazawa I, Nakashima I, Petzold A, Fujihara K, Sato S, Itoyama Y. High CSF neurofilament heavy chain levels in neuromyelitis optica. *Neurology* 2007; 68(11): 865-7.

Murk JL, Stoorvogel W, Kleijmeer MJ, Geuze HJ. The plasticity of multivesicular bodies and the regulation of antigen presentation. *Semin Cell Dev Biol* 2002; 13(4): 303-11.

Nakadate K, Noda T, Sakakibara S, Kumamoto K, Matsuura T, Joyce JN, *et al.* Progressive dopaminergic neurodegeneration of substantia nigra in the zitter mutant rat. *Acta neuropathologica* 2006; 112(1): 64-73.

Nixon RA. Dynamic behavior and organization of cytoskeletal proteins in neurons: reconciling old and new findings. *Bioessays* 1998; 20(10): 798-807.

Nixon RA. Autophagy in neurodegenerative disease: friend, foe or turncoat? *Trends in neurosciences* 2006; 29(9): 528-35.

Nixon RA, Lewis SE, Mercken M, Sihag RK. [32P]orthophosphate and [35S]methionine label separate pools of neurofilaments with markedly different axonal transport kinetics in mouse retinal ganglion cells in vivo. *Neurochem Res* 1994a; 19(11): 1445-53.

Nixon RA, Logvinenko KB. Multiple fates of newly synthesized neurofilament proteins: evidence for a stationary neurofilament network distributed nonuniformly along axons of retinal ganglion cell neurons. *J Cell Biol* 1986; 102(2): 647-59.

Nixon RA, Marotta CA. Degradation of neurofilament proteins by purified human brain cathepsin D. *Journal of neurochemistry* 1984; 43(2): 507-16.

Nixon RA, Paskevich PA, Sihag RK, Thayer CY. Phosphorylation on carboxyl terminus domains of neurofilament proteins in retinal ganglion cell neurons in vivo: influences on regional neurofilament accumulation, interneurofilament spacing, and axon caliber. *J Cell Biol* 1994b; 126(4): 1031-46.

Nixon RA, Sihag RK. Neurofilament phosphorylation: a new look at regulation and function. *Trends Neurosci* 1991; 14(11): 501-6.

Nixon RA, Wegiel J, Kumar A, Yu WH, Peterhoff C, Cataldo A, *et al.* Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. *Journal of neuropathology and experimental neurology* 2005; 64(2): 113-22.

Norgren N, Sundstrom P, Svenningsson A, Rosengren L, Stigbrand T, Gunnarsson M. Neurofilament and glial fibrillary acidic protein in multiple sclerosis. *Neurology* 2004; 63(9): 1586-90.

Novakova L, Zetterberg H, Sundstrom P, Axelsson M, Khademi M, Gunnarsson M, *et al.* Monitoring disease activity in multiple sclerosis using serum neurofilament light protein. *Neurology* 2017; 89(22): 2230-7.

Nylen K, Csajbok LZ, Ost M, Rashid A, Karlsson JE, Blennow K, *et al.* CSF -neurofilament correlates with outcome after aneurysmal subarachnoid hemorrhage. *Neuroscience letters* 2006; 404(1-2): 132-6.

Parfitt DA, Michael GJ, Vermeulen EG, Prodromou NV, Webb TR, Gallo JM, *et al.* The ataxia protein saccin is a functional co-chaperone that protects against polyglutamine-expanded ataxin-1. *Human molecular genetics* 2009; 18(9): 1556-65.

Paterson RW, Gabelle A, Lucey BP, Barthelemy NR, Leckey CA, Hirtz C, *et al.* SILK studies - capturing the turnover of proteins linked to neurodegenerative diseases. *Nature reviews Neurology* 2019; 15(7): 419-27.

Pennington K, Beasley CL, Dicker P, Fagan A, English J, Pariante CM, *et al.* Prominent synaptic and metabolic abnormalities revealed by proteomic analysis of the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder. *Mol Psychiatry* 2008; 13(12): 1102-17.

Perez-Olle R, Jones ST, Liem RK. Phenotypic analysis of neurofilament light gene mutations linked to Charcot-Marie-Tooth disease in cell culture models. *Human molecular genetics* 2004; 13(19): 2207-20.

Perez-Olle R, Leung CL, Liem RK. Effects of Charcot-Marie-Tooth-linked mutations of the neurofilament light subunit on intermediate filament formation. *J Cell Sci* 2002; 115(Pt 24): 4937-46.

Perrot R, Berges R, Bocquet A, Eyer J. Review of the multiple aspects of neurofilament functions, and their possible contribution to neurodegeneration. *Molecular neurobiology* 2008; 38(1): 27-65.

Peterson AC, Russell JD, Bailey DJ, Westphall MS, Coon JJ. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol Cell Proteomics* 2012; 11(11): 1475-88.

Petzold A, Altintas A, Andreoni L, Bartos A, Berthele A, Blankenstein MA, *et al.* Neurofilament ELISA validation. *J Immunol Methods* 2010; 352(1-2): 23-31.

Petzold A, Hinds N, Murray NM, Hirsch NP, Grant D, Keir G, *et al.* CSF neurofilament levels: a potential prognostic marker in Guillain-Barre syndrome. *Neurology* 2006; 67(6): 1071-3.

Piehl F, Kockum I, Khademi M, Blennow K, Lycke J, Zetterberg H, *et al.* Plasma neurofilament light chain levels in patients with MS switching from injectable therapies to fingolimod. *Multiple sclerosis* 2018; 24(8): 1046-54.

Pini L, Pievani M, Bocchetta M, Altomare D, Bosco P, Cavedo E, *et al.* Brain atrophy in Alzheimer's Disease and aging. *Ageing Res Rev* 2016; 30: 25-48.

Preisich O, Schultz SA, Apel A, Kuhle J, Kaeser SA, Barro C, *et al.* Serum neurofilament dynamics predicts neurodegeneration and clinical progression in presymptomatic Alzheimer's disease. *Nature medicine* 2019; 25(2): 277-83.

Prineas JW, Ouvrier RA, Wright RG, Walsh JC, McLeod JG. Giant axonal neuropathy--a generalized disorder of cytoplasmic microfilament formation. *Journal of neuropathology and experimental neurology* 1976; 35(4): 458-70.

Rao MV, Campbell J, Yuan A, Kumar A, Gotow T, Uchiyama Y, *et al.* The neurofilament middle molecular mass subunit carboxyl-terminal tail domains is essential for the radial growth and cytoskeletal architecture of axons but not for regulating neurofilament transport rate. *J Cell Biol* 2003; 163(5): 1021-31.

Rao MV, Houseweart MK, Williamson TL, Crawford TO, Folmer J, Cleveland DW. Neurofilament-dependent radial growth of motor axons and axonal organization of neurofilaments does not require the neurofilament heavy subunit (NF-H) or its phosphorylation. *J Cell Biol* 1998; 143(1): 171-81.

Rao MV, Yuan A, Campbell J, Kumar A, Nixon RA. The C-terminal domains of NF-H and NF-M subunits maintain axonal neurofilament content by blocking turnover of the stationary neurofilament network. *PloS one* 2012; 7(9): e44320.

Ratnam J, Teichberg VI. Neurofilament-light increases the cell surface expression of the N-methyl-D-aspartate receptor and prevents its ubiquitination. *Journal of neurochemistry* 2005; 92(4): 878-85.

Rebelo AP, Abrams AJ, Cottenie E, Horga A, Gonzalez M, Bis DM, *et al.* Cryptic Amyloidogenic Elements in the 3' UTRs of Neurofilament Genes Trigger Axonal Neuropathy. *Am J Hum Genet* 2016; 98(4): 597-614.

Rojas JC, Karydas A, Bang J, Tsai RM, Blennow K, Liman V, *et al.* Plasma neurofilament light chain predicts progression in progressive supranuclear palsy. *Ann Clin Transl Neurol* 2016; 3(3): 216-25.

Rooke K, Figlewicz DA, Han FY, Rouleau GA. Analysis of the KSP repeat of the neurofilament heavy subunit in familiar amyotrophic lateral sclerosis. *Neurology* 1996; 46(3): 789-90.

Roots BI. Neurofilament accumulation induced in synapses by leupeptin. *Science (New York, NY)* 1983; 221(4614): 971-2.

Rosengren LE, Karlsson JE, Karlsson JO, Persson LI, Wikkelso C. Patients with amyotrophic lateral sclerosis and other neurodegenerative diseases have increased levels of neurofilament protein in CSF. *Journal of neurochemistry* 1996; 67(5): 2013-8.

Roy S, Coffee P, Smith G, Liem RK, Brady ST, Black MM. Neurofilaments are transported rapidly but intermittently in axons: implications for slow axonal transport. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2000; 20(18): 6849-61.

Sandelius A, Zetterberg H, Blennow K, Adiuatori R, Malaspina A, Laura M, *et al.* Plasma neurofilament light chain concentration in the inherited peripheral neuropathies. *Neurology* 2018; 90(6): e518-e24.

Sanelli T, Ge W, Leystra-Lantz C, Strong MJ. Calcium mediated excitotoxicity in neurofilament aggregate-bearing neurons in vitro is NMDA receptor dependant. *Journal of the neurological sciences* 2007; 256(1-2): 39-51.

Sasaki T, Gotow T, Shiozaki M, Sakaue F, Saito T, Julien JP, *et al.* Aggregate formation and phosphorylation of neurofilament-L Pro22 Charcot-Marie-Tooth disease mutants. *Human molecular genetics* 2006; 15(6): 943-52.

Sato C, Barthelemy NR, Mawuenyega KG, Patterson BW, Gordon BA, Jockel-Balsarotti J, *et al.* Tau Kinetics in Neurons and the Human Central Nervous System. *Neuron* 2018; 97(6): 1284-98 e7.

Schwartz ML, Bruce J, Shneidman PS, Schlaepfer WW. Deletion of 3'-untranslated region alters the level of mRNA expression of a neurofilament light subunit transgene. *J Biol Chem* 1995; 270(44): 26364-9.

Schwartz ML, Shneidman PS, Bruce J, Schlaepfer WW. Stabilization of neurofilament transcripts during postnatal development. *Brain Res Mol Brain Res* 1994; 27(2): 215-20.

Scrivo A, Codogno P, Bomont P. Gigaxonin E3 ligase governs ATG16L1 turnover to control autophagosome production. *Nat Commun* 2019; 10(1): 780.

Shabanzadeh AP, D'Onofrio PM, Monnier PP, Koeberle PD. Targeting caspase-6 and caspase-8 to promote neuronal survival following ischemic stroke. *Cell Death Dis* 2015; 6: e1967.

Shahim P, Gren M, Liman V, Andreasson U, Norgren N, Tegner Y, *et al.* Serum neurofilament light protein predicts clinical outcome in traumatic brain injury. *Sci Rep* 2016; 6: 36791.

Shahim P, Zetterberg H, Tegner Y, Blennow K. Serum neurofilament light as a biomarker for mild traumatic brain injury in contact sports. *Neurology* 2017; 88(19): 1788-94.

Shea TB, Flanagan LA. Kinesin, dynein and neurofilament transport. *Trends in neurosciences* 2001; 24(11): 644-8.

Shen H, Barry DM, Dale JM, Garcia VB, Calcutt NA, Garcia ML. Muscle pathology without severe nerve pathology in a new mouse model of Charcot-Marie-Tooth disease type 2E. *Human molecular genetics* 2011; 20(13): 2535-48.

Sihag RK, Inagaki M, Yamaguchi T, Shea TB, Pant HC. Role of phosphorylation on the structural dynamics and function of types III and IV intermediate filaments. *Experimental cell research* 2007; 313(10): 2098-109.

Siller N, Kuhle J, Muthuraman M, Barro C, Uphaus T, Groppa S, *et al.* Serum neurofilament light chain is a biomarker of acute and chronic neuronal damage in early multiple sclerosis. *Multiple sclerosis* 2018; 1352458518765666.

Smerjac SM, Zheng J, Hu CL, Bizzozero OA. The Role of Calpain and Proteasomes in the Degradation of Carbonylated Neuronal Cytoskeletal Proteins in Acute Experimental Autoimmune Encephalomyelitis. *Neurochem Res* 2018; 43(12): 2277-87.

Smith MD, Morris PJ, Dawson SJ, Schwartz ML, Schlaepfer WW, Latchman DS. Coordinate induction of the three neurofilament genes by the Brn-3a transcription factor. *J Biol Chem* 1997; 272(34): 21325-33.

Sormani MP, Haering DA, Kropshofer H, Leppert D, Kundu U, Barro C, *et al.* Blood neurofilament light as a potential endpoint in Phase 2 studies in MS. *Ann Clin Transl Neurol* 2019; 6(6): 1081-9.

Sproviero D, La Salvia S, Giannini M, Crippa V, Gagliardi S, Bernuzzi S, *et al.* Pathological Proteins Are Transported by Extracellular Vesicles of Sporadic Amyotrophic Lateral Sclerosis Patients. *Front Neurosci* 2018; 12: 487.

Steinacker P, Feneberg E, Weishaupt J, Brettschneider J, Tumani H, Andersen PM, *et al.* Neurofilaments in the diagnosis of motoneuron diseases: a prospective study on 455 patients. *Journal of neurology, neurosurgery, and psychiatry* 2016; 87(1): 12-20.

Strong MJ, Volkening K, Hammond R, Yang W, Strong W, Leystra-Lantz C, *et al.* TDP43 is a human low molecular weight neurofilament (hNFL) mRNA-binding protein. *Mol Cell Neurosci* 2007; 35(2): 320-7.

Szentistvanyi I, Patlak CS, Ellis RA, Cserr HF. Drainage of interstitial fluid from different regions of rat brain. *Am J Physiol* 1984a; 246(6 Pt 2): F835-44.

Szentistvanyi I, Patlak CS, Ellis RA, Cserr HF. Drainage of interstitial fluid from different regions of rat brain. *American Journal of Physiology* 1984b; 246: F835-44.

Terry-Lorenzo RT, Inoue M, Connor JH, Haystead TA, Armbruster BN, Gupta RP, *et al.* Neurofilament-L is a protein phosphatase-1-binding protein associated with neuronal plasma membrane and post-synaptic density. *J Biol Chem* 2000; 275(4): 2439-46.

Teunissen CE, Iacobaeus E, Khademi M, Brundin L, Norgren N, Koel-Simmelink MJ, *et al.* Combination of CSF N-acetylaspartate and neurofilaments in multiple sclerosis. *Neurology* 2009; 72(15): 1322-9.

Thelin EP, Zeiler FA, Ercole A, Mondello S, Buki A, Bellander BM, *et al.* Serial Sampling of Serum Protein Biomarkers for Monitoring Human Traumatic Brain Injury Dynamics: A Systematic Review. *Frontiers in neurology* 2017; 8: 300.

Tomkins J, Usher P, Slade JY, Ince PG, Curtis A, Bushby K, *et al.* Novel insertion in the KSP region of the neurofilament heavy gene in amyotrophic lateral sclerosis (ALS). *Neuroreport* 1998; 9(17): 3967-70.

Tradewell ML, Durham HD, Mushynski WE, Gentil BJ. Mitochondrial and axonal abnormalities precede disruption of the neurofilament network in a model of charcot-marie-tooth disease type 2E and are prevented by heat shock proteins in a mutant-specific fashion. *Journal of neuropathology and experimental neurology* 2009; 68(6): 642-52.

Trivedi N, Jung P, Brown A. Neurofilaments switch between distinct mobile and stationary states during their transport along axons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2007; 27(3): 507-16.

Truant R, Atwal RS, Desmond C, Munsie L, Tran T. Huntington's disease: revisiting the aggregation hypothesis in polyglutamine neurodegenerative diseases. *FEBS J* 2008; 275(17): 4252-62.

van Elst LT, Valerius G, Buchert M, Thiel T, Rusch N, Bubl E, *et al.* Increased prefrontal and hippocampal glutamate concentration in schizophrenia: evidence from a magnetic resonance spectroscopy study. *Biological psychiatry* 2005; 58(9): 724-30.

van Lieverloo GGA, Wieske L, Verhamme C, Vrancken AFJ, van Doorn PA, Michalak Z, *et al.* Serum neurofilament light chain in chronic inflammatory demyelinating polyneuropathy. *J Peripher Nerv Syst* 2019; 24(2): 187-94.

Vechio JD, Bruijn LI, Xu Z, Brown RH, Jr., Cleveland DW. Sequence variants in human neurofilament proteins: absence of linkage to familial amyotrophic lateral sclerosis. *Annals of neurology* 1996; 40(4): 603-10.

Veyrat-Durebex C, Corcia P, Dangoumau A, Laumonnier F, Piver E, Gordon PH, *et al.* Advances in cellular models to explore the pathophysiology of amyotrophic lateral sclerosis. *Molecular neurobiology* 2014; 49(2): 966-83.

Volkening K, Leystra-Lantz C, Yang W, Jaffee H, Strong MJ. Tar DNA binding protein of 43 kDa (TDP-43), 14-3-3 proteins and copper/zinc superoxide dismutase (SOD1) interact to modulate NFL mRNA stability. Implications for altered RNA processing in amyotrophic lateral sclerosis (ALS). *Brain Res* 2009; 1305: 168-82.

Von Bartheld CS, Altick AL. Multivesicular bodies in neurons: distribution, protein content, and trafficking functions. *Progress in neurobiology* 2011; 93(3): 313-40.

Walker CL, Uchida A, Li Y, Trivedi N, Fenn JD, Monsma PC, *et al.* Local Acceleration of Neurofilament Transport at Nodes of Ranvier. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2019; 39(4): 663-77.

Wang JT, Medress ZA, Barres BA. Axon degeneration: molecular mechanisms of a self-destruction pathway. *J Cell Biol* 2012; 196(1): 7-18.

Wang L, Ho CL, Sun D, Liem RK, Brown A. Rapid movement of axonal neurofilaments interrupted by prolonged pauses. *Nat Cell Biol* 2000; 2(3): 137-41.

Wang QJ, Ding Y, Kohtz DS, Mizushima N, Cristea IM, Rout MP, *et al.* Induction of autophagy in axonal dystrophy and degeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2006; 26(31): 8057-68.

Weller RO, Hawkes CA, Carare RO, Hardy J. Does the difference between PART and Alzheimer's disease lie in the age-related changes in cerebral arteries that trigger the accumulation of A β and propagation of tau? *Acta neuropathologica* 2015; 129(5): 763-6.

Weston PSJ, Poole T, O'Connor A, Heslegrave A, Ryan NS, Liang Y, *et al.* Longitudinal measurement of serum neurofilament light in presymptomatic familial Alzheimer's disease. *Alzheimers Res Ther* 2019; 11(1): 19.

Weston PSJ, Poole T, Ryan NS, Nair A, Liang Y, Macpherson K, *et al.* Serum neurofilament light in familial Alzheimer disease: A marker of early neurodegeneration. *Neurology* 2017; 89(21): 2167-75.

Yabe JT, Jung C, Chan WK, Shea TB. Phospho-dependent association of neurofilament proteins with kinesin in situ. *Cell Motil Cytoskeleton* 2000; 45(4): 249-62.

Yaworsky PJ, Gardner DP, Kappen C. Transgenic analyses reveal developmentally regulated neuron- and muscle-specific elements in the murine neurofilament light chain gene promoter. *J Biol Chem* 1997; 272(40): 25112-20.

Ylikallio E, Poyhonen R, Zimon M, De Vriendt E, Hilander T, Paetau A, *et al.* Deficiency of the E3 ubiquitin ligase TRIM2 in early-onset axonal neuropathy. *Human molecular genetics* 2013; 22(15): 2975-83.

Yuan A, Nixon RA. Specialized roles of neurofilament proteins in synapses: Relevance to neuropsychiatric disorders. *Brain Res Bull* 2016; 126(Pt 3): 334-46.

Yuan A, Rao MV, Kumar A, Julien JP, Nixon RA. Neurofilament transport in vivo minimally requires hetero-oligomer formation. *J Neurosci* 2003; 23(28): 9452-8.

Yuan A, Rao MV, Sasaki T, Chen Y, Kumar A, Veeranna, *et al.* Alpha-internexin is structurally and functionally associated with the neurofilament triplet proteins in the mature CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2006; 26(39): 10006-19.

Yuan A, Rao MV, Veeranna, Nixon RA. Neurofilaments at a glance. *J Cell Sci* 2012a; 125(Pt 14): 3257-63.

Yuan A, Rao MV, Veeranna, Nixon RA. Neurofilaments and Neurofilament Proteins in Health and Disease. *Cold Spring Harb Perspect Biol* 2017; 9(4).

Yuan A, Sasaki T, Kumar A, Peterhoff CM, Rao MV, Liem RK, *et al.* Peripherin is a subunit of peripheral nerve neurofilaments: implications for differential vulnerability of CNS and peripheral nervous system axons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2012b; 32(25): 8501-8.

Yuan A, Sasaki T, Rao MV, Kumar A, Kanumuri V, Dunlop DS, *et al.* Neurofilaments form a highly stable stationary cytoskeleton after reaching a critical level in axons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2009; 29(36): 11316-29.

Yuan A, Sershen H, Veeranna, Basavarajappa BS, Kumar A, Hashim A, *et al.* Functions of neurofilaments in synapses. *Mol Psychiatry* 2015a; 20(8): 915.

Yuan A, Sershen H, Veeranna, Basavarajappa BS, Kumar A, Hashim A, *et al.* Neurofilament subunits are integral components of synapses and modulate neurotransmission and behavior in vivo. *Mol Psychiatry* 2015b; 20(8): 986-94.

Yuan A, Veeranna, Sershen H, Basavarajappa BS, Smiley JF, Hashim A, *et al.* Neurofilament light interaction with GluN1 modulates neurotransmission and schizophrenia-associated behaviors. *Translational psychiatry* 2018a; 8(1): 167.

Yuan A, Veeranna S, Sershen H, Basavarajappa B, Smiley J, Hashim A, *et al.* Neurofilament light interaction with GluN1 modulates neurotransmission and schizophrenia-associated behaviors. *Translational Psychiatry* 2018b; 8.

Yum SW, Zhang J, Mo K, Li J, Scherer SS. A novel recessive Nefl mutation causes a severe, early-onset axonal neuropathy. *Annals of neurology* 2009; 66(6): 759-70.

Zanier ER, Refai D, Zipfel GJ, Zoerle T, Longhi L, Esparza TJ, *et al.* Neurofilament light chain levels in ventricular cerebrospinal fluid after acute aneurysmal subarachnoid haemorrhage. *Journal of neurology, neurosurgery, and psychiatry* 2011; 82(2): 157-9.

Zetterberg H. Neurofilament Light: A Dynamic Cross-Disease Fluid Biomarker for Neurodegeneration. *Neuron* 2016; 91(1): 1-3.

Zetterberg H, Skillback T, Mattsson N, Trojanowski JQ, Portelius E, Shaw LM, *et al.* Association of Cerebrospinal Fluid Neurofilament Light Concentration With Alzheimer Disease Progression. *JAMA neurology* 2016; 73(1): 60-7.

Zhai J, Lin H, Julien JP, Schlaepfer WW. Disruption of neurofilament network with aggregation of light neurofilament protein: a common pathway leading to motor neuron degeneration due to Charcot-Marie-Tooth disease-linked mutations in NFL and HSPB1. *Human molecular genetics* 2007; 16(24): 3103-16.

Zhu Q, Couillard-Despres S, Julien JP. Delayed maturation of regenerating myelinated axons in mice lacking neurofilaments. *Experimental neurology* 1997; 148(1): 299-316.

Zhu Q, Lindenbaum M, Levavasseur F, Jacomy H, Julien JP. Disruption of the NF-H gene increases axonal microtubule content and velocity of neurofilament transport: relief of axonopathy resulting from the toxin beta,beta'-iminodipropionitrile. *J Cell Biol* 1998; 143(1): 183-93.

Zuchner S, Vorgerd M, Sindern E, Schroder JM. The novel neurofilament light (NEFL) mutation Glu397Lys is associated with a clinically and morphologically heterogeneous type of Charcot-Marie-Tooth neuropathy. *Neuromuscul Disord* 2004; 14(2): 147-57.