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Review of the Multiple Aspects of Neurofilament Functions, and their Possible Contribution to Neurodegeneration

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Abstract Neurofilaments (NF) are the most abundant cytoskeletal component of large myelinated axons from adult central and peripheral nervous system. Here, we provide an overview of the complementary approaches, including biochemistry, cell biology and transgenic technology that were used to investigate the assembly, axonal transport and functions of NF in normal and pathological situations. Following their synthesis and assembly in the cell body, NFs are transported along the axon. This process is finely regulated via phosphorylation of the carboxy-terminal part of the two high-molecular-weight subunits of NF. The correct formation of an axonal network of NF is crucial for the establishment and maintenance of axonal calibre and consequently for the optimisation of conduction velocity. The frequent disorganisation of NF network observed in several neuropathologies support their contribution. However, despite the presence of NF mutations found in some patients, the exact relations between these mutations, the abnormal NF organisation and the pathological process remain a challenging field of investigation.

Keywords Neurofilament · Intermediate filaments · Cytoskeleton · Axonal transport · Neurodegenerative diseases

Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
BPAG1n	bullous pemphigoid antigen 1 neural isoform
CaMKII	calcium–calmodulin-dependent protein kinase II
CKI/II	casein kinase I and II
CMT	Charcot-Marie-Tooth disease
CNS	central nervous system
GAN	giant axonal neuropathy
GSK3	glycogen synthetase kinase 3
IDPN	β, β'-iminodipropionitrile
IF	intermediate filaments
JNK1/3	c-Jun N-terminus kinase 1 and 3
KSP	lysine–serine–proline
MAG	myelin-associated glycoprotein
MAP	microtubule-associated protein
MF	microfilaments
MT	microtubules
NF	neurofilaments
NFH	heavy neurofilament subunit
NFL	light neurofilament subunit
NFM	medium-sized neurofilament subunit
NFT	neurofibrillary tangles
NIFID	neuronal intermediate filament inclusion disease
O-GlcNAc	O-linked *N*-acetyl glucosamine
PD	Parkinson's disease

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PKA	protein kinase A
PKC	protein kinase C
PKN	protein kinase N
PNS	peripheral nervous system
PP2A	phosphatase 2A
SAPK	stress-activated protein kinase
SC	slow component
SOD1	superoxide dismutase 1
STOP	stable tubule only polypeptide
TBCB	tubulin folding cofactor B

Introduction

The neuronal cytoskeleton is composed of three interconnected structures: the actin microfilaments (MFs), microtubules (MTs) and intermediate filaments (IFs). The diameter of IFs (10 nm) is "intermediate" between microfilaments (6 nm) and myosin filaments (15 nm) [1]. Neurofilaments (NF) are the major IFs present in adult neurons and their expression is restricted to neuronal cell types. Neurons express differentially several IF proteins depending on their developing stage or their localisation in the nervous system: nestin (200 kDa), three NF subunits (called NFL (light, 68 kDa), NFM (medium, 160 kDa) and NFH (heavy, 205 kDa)), α-internexin (66 kDa), peripherin (57 kDa) and synemin (41 kDa) [2–6].

The main role recognised for NF is to increase the axonal calibre of myelinated axons and consequently their conduction velocity. They also contribute to the dynamic properties of the axonal cytoskeleton during neuronal differentiation, axon outgrowth, regeneration and guidance [7]. Perturbations of their metabolism and organisation are frequently associated with various neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and Charcot-Marie-Tooth disease (CMT). An intense production and investigation of several transgenic models revealed new mechanisms underlying the normal and pathological biology of NF. However, different aspects concerning NF biology and function are still unsolved. In this review, we tempted to cover the current knowledge related to NF structure, assembly, expression, metabolism and functions. We will also discuss various transgenic models and NF involvement in neurodegenerative disorders.

Composition and Structure of Neurofilaments

Fibrous networks within neurons, initially named neurofibrils, were first described in the nineteenth century. With the development of electron microscopy, it was shown that neurofibrils were comprised of ~10-nm-diameter filaments, which were called NF and later classified in the IF family. IF proteins form a large family of proteins of various sizes (40–280 kDa) and primary structure, expressed differentially according to cell types (for recent reviews, see [8–10]). They are classified into six types on the basis of similarities in sequence and gene structure. IFs expressed by mammalian neurons consist of α-internexin, the NF triplet proteins designated as NFL, NFM and NFH for, respectively, low-, medium- and high-molecular-weight subunits (type IV), peripherin, synemin (type IV) and nestin (type VI). Originally, it was assumed that NF were composed only by NFL, NFM and NFH, but recent studies indicated that other proteins such as α-internexin and peripherin are also co-assembled with NF [11, 12]. This review is focused on NF triplet proteins hereafter referred to as NF subunits or NF proteins, but it is important to keep in mind that other IF proteins also participate to the formation of neuronal intermediate filaments.

NFs represent the main cytoskeletal elements in mature neurons. They account for 13% of total proteins and 54% of the Triton-insoluble proteins [13]. The molecular weights of NFL, NFM and NFH predicted from the DNA sequences are respectively 61.5, 102.5 and 112.5 kDa in human. However, due to their high content of negatively charged amino acids (glutamic acids) in their sequences and their extensive post-translational modifications (phosphorylation and glycosylation), they display higher apparent molecular weights on sodium dodecyl sulphate polyacrylamide gel electrophoresis (68, 160 and 205 kDa, respectively, for NFL, NFM and NFH). NFM and NFH alone or combinations of NFM and NFH fail to form filaments in the absence of NFL. NFL alone is able to form homopolymers in vitro and in cells transfected with NFL but not in rodents [14–20]. As opposed to mouse or rat NFL, human NFL has been reported to form homopolymers in vivo [19]. In rodent, NF are obligate heteropolymers composed of the NFL, NFM and NFH subunits [21] with a subunit stoichiometry of 4:2:1 [22]. This ratio varies during neuronal development. Moreover, others IF proteins such as peripherin in the peripheral nervous system (PNS) and α-internexin in the central nervous system (CNS) can co-assemble with NFL, NFM and NFH to form NF [11, 12]. Today, it must be admitted that these proteins are integral components of NF, as well as NFL, NFM and NFH. Yuan et al. [12] demonstrated that α-internexin can be considered as a fourth subunit of NF in adult CNS. They co-purified α-internexin with the NF triplet proteins from Triton-insoluble fraction in amounts comparable with those of the others subunits. Moreover, α-internexin with all three NF subunits co-assemble into single filamentous network in SW13vim (−) cells and is co-localised with NFM on the same NF in optic nerve in mice. They calculated a subunit stoichiometry of 4:2:2:1 (NFL–α-internexin–NFM–NFH) in optic

nerve and spinal cord. Therefore, NF must be considered as the IFs of neurons, which can be comprised of up to four or more different proteins depending on neuronal type and developmental stage.

As all IF proteins, NF triplet proteins share a common tripartite structure, with non-helical amino and carboxy-terminal regions (called the head and tail domains) flanking a ~46-nm-long central α-helical rod domain composed of approximately 310 amino acids [23] (Fig. 1). This rod domain contains highly conserved motifs. Every seventh residue of its more central portion is hydrophobic, providing a hydrophobic seal on the helical surface necessary for the formation of coiled coil structures. Head and tail domains of the NF subunits are less conserved. The head domain is short and rich for Ser and Thr. The tail domain is the distinctive feature of NF proteins. For NFL, this region is short and contains many glutamic acid residues (E segment) while tail domains of NFM and NFH are longer and also contain E segments. Moreover, the carboxy-terminal domains of NFM and NFH contain numerous repeats of phosphorylation sites Lys–Ser–Pro (KSP, up to 51 repeats are present in mouse NFH; Fig. 1). The Ser residue of these repeats is highly phosphorylated in vivo [24, 25].

Assembly of Neurofilaments

As soon as the different NF subunits are expressed, they co-assemble to form an IF. This assembly does not require

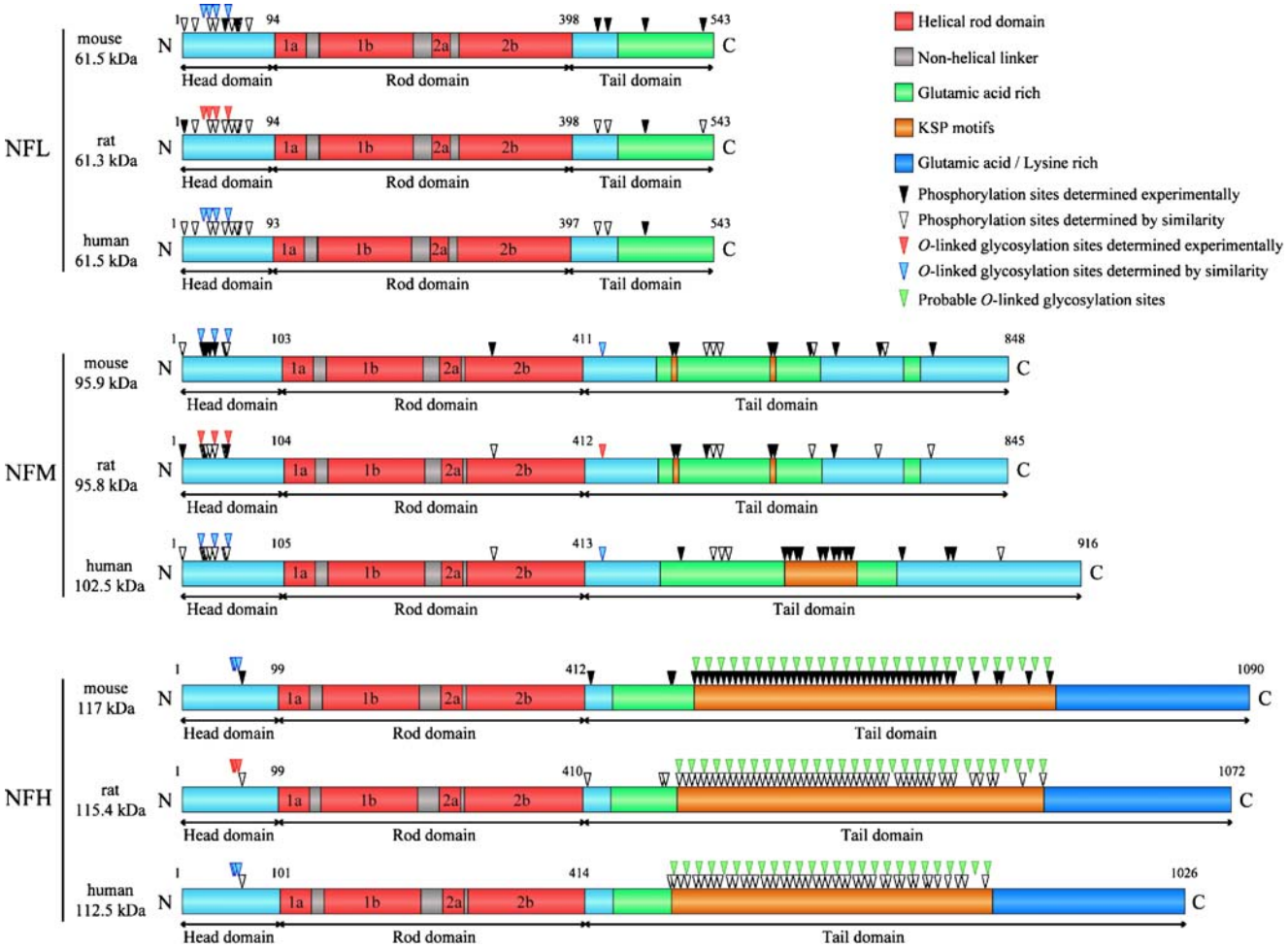

Fig. 1 Schematic representation of mouse, rat and human NF subunits**.** The molecular weights predicted from the DNA sequences are shown. The three subunits share a highly conserved central α-helical domain of approximately 310 amino acids that is flanked by non-α-helical amino and carboxy-terminal end domains called, respectively, heads and tails. The tail domains are of variable size and contain many glutamic acid residues (*green*) and lysine for NFH (*deep blue*). The tail domains of NFM and NFH also contain multiple KSP repeats (*orange*) which are heavily phosphorylated (*arrowheads*). This graph is made according to the sequences and phosphorylation sites (determined experimentally or by similarity) provided by UniProtKB/Swiss-Prot database (mouse NFL: P08551, rat NFL: P19527, human NFL: P07196, mouse NFM: P08553, rat NFM: P12839, human NFM: P07197, mouse NFH: P19246, rat NFH: P16884, human NFH: P12036)

nucleotide binding or hydrolysis but is strongly dependent on ionic strength, pH and temperature [26]. The first step of NF formation is the dimerisation of NFL with either NFM or NFH via the association of their conserved rod domains to form parallel side-to-side coiled coil dimers. Two coiled coil dimers line up in a half-staggered manner, forming an anti-parallel tetramer [27]. Tetramers combine to form protofilaments, which finally assemble to constitute the final 10-nm filament.

It has long been considered that NFL subunits constitute the core of the NF whereas NFM and NFH are arranged around this core. However, immunoelectron microscopy using antibodies directed against head, rod or tail domains of individual NF subunits showed that all three NF proteins are incorporated integrally into filaments [28, 29]. Tail domains of NFM and NFH form lateral projections extending from the filament backbone [17, 30]. These sidearms participate to the stabilisation of the filament network by forming cross-bridges between NF and other cytoskeletal elements or organelles.

Head and rod domains of NFL and NFM, and especially their post-translational modifications, are essential for the NF assembly [31–35]. Phosphorylation of Ser-51 and Ser-55 on NFL [36] and phosphorylation of Ser-23 on NFM by protein kinase A (PKA) and protein kinase C (PKC) [37] were shown to regulate in vivo NF assembly. Indeed, the phosphorylation of NFL head domain prevents their assembly or cause disassembly when incorporated into filaments [38]. It is also interesting to note that the phosphate present on Ser-55 of NFL is turned over rapidly following NFL synthesis in neurons [36], suggesting a possible role in the blockade of NF assembly before their transport into neurites. The generation of a transgenic mouse with a mutant NFL transgene in which Ser-55 was mutated to Asp to mimic permanent phosphorylation resulted in pathological accumulation of NF in brain neuronal cell bodies [39]. Finally, polymerisation of NFL protein in vitro was inhibited by phosphorylation of NFL head domain by protein kinase N (PKN) [40]. Together, these results indicate that the transient phosphorylation of head domains in perikarya prevents the polymerisation of NF subunits [35]. NFM and NFH head domains are also modified by O-linked *N*-acetyl glucosamine (O-GlcNAc) on sites close to phosphorylation sites [41]. Similar sites are present in the NFH head and tail domains [42]. This proximity could suggest that these post-translational modifications may influence each other and play a yet unknown role in filament assembly.

Finally, it was shown that NUDEL, a mammalian homologue of the *Aspergillus nidulans* nuclear distribution molecule NudE, is involved in NF assembly [43]. This protein associates directly with soluble pool of NFL and indirectly with NFH subunit. By interacting with NFL, NUDEL promotes the incorporation of NF subunits in the network during NF assembly but does not assemble with NF proteins. Moreover, genetic knockdown of NUDEL disrupts NF stoichiometry which, in turn, results in impaired NF assembly and transport [43].

In vitro interactions occurring between NF allow the formation of a viscoelastic network resistant to important deformation, suggesting the importance of cross-bridges for NF mechanical properties [44, 45]. Kreplak et al. [46] used atomic force microscopy to test the mechanical properties of single NF. They showed that NF can be stretched more than threefold, with an average of 2.6-fold, suggesting that NF may indeed function as mechanical shock absorbers in vivo.

Expression of Neurofilaments

In human, genes coding for NFL and NFM (*NEFL* and *NEFM* genes) are very closely linked on chromosome 8 (8p21) [47, 48] while NFH gene (*NEFH* gene) is located on chromosome 22 (22q12.2) [49]. In mice, *NEFL* and *NEFM* genes are located on chromosome 14 (14 D3 for *NEFL* and 14 D1 for *NEFM*) while *NEFH* gene is located on chromosome 11 (11 A1–A5). The expression of the three NF subunits is finely regulated during nervous system development and coincides with neuron differentiation [7]. This expression is controlled at the transcriptional level but also through post-transcriptional regulation of mRNA localisation, stability and translational efficiency (for recent review, see [50]). When axons reached their targets, NFL is the first subunit to be expressed, together with α-internexin and peripherin, and then it is rapidly followed by NFM expression [51, 52]. At this early stage, the axoskeleton is composed mainly by MTs while NFL and NFM are expressed only at low levels. This unique composition of the cytoskeleton is also found during axonal regeneration in the PNS, and thus it has been suggested that it may improve growth [52–57]. The appearance of NFH occurs after synaptogenesis and is accompanied by robust up-regulation of NFL and NFM expression [52]. At this advanced stage, NF of mature composition may further enhance axon stability and calibre. Interestingly, the increased NF density is accompanied by a decreased MT density [58], suggesting a fine balance between these two networks. In cultured neuroblastoma cells, the level of mRNAs coding for NF is proportional to their protein levels, and NFL and NFM mRNAs appear several days before the expression of NFH mRNA. The sequential appearance of NF proteins follows that of their mRNAs, with an early expression of NFL and NFM and a later expression of NFH [59]. Apart from Giasson and Mushynski [60], several studies observed that the expression levels of NFL and NFM are mutually

regulated and independent from NFH [61, 62]. This could be related to their genomic localisation, but the molecular mechanisms are still unknown.

Axotomy is followed by a strong down-regulation of NF mRNAs and proteins in PNS [63–65] and CNS [66–69], leading to reduced levels of axonally transported NF in injured neurons [65, 70, 71]. Then, during regeneration of injured axons in PNS, the expression of NF subunits is strongly up-regulated [71–74]. This reversion does not occur when regeneration is prevented [75, 76] as well as in mammalian CNS axons which normally do not regenerate [66, 67]. However, in transected axons of the spinal cord from lamprey, where NFM expression is initially suppressed, Jacobs et al. [77] observed an up-regulation of this subunit only in axons that successfully regenerate, while NFM levels remain low in those that do not regenerate. Similarly, increased amounts of NFM mRNA and protein are observed during successful optic nerve regeneration in *Xenopus laevis* [78].

Post-translational Modifications of Neurofilaments

Phosphorylation of Neurofilaments

The phosphorylation is the best-documented post-translational modification of NF proteins. Multiple aspects of NF's biology, including their assembly and their axonal transport, are regulated by their phosphorylation status. Moreover, aberrant NF phosphorylation is a pathological hallmark of many human neurodegenerative disorders (for detailed reviews, see [79, 80]).

NF proteins are the most extensively phosphorylated proteins in neurons with up to 51 sites of phosphorylation located on the C-terminal domain of NFH [24, 81–85] (Fig. 1). This phosphorylation is topographically regulated, with a proximo-distal gradient consisting of an intense phosphorylation in axons and little or no phosphorylation in cell bodies and dendrites [7, 86–89]. Phosphorylation sites are located on the amino-terminal and carboxy-terminal domains of the three NF subunits. These sites are the targets of, respectively, second messenger-dependent kinases [90–92] and second messenger-independent kinases [25, 91, 92]. Phosphorylation of head domain arises mainly in cell body soon after the synthesis of NF proteins while the phosphorylation of tails domains coincides with their entry into the axon [93–95].

As mentioned above, phosphorylation of NFL and NFM head domains by PKA, PKC and PKN prevents the assembly of NF or leads to their disassembly [36–38, 40]. In vitro studies also revealed Rho-associated kinases and calcium–calmodulin-dependent protein kinase II phosphorylation sites in the NFL head domain [96, 97].

NFM and NFH tail domains are the most extensively phosphorylated regions. Most of these phosphorylation sites are KSP repeats, although other Ser–Thr-containing motifs are also phosphorylated. Many roles have been attributed to the phosphorylation of KSP motifs, including the formation of cross-bridges between NF or with MTs, the expansion of the axonal calibre, the slowing of the NF axonal transport and the integration of NF in a stationary pool [18, 47, 87, 98–103]. Phosphorylation of KSP sites depends on two families of Pro-directed kinases: the cyclin-kinase Cdk5 and the microtubule-associated protein (MAP) kinases. Signal transduction cascades leading to the activation of these kinases could be triggered by growth factors [104, 105], Ca^{2+} influx [106], integrins [107] and myelination [89]. The link between the myelination and the phosphorylation of NFM and NFH sidearms was first suggested by the decreased phosphorylation of NF in the dysmyelinated mouse mutant *Trembler* [108] and by their poor phosphorylation in non-myelinated axonal domains like the initial segment and nodes of Ranvier [108–111]. The binding of myelin-associated glycoprotein (MAG) to axonal receptors was proposed to activate a signalisation cascade leading to the phosphorylation of NF in myelinated regions [112, 113], but the molecular cascade of this process as well as its regulation are still unclear.

Cdk5 preferentially phosphorylates KSPXK motifs of NFH in vitro and in vivo [114–121], preventing the binding of dephosphorylated NFH to MTs [122]. But the majority of KSP repeats in rat–mouse NF tail domains are phosphorylated by MAP kinases, including extracellular signal-regulated kinases 1 and 2 which phosphorylates KSPXXK and KSPXXXK motifs of NFH [120], stress-activated protein kinase which is responsible for NFH tail domain phosphorylation on KSPXE motifs in cell body under stress-activated conditions [60, 123], glycogen synthetase kinase 3 (GSK3) which phosphorylates some of the KSP sites in bovine NFM [124] and few sites on NFH [117], p38 kinase [125, 126] and c-Jun N-terminus kinase 1 and 3 (JNK1/3) [123, 127, 128].

Ser–Thr residues in the glutamic acid region of the three subunits tail domains are phosphorylated by casein kinase I (CKI) [91, 129–133] while casein kinase II (CKII) also phosphorylates Ser-473 on the short-tail domain of NFL [134, 135].

Phosphorylation of head and tail domains are intimately related. Zheng et al. [136] showed that phosphorylation of NFM head domain by PKA reduces the phosphorylation of tail domain by MAP kinases in vitro and in vivo. Moreover, mutation of Ser-1, 23 and 46 residues to Ala in the head domain of NFM prevents PKA phosphorylation in transfected NIH3T3 cells and fails to inhibit tail domain phosphorylation by MAP kinases [136]. These results

highlight a regulatory mechanism by which phosphorylation of NF head domains could prevent NF assembly and C-terminal phosphorylation in cell body, protecting the neuron from abnormal accumulation of phosphorylated NF in perikarya.

The phosphorylation state of NF proteins in the different neuronal compartments depends on a dynamic balance between the activities of kinases and phosphatases. Since NF head domain phosphorylation inhibits NF assembly [36–38], their dephosphorylation is necessary to allow the polymerisation of NF proteins prior to their transport into the axon. The extensive enzymatic dephosphorylation of NF induces a progressive loss of their capacity to interconnect in vitro into a reticulated network, measured by the formation of highly viscous gels in purified preparations of NF [137]. Finally, dephosphorylation of NF tail domains facilitates their degradation at the terminals [138] and regulates their interaction with other cytoskeletal proteins. Dephosphorylation of head and tail domains of NF subunits is mainly (60%) catalysed by phosphatase 2A [139–142]. phosphatase 1 also contributes to the dephosphorylation of NF but to a lower extent (10–20%) [142].

Glycosylation and Glycation of Neurofilaments

NFs are also post-translationally modified by attachment of O-GlcNAc to individual Ser and Thr residues. O-GlcNAc is a common modification of cytosolic and nuclear proteins that regulates protein stability, subcellular localisation and protein–protein interactions [143]. Like phosphorylation, O-glycosylation is dynamic and often reciprocal to phosphorylation at the same sites or adjacent to them (Fig. 1). Dong et al. [41, 42] identified several O-GlcNAc sites on NFL head domain (Thr-21, Ser-27, Ser-34 and Ser-48) and NFM head (Thr-19, Ser-34 and Thr-48) and tail domains (Thr-431). NFH is also extensively modified by O-GlcNAc in the head domain (Thr-53, Ser-54 and Ser-56) and at multiple sites within the KSP repeat motifs in the tail domain, although the exact sites remain to be identified. In purified NF proteins, the O-GlcNAc modifications occur at a stoichiometry of approximately 0.1, 0.15 and 0.3 mol of GlcNAc per mole of, respectively, NFL, NFM and NFH [41, 42]. The function of these modifications is still elusive, but several clues suggest a role in the NF assembly. For example, all O-glycosylation sites within head domains are located in regions essential for in vivo NF assembly, close to the phosphorylation sites involved in this process. O-glycosylation of NF head domains could reciprocally modulate its phosphorylation and consequently the assembly and dynamics of NF. Further investigations are necessary to elucidate the precise mechanism regulating NF O-glycosylation, the relation between NF phosphorylation and O-glycosylation and the distribution of O-glycosylated NF. Antibodies that specifically recognise O-glycosylated epitopes in NF subunits could be an important tool to elucidate these questions. To this end, Lüdemann et al. [144] generated a monoclonal antibody specifically directed against an O-glycosylated epitope in the tail domain of NFM. They showed that O-glycosylated NFM is enriched in the axons of human neurons in situ, together with hyperphosphorylated NF, indicating a synchronous phosphorylation and O-glycosylation of the tail domain of NFM within the axon. However, the O-glycosylation of NFM and the activity of MAP kinases are reversibly regulated, suggesting reciprocal regulation between phosphorylation of the KSP region and O-glycosylation.

The first evidence of NF glycation, also called non-enzymatic glycosylation, was reported in peripheral nerves in diabetes mellitus [145]. A possible role for this modification in familial and sporadic ALS was also suggested [146].

Nitration, Oxidation and Ubiquitination of Neurofilaments

In addition to phosphorylation and O-glycosylation, NF undergo nitration, oxidation and ubiquitination. Nitration of NFL subunit was reported in the normal rat brain using a proteomic analysis [147]. NF nitration was also detected in NF-rich inclusions in motoneurons of sporadic ALS cases [148]. This modification is catalysed by superoxide dismutase 1 (SOD1) in vitro on four Tyr residues of NFL, one in the head domain (Tyr-17) and three in the rod domain (Tyr-138, Tyr-177 and Tyr-265) [149]. It is interesting to note that Tyr-17 is essential for the polymerisation of NF while the other three Tyr residues are located within the coiled coil structure of the rod domain and are likely involved in intermolecular hydrophobic interactions. The nitration change normally hydrophobic residues into negatively charged hydrophilic residues, thereby disrupting the assembly and stability of NF. Consequently, it was proposed that SOD1-catalysed nitration of NF may contribute to motoneurons dysfunction in ALS [149, 150].

The oxidation of NF arises during ageing and Wallerian degeneration and was found in neurodegenerative disorders. Oxidised NFs are more susceptible to calpain proteolysis and form dense aggregates and bundles of laterally aggregated filaments [151]. The incubation of disassembled NFL with SOD1 and H_2O_2 causes the formation of dityrosine crosslink and the aggregation of NFL protein proportionally to the concentration of hydrogen peroxide [152]. Antioxidant molecules inhibit these effects. Finally, ubiquitination of NF proteins has been suggested by Gou and Leterrier [153] as a possible mechanism for NF degradation.

Degradation of Neurofilaments

After their assembly in the perikaryon, NFs are slowly transported by the slow axonal transport toward the nerve terminal [154] where they are degraded. A degradation of NF over the entire length of axons in mouse sciatic nerve has also been reported during Wallerian degeneration in transected fragments of nerve [155] and in a context of axonal NF deficiency [156]. Nixon and Logvinenko [103] suggested that such a degradation of NF proteins in axons could account for non-homogeneous distribution of NF in axons.

Calcium-activated proteases such as calpain are found in human tissues and degrade NF from squid, rat, bovine and worm [157–161]. NF proteins that are usually absent in synaptic terminals accumulate following leupeptin treatment, a protease inhibitor. This indicates that their normal absence in synapses is due to degradation by calcium-activated proteases [162]. The initial evidence that calcium plays a role in the degradation of NF came from observations showing disintegration of NF in rat peripheral nerve fibres after exposure to calcium [163]. Purification of calcium-activated neutral proteases in rat peripheral nerve or spinal cord resulted in the identification of calpain I (or μ-calpain) and calpain II (or m-calpain), as defined respectively by the micromolar or millimolar levels of calcium required for their activation [164, 165]. Calpain II is present in glial cells while calpain I is predominantly expressed in neurons [166]. At endogenous sub-micromolar calcium concentrations, Nixon et al. [167] demonstrated a limited proteolysis of NFM as a post-translational modification during the axonal transport.

Most calcium-activated neutral proteases show a high degree of substrate specificity with IFs [168]. Unlike many proteases, calpain specificity appears to be determined by conformational factors and primary amino acid sequences. It usually catalyses limited cleavage of its substrates. Participation of calpain and calcium-activated neutral proteases in NF turnover is plausible as reflected by the occurrence in normal brain of characteristic NF protease-resistant fragments throughout the neuraxis. These fragments could be retrogradely transported and could regulate the synthesis, assembly and delivery of NF in accordance with their turnover level at remote sites [158, 162]. Inhibition of calpain proteolytic activity in transected axon abolishes growth cone formation suggesting a central role in the reorganisation of the axonal cytoskeleton during its transition from a stable differentiated state into a dynamically extending structure [169].

NF are also degraded by non-specific proteases like lysosomal cathepsin D, trypsin and α-chymotrypsin. Such trypsin proteolytic strategies were used to analyse the spatial architecture of NF [170, 171]. Cathepsin D plays an important role in NF metabolism. The content of cathepsin D is probably more than 1,000-fold greater than that of a calcium-activated neutral proteases [172]. Purified brain cathepsin D was shown to degrade NF proteins from rat, mouse, bovine and human tissues, and some characteristic fragments produced by this hydrolysis were shown to be normally present in brain [173–175].

In addition to their participation in the turnover process, proteases produce NF-derived peptides that could be active. The hypothesis of a possible regulation of gene expression by NF proteins led Traub et al. [176] to show that subunit proteins of NF bind to RNA and single-stranded DNA. The DNA-binding sites are located in the amino-terminal domain [177] and are preserved during the digestion of NF by calcium-activated proteases. However, the capacity of NF to affect the DNA or RNA fragments to which they bind is unknown.

Post-translational modifications can regulate proteolysis of NF, as illustrated for phosphorylation which protect NF from proteolysis by calpains [138, 178]. Aluminium, a neurotoxin which causes NF protein phosphorylation and accumulation in neuronal perikarya [179, 180], inhibits calpain-mediated proteolysis of NF [181]. These results raise the possibility that kinase or phosphatase activity might determine the rate of turnover of NF proteins. Gou and Leterrier [153] also indicated that ubiquitination facilitates the proteolysis of NF.

Calcium-activated neutral proteases play important roles in tissue injury. Many pathological states induce an increase of free calcium within the axon leading to a massive proteolysis of NF [182, 183]. The degradation of NF in transected rat sciatic nerve is reduced if the influx of calcium into the axoplasm is prevented or if calcium-activated neutral proteases activities are inhibited [155]. Such enzymatic fragmentation in transected nerves generates protease-resistant NF fragments which may represent, after externalisation into the endoneurium, a mechanism responsible for the generation of auto-antibodies to NF proteins detected in Parkinson's disease and in several neurological diseases [184–187].

Abnormal aggregations of NF are a hallmark of several human neuropathological situations. In ALS, they accumulate in cell bodies or in the proximal part of axons from motor neurons [188, 189]. They accumulate in Lewy bodies of PD [190], in neurofibrillary tangles (NFTs) of AD [191] and, following intoxication by aluminium, hexanedione, acrylamide or β, β'-iminodipropionitrile (IDPN) [192–195]. In all these pathological situations, the cellular and molecular mechanisms used to eliminate the neurofilamentous aggregates are still unknown. It has been shown that trypsin-like proteases are expressed in neurons [196–198], and they accumulate within pathological neurofilamentous aggregates [199]. Tsuji et al. [200] showed increased levels

of calpains in the cytosolic fraction of AD brains when compared to control brains. Fasani et al. [201] showed that NF isolated from NFH-LacZ transgenic mice (in which NF are sequestered in cell bodies [202]) are more sensitive to exogenous trypsin and α-chymotrypsin than normal NF. Moreover, an increased trypsin immuno-labelling is detected in perikarya from such mice compared to wild-type animals. These results suggest that when NFs are sequestered in the cell body, their amount is controlled by an increased susceptibility to trypsin-like proteolysis and an increased production of proteases [201].

As mentioned above, it has long been considered that NF degradation only occurs at the axon terminals. However, a degradation of NF over the entire length of axons in mouse sciatic nerve was recently highlighted by Millecamps et al. [156]. They generated transgenic mice with doxycycline-regulated expression of human NFL (hNFL) with or without endogenous mouse NFL proteins (respectively, tTA;hNFL;$NFL^{+/-}$ and tTA;hNFL;$NFL^{-/-}$ mice). The doxycycline administration in drinking water of tTA;hNFL;$NFL^{-/-}$ mice silences the expression of hNFL and the pre-existing protein subsequently disappears in their sciatic nerve, with an estimated half-life of 3 weeks. This loss is synchronised over the entire length of the sciatic nerve, suggesting a homogeneous and not a local degradation of hNFL. In contrast, no detectable loss of hNFL protein was observed in presence of stationary NF network in sciatic nerve from tTA;hNFL;$NFL^{+/-}$ mice, even after 4.5 months of doxycycline treatment. After 8 months of treatment, 35% of hNFL protein was still remaining [156]. These results show that a pre-existing NF network is a key determinant of half-lives of NF proteins by reducing their turnover rate and support the view that NF proteins can spend several months, if not years, in long NF-rich peripheral axons. This long life makes them potential targets for oxidation or other harmful modifications, which in turn may cause NF disorganisation.

Interaction of Neurofilaments with Proteins and Organelles

As parts of a complex and dynamic network, NF interact with several partners and these interactions are principally mediated through NF-associated proteins that can modulate function (enzyme) and structure (linker protein) of NF (Table 1). Linker proteins are responsible for the interaction between the different filaments or organelles, whereas enzymes (principally kinases and phosphatases) modulate NF architecture, assembly and spacing.

In neurons, NF and MTs are two major components of the cytoskeleton. Dynamic interactions between these elements are crucial for the axoskeleton and are regulated mostly by phosphorylation. This was documented by several biochemical studies showing that tubulin and/or MTs are able to interact directly with NF both in vitro and in vivo [203–209]. This direct interaction was also illustrated by quick freeze deep etch electron microscopy [30, 210]. Dephosphorylation of NF by alkaline phosphatase promotes the NF–MT interaction mediated by carboxy-terminal domain of NFH [18] suggesting spatio-temporal regulation of NF–MT interaction by kinases. Tau protein kinase II specific phosphorylation of NFH tail domain has been described to dissociate NF and MTs [122]. NF–MT interaction was also shown to be mediated by MAPs such as MAP2 [30].

Stable tubule only polypeptide (STOP) proteins (named also MTAP6 or MAP6) are a family of cytoskeleton associated proteins responsible for the MT cold stability. These proteins were initially isolated from rat brain cold-stable MTs [211, 212] and were shown to induce MT cold stability in vitro when added to labile MTs [213, 214] or when expressed in cells normally devoid of stable MTs [215]. They are abundant in neurons, which contain a large amount of stable MTs [216, 217], and associate preferentially to cold- and drug-stable polymers [218]. STOP proteins were shown to be associated with NF by both biochemical co-purification of NF and their co-precipitation with NF in axonal spheroids of ALS [219]. In NFH-LacZ transgenic mice, STOP proteins were also found to co-accumulate with NF in the perikaryon. While further investigations are necessary to understand the mechanism of STOP–NF interactions and their functional relevance, these data indicate that STOP proteins could be considered as a cytoskeletal integrator and a marker of ALS spheroids [219].

Several studies indicate a direct interaction between motor protein dynein and kinesin with NF [220–225], mediated principally by the phosphorylated carboxy-terminal domains of NFM and NFH [222, 223, 225]. These motor proteins could contribute to the transport of NF along axons and dendrites (for review, see [226]). For example, co-immunoprecipitation experiments with anti-dynein antibody induced selectively co-precipitation of phosphorylated NF, while anti-kinesin selectively co-precipitated hypo-phosphorylated NF [225]. Recently, atomic force microscopy allowed a direct evaluation of the interaction between NF and cytoplasmic dynein [223]. Yeast two-hybrid and affinity chromatography assays also identified a direct binding between dynein intermediate chain and NFM [223], possibly involved in the saltatory bi-directional axonal transport of NF in the neuron.

Associated proteins mediate interaction of NF with vesicles. Biochemical preparations such as synapsin I immunoprecipitation [227] or nearest neighbour analysis for brain synapsin I [228] have shown that this protein can link non-secretory vesicles directly to NFL subunit.

Table 1 Summary of proteins interacting with neurofilaments

Partners	Known or possible functions	References
Fodrin (brain spectrin)	Interaction cytoskeleton-organelle	[455, 456]
G-actin	Maintenance of the neuronal structure	[457]
H1 histone	DNA synthesis regulation	[237]
Hamartin	NF-actin interaction	[458]
MAP2	Cross-bridging between NF and MT	[30, 210]
NUDEL	NF assembly	[43]
STOP	NF–tubulin interaction	[219]
Synapsin I	Interaction NF-non-secretory vesicle	[227, 228]
Tubulin	Cytoskeletal integrity	[18, 122]
Kinases–phosphatases		
CaMKII	Phosphorylation of N-terminal domain of NFL	[97]
Casein kinase I and II	Phosphorylation of C-terminal domain of NFL and NFM	[91, 129–135]
Cdk5	Phosphorylation of KSP motifs of NFM and NFH (preferentially human NFH)	[114–121]
Erk1/2	Phosphorylation of KSP motifs of NFM and NFH	[120]
GSK3	Phosphorylation of KSP sites in NFM and NFH	[117, 124]
PKA, PKC, PKN	Phosphorylation of N-terminal domain of NFL, NFM and NFH	[18, 36, 37, 40, 80]
SAPK	Phosphorylation of C-terminal domain of NFH under stress-activated conditions	[123, 125, 127]
PP1 and PP2A	Dephosphorylation of NFs	[142]
Molecular motors		
Dynein	NFs axonal transport	[222, 223]
Kinesin	NFs axonal transport	[220, 221, 317, 319]
Myosin	Myosin Va NFs axonal transport	[459]
Receptor		
D(1) dopamine receptor	Regulation of cell surface expression and desensitization	[460]
NMDA receptor subunit NR1	Anchoring or localization	[355]
Other		
DNA–RNA	DNA synthesis regulation	[176, 177]

Bullous pemphigoid antigen 1 neural isoform (BPAG1n, 280 kDa) has originally been described as a major cytoskeletal integrator connecting actin filaments to NF [229]. In BPAG1$^{-/-}$ mice, electron microscope analysis along sensory axons revealed regional swellings filled with lysosomal vesicles and disorganised arrays of NF [229, 230]. Co-transfection of BPAG1n with NFL and NFH in SW13 cells show that BPAG1n is able to link NF and actin networks [229]. Natural mutations of BPAG1 [231] cause the well-known mouse model of neurological disorder dystonia muscularum (dt/dt) characterised by a disorganised cytoskeleton in the sensory nervous system. It was thus suggested that a loss of BPAG1 interactions with IFs is important to the pathogenesis in dt/dt mice. However, Eyer et al. [232] showed that pathogenesis of axonopathy in dt/dt mice was independent of axonal NF as demonstrated by matting dt/dt mice and NFH-LacZ transgenic mice in which NF aggregated in the cell body of neurons [202]. Similar results were obtained by matting dt/dt mice with knockout NFL mice [233], confirming that the presence or absence of NF in the axon does not affect the appearance of the dt/dt phenotype. Moreover, the ability of the neuronal splice isoform of BPAG1 to connect actin filaments to NF is now called into question by works of Leung et al. [234]. Indeed, they found that the only neuronal isoform of BPAG1 (named BPAG1a) lacks an IF binding domain. However, BPAG1a has an actin binding domain and a microtubule actin domain, suggesting that this protein could play a role in maintaining the structural organisation of the neuronal cytoskeleton [235].

Electron microscopy investigation in Dieters' neurons from lateral vestibular nucleus of rabbit brain allowed to visualise MF-mediated interaction of NF with the plasma membrane and the nuclear pores [236]. These authors suggested that NF may play a linking role between plasma membrane and nucleus. As a matter of fact, during membrane depolarisation, early transduction signal mediator such as Ca^{2+} or cyclic adenosine monophosphate could mediate NF rearrangement and contribute to a modification of the DNA transcription at the site of the nuclear pore complex [236]. Moreover, it has been shown that NF were able to bind DNA, RNA and histone H1 nuclear protein, suggesting a possible role of NF in the regulation of transcription processes [176, 177, 237].

Finally, using electron microscopy on transected squid giant axons, it has been shown that NF formed dynamic complex with smooth endoplasmic reticulum. Modification and rearrangement of such complex could be involved in some neurodegenerative diseases [238]. Cross-bridges between mitochondria and NF were also emphasised by ultrastructural studies [210, 239]. Since mitochondria do not move along NF in neuronal processes deprived of MTs and MFs [240], it was postulated that NF serve as a docking site for these organelles and regulate their spatial distribution along axons. The overexpression of NFH in culture cells induces the selective perikaryal retention of mitochondria [241, 242], suggesting that regulated NF–mitochondria binding is required for normal translocation of mitochondria in axons. Wagner et al. [243] provided evidences that NFM and NFH sidearms mediate this interaction between NF and mitochondria and proposed that porin molecules or other cytoskeleton-binding proteins of the mitochondrial outer membrane mediate this interaction. They also revealed that the binding of NF to mitochondria depends on the potential of the mitochondrial membranes, suggesting that the conformation or the organisation of the partner of NF on the mitochondrial outer membrane is modified by the membrane potential of the organelle. Finally, it seems that phosphorylation of NF may regulate their binding affinity for mitochondria in a potential-dependent manner [243].

Neurofilaments Contribute to and Modulate the Axonal Calibre

NFs play an essential role in growth and maintenance of axonal calibre. This was first suggested when Friede and Samorajski [244] observed that increased NF numbers and densities are correlated with increased axonal calibres. Moreover, the axonal radial growth coincides with the entry of NF into axons during axonal development or regeneration [245, 246]. The recent use of various animal models clearly showed the importance of NF in the control of the axonal diameter. These models include mice knockout for NF genes (Table 2), mice expressing human (Table 3), mouse (Table 4) and modified NF subunits (Table 5). But the first evidence of the implication of NF in axonal radial growth in an animal model was obtained in Japanese quails. Indeed, an important axonal atrophy was observed in *quiver* quails characterised by the absence of NF in their axons caused by non-sense mutation in *NEFL* gene [247–249]. The first evidence in mice was provided by the production of transgenic mice overexpressing human NFH [250] (Table 3). These mice develop perikaryal accumulations of NF, resulting in a deficiency of axonal NF and in an axonal atrophy. This was further confirmed by the generation of NFH-LacZ transgenic mice in which expression of a NFH-β-galactosidase fusion protein provokes the perikaryal aggregation of NF, leading to a 50% reduction of axonal calibres [202, 251] (Fig. 2; Table 5). Finally, the targeted disruption of *NEFL* gene in mice caused the lack of axonal NF and strongly reduced diameter of myelinated axons [61] (Table 2). Multiple studies analysed the importance of the number and stoichiometric proportion of each NF subunit in the axonal calibre determination. Transgenic mice overexpressing murine NFL have a two to threefold increase in the number of NF but the diameter of their axons is only slightly modified [252, 253] (Table 4), suggesting that the number of NF by itself is not the main determinant of axonal diameter. Yet, triple heterozygous knockout mice (NFL+/−; NFM+/−; NFH+/−) in which integrity of NF network and normal subunit stoichiometry are preserved exhibit a 40% decrease of NF content and a 50% decrease of axonal diameter in L5 ventral root [254]. The individual increase in each of the three NF subunits inhibits radial axonal growth, and the simultaneous increase of NFM and NFH exacerbates this axonal atrophy [253]. In contrast, the co-overexpression of either NFL–NFM or NFL–NFH increases the axonal calibre [253, 255], suggesting that NFL in combination with either NFM or NFH is sufficient to promote radial growth. Altogether, these results indicate that both number of NF and a precise stoichiometry of their subunits are essential in the expansion of axonal diameter. To determine the specific contributions of NFM and NFH subunits in the axonal size, Elder et al. [62] made null mutant mice deficient for NFM. The axonal calibre was strongly reduced as well as NFL mRNA and protein levels. Modification of NFM expression was also reported in NFL−/− mice [61] suggesting that the levels of NFL and NFM are mutually regulated, reinforcing the view that the stoichiometry of each NF subunit is crucial for the establishment of a proper axonal calibre. Three different NFH-null mice were produced and revealed that this subunit contributes to a lesser extent than NFM to the determination of the axonal diameter [256–258]. However, subtle differences exist between these models. Indeed, Elder et al. [256] reported no modification in NFM protein level but a significant reduction of the axonal calibre, while Rao et al. [257] and Zhu et al. [258] observed an up-regulation of NFM (respectively, 100% and 20%) but only minor modification of the axonal diameter, with a slight decrease in the large-calibre axons. As suggested by Hirokawa and Takeda [259], this divergence could be explained in part by the chronological differences between the data ([257] and [258] 3 months; [256] 4 months), suggesting a later effect of NFH on the axonal radial growth. It is also important to note that Rao et al. [257] and Zhu et al. [258] reported a compensatory increase in MT density and NFM

Table 2 Mice knockout for NF genes

Mice	NF inclusions	Axonal calibre	Axonal cytoskeleton	Conduction velocity	Axonal transport	Others
NFL−/− [61, 292, 328, 461]	Perikaryal accumulation of NFM and NFH	Decreased of ~50% in L5 VR	Scarcity of IFs Increased MT density ~5% of normal level of NFM–NFH in SN 52% of normal level of NFM and 13% of normal level of NFH in ON	Decrease of 70%	Normal rate of axonal transport of NFM in ON	~20% loss of motor axons Delayed axonal regeneration Decreased g-ratio in L5 VR Mild sensorimotor dysfunction
NFM−/− C57BL/6 [20, 292, 330]	No	Decreased of ~50% in L4 VR at 3 months old	Decreased NF density Increased MT density 68%, 35% and 22% of normal level of NFL in SC, SN and ON, respectively 121% and 33% of normal level of NFH in SC and ON, respectively Increased phosphorylation of NFH Decreased level of α-internexin in brain, cerebellum, SC and ON	Decrease of 40%	Axonal transport of NFL and NFH is increased by twofold in sciatic nerve Axonal transport of NFL, NFH and α-internexin is increased by fivefold in ON	~10% loss of motor axons
NFM−/− 129 Sv/J [62, 256, 296]	No	Decrease in SC, decrease of 20% in L5 VR, 24% in SN and 20% in ON at 4 months old	Decreased NF density	Not determined	Not determined	~10% loss of motor axons
		Decreased of ~55% in L5 VR and ~10% in L5 DR at 2 years old	Increased MT density 13% and 23% of normal level of NFL in cortex and SC, respectively 140% of normal level of heavily phosphorylated NFH in cortex Normal level of β-tubulin			Decreased g-ratio in PNS and unchanged g-ratio in CNS
NFH−/− C57BL/6 [20, 257, 258, 292]	No	Minor change in L5 VR	Unchanged NF density ~Twofold increased of MT density Normal level of NFL 120–200% of normal level of NFM Increased phosphorylation of NFM Fourfold increase of βIII-tubulin	Decrease of 43%	Increased velocity of NF transport in SN	No axonal loss for [254] but 13% loss of motor axons and 19% loss of sensory axons for [253] Prevention of IDPN toxicity
NFH−/− 129 Sv/J [256]	No	Decreased of ~20% in L5 VR, SN, SC and ON	Slight decrease of NF density Unchanged MT density 75–90% of normal level of NFL Normal levels of NFM and tubulin	Not determined	Not determined	No overt phenotype

Table 2 (continued)

Mice	NF inclusions	Axonal calibre	Axonal cytoskeleton	Conduction velocity	Axonal transport	Others
NFM;NFH−/− [12, 20, 328, 462, 463]	Enhanced NFL staining in the perikaryon of motor neurons	Decrease of ~30–50% in L4 and L5 VR at 3–4 months old Decrease of ~60% in L5 VR and ~41% in L5 DR at 2 years old	Scarcity of IFs Twofold increase of MT density 23%, 11–17% and 12% of normal level of NFL in SC, SN and L5 VR, respectively, and barely detectable in ON <10% of normal level of α-internexin in ON Normal levels of peripherin, actin and tubulin	Not determined	Elevated velocity of a small pool of NFL proteins in SN Barely detectable NFL and α-internexin proteins in ON	24% loss of motor axons
NFL;NFH−/− [328]	No	Decrease	Small number of IFs in ON Small increase in MT number ~30%, 40% and 85% of normal level of NFM in brain, ON and SC, respectively Normal level of α-internexin	Not determined	NFM protein is transported at a normal rate along ON	NFM transport is abolished with deletion of both NFL and α-internexin
NFL,M,H+/− [254]	No	Decrease of ~50% in L5 VR at 7 months old	~60% of normal level of NFL, NFM and NFH in SC Normal levels of α-internexin, peripherin and β-tubulin	Not determined	Not determined	No loss of motor axons The reduced axonal calibre in NFL+/−;NFM+/−;NFH+/− mice expressing mutant SOD1G37R does not alleviate motor neuron disease caused by mutant SOD1

DR Dorsal root, *ON* optic nerve, *SC* spinal cord, *SN* sciatic nerve, *VR* ventral root

Table 3 Transgenic mice expressing human NF subunits

Mice	NF inclusions	Axonal caliber	Axonal cytoskeleton	Axonal transport	Others
Human NFL [255, 464–468]	Perikaryal accumulation of disarrayed filaments in thalamic neurons and in cortical neurons but not in motor neurons	Normal in L5 VR at 6 months old	Increased NF density in motor axons 115% of normal level of NFL in SC 110% of normal level of NFM in SC 97% of normal level of NFH in SC hNFL constitutes 80% of total NFL proteins Delayed developmental expression of hNFL Normal levels of actin and tubulin in SC	Not determined	Severe neuronal losses with age in the parietal cortex and ventrobasal thalamus
Human NFM (low–mid level) [469–472]	Neurofilamentous accumulations in the cerebral cortex at 12 months old No accumulation in SC	Normal	Unchanged NF density Not determined Increased NFL level in CNS Decreased phosphorylation of NFH	Not determined	No motor dysfunction
Human NFM (high-level) [473]	Neurofilamentous accumulations in ventral horn motor neurons	Normal in L5 VR at 2 months old	Increased NF density in motor axons Fourfold increase of total NFM in brain and twofold increase in SC Mouse NFM is less phosphorylated Peripherin is driven out of filaments	Not determined	Hind limb paralysis ~50% loss of motor axons at 2 months old
Human NFH [250, 255, 474]	Abnormal accumulations of NFs in the perikarya and proximal axons of spinal motor neurons	Dramatic axonal atrophy in distal region of the SN at 3 month old and in L5 VR at 6 months old	Reduced number of normal NF structures in motor axons 87% of normal level of NFL in SC 61% of normal level of NFM in SC 208% of normal level of NFH in SC Normal levels of actin and tubulin in SC	Slowing of NF, actin and tubulin transport in SN at 2 and 3 months old	Progressive motor dysfunction and weaknesses Reduction of body weight with aging Massive loss of motor axons at 2 years old
Human NFL–human NFH [466]	Reduced number and size of perikaryal accumulations in motor neurons compared to hNFH mice	Normal in L5 VR at 6 months old	Normal NF density in motor axons 130% of normal level of NFL in SC 64% of normal level of NFM in SC 251% of normal level of NFH in SC Normal levels of actin and tubulin in SC	Normal velocity of NF and tubulin transport in SN at 2 month old	No overt phenotype

Table 4 Transgenic mice overexpressing mouse NF subunits

Mice	NF inclusions	Axonal calibre	Axonal cytoskeleton	Axonal transport	Others
Mouse NFL (mid level) [252]	Accumulations of NFs in kidney	Normal in SN at 6 months old	2.2-fold increased NF density in SN at 6 months old Normal levels of NFM and NFH in SN	Not determined	No overt phenotype except the formation of cataracts
Mouse NFL (high-level) [253, 338]	Massive accumulations of NFs in perikarya, axons and dendrites from motor neurons and in some dorsal root ganglion neurons	Decreased	Twofold increased NF density in large myelinated axons in VR 115% and 110% of normal level of NFM and NFH in SN Decreased NF spacing	Not determined	Death before 3 weeks Proximal axon swelling Severe skeletal muscle atrophy
Mouse NFM [253, 264, 475]	Neurofilamentous accumulations in perikarya from various types of neurons	Decrease of ~50% in L5 VR and DR at 1.5 months old	Normal NF density in L5 VR at 1.5 months old Transgenic NFM accumulates up to ~130% and ~180%, the level of endogenous NFM at 1.5 months old in L5 VR and SN, respectively	Acceleration of NF transport in SN	aNo overt phenotype
	Aggregates in motor axons		Levels of endogenous NFM and NFH decreased by ~50% in SN at 1.5 and 9 months old ~90% and ~125% of normal l evel of NFL at 1.5 months old in L5 VR and SN, respectively	Normal velocity of tubulin transport in SN	No axonal loss
Mouse NFH [253, 265]	High level in NFH results in perikaryal and proximal axonal accumulations of NFs in both SC and dorsal root ganglion neurons	Modest increase of NFH leads to a slight increase of axonal calibre	Normal NF density in L5 VR Modest increase in NFH expression result in an elevation in both NFL and NFM levels in SN at 9 weeks old	Slowing of NF transport and acceleration of tubulin and actin transport in motor and sensory axons at 8 weeks old	No overt phenotype
		High level of NFH induces an important axonal atrophy in L5 VR at 4, 9 weeks and 1 year old	High level in NFH results in a decrease in both NFL and NFM levels in SN at 9 weeks old Normal level of βIII-tubulin Normal NF spacing		No axonal loss
Mouse NFL–mouse NFH [253]	Not reported	Increase of 46% in L5 VR at 8 weeks old	300%, 360% and 90% of normal level of NFL, NFM and NFH in SN Decreased NF spacing	Not determined	–
Mouse NFL–mouse NFH [253]	Not reported	Increase of 30% in L5 VR at 8 weeks old	320%, 90% and 300% of normal level of NFL, NFM and NFH in SN Increased NF spacing	Not determined	–
Mouse NFL–mouse NFH [253]	Accumulation of NFs in motor neuron cell bodies	Decrease of 55% in L5 VR at 8 weeks old	Decreased NF density 45%, 60% and 60% of normal level of NFL, NFM and NFH in SN Decreased NF spacing	Not determined	–

Table 5 Other transgenic mice used for the analysis of NF biology

Mice	NF inclusions	Axonal calibre	Axonal cytoskeleton	Conduction velocity	Axonal transport	Others
NFH-LacZ [12, 202, 251, 294, 476]	Massive accumulations of NFs in perikarya from various types of CNS neurons	Decrease of ~50% in L4 VR, SN and SC at 4–5 months old	Scarcity of IFs Increased MT density Decreased levels of NFL, NFM and NFH in SC and in various brain regions Decreased phosphorylation of NFH Normal level of tubulin	Decrease of 35% in caudal nerve at 3 months old	Decreased	Normal life span Degeneration of Purkinje cells with ageing Loss of motor axons in oldest mice Decreased g-ratio in PNS and unchanged g-ratio in CNS Unchanged internodal length Normal molecular organisation of nodes of Ranvier in CNS and PNS
NFH-GFP [477]	No	Normal in SN	Normal NF–MT density Normal levels of NFL, NFM and NFH in brain, SC, SN and ON	Normal	Not determined	Normal g-ratio in SN Removal of a significant portion of NFH tail domain does not affect the capacity of NFH to assemble and to be transported into axons
NFL(Pro) mutant mice [341]	Perikaryal and proximal axonal NF accumulations in motor and sensory neurons	Decrease of ~50% in L5 VR at 6 months old	Disorganised axonal cytoskeleton Mutant NFL accumulates up to ~50% of the endogenous NFL The overall NF content is reduced by ~25–50% of the normal level in the SN	Not determined	Not determined	Massive degeneration of spinal motor neurons but no degeneration of sensory neurons Severe neurogenic atrophy of skeletal muscle resulting in a severe fore and hind limb weakness Early death
NFM tail-deleted (NFMtailΔ) [267, 268]	No	Decrease in L5 VR and DR at 2 and 6 months old	Increased NF density in L5 VR Increased MT density in L5 VR NFMtailΔ accumulates to the normal NFM level Normal levels of NFL and NFH in SN and ON Compensatory phosphorylation of NFH Increased levels of α and βIII-tubulin in SN and ON for [263] but unchanged βIII-tubulin level for [262] Decreased NF spacing	Decreased of 30% in SN at 5–6 months old	Normal velocity of NF, actin and tubulin transport in ON at 4 months old	No overt phenotype No axonal loss

Table 5 (continued)

Mice	NF inclusions	Axonal calibre	Axonal cytoskeleton	Conduction velocity	Axonal transport	Others
NFH tail-deleted (NFHtailΔ) [266, 267, 330]	No	Normal in L5 VR and DR at 3 and 6 months old	Normal NF–MT density NFHtailΔ accumulates to the normal NFH level Normal levels of NFL, NFM and tubulin in SN at 3 months old Compensatory phosphorylation of NFM Normal NF spacing	Normal in SN at 5–6 months old	Normal NF, actin and tubulin transport rates in ON	No overt phenotype No axonal loss
NFM/HtailΔ [267]	No	Decrease in L5 VR and at 2 and 6 months old Delayed axonal radial growth	NFMtailΔ and NFHtailΔ accumulate to the normal NFM and NFH levels Irregular NF arrays Decreased NF spacing Normal levels of NFL and tubulin in SN	Decrease of 30% in SN at 5–6 months old	Not determined	No overt phenotype No axonal loss

DR Dorsal root, *ON* optic nerve, *SC* spinal cord, *SN* sciatic nerve, *VR* ventral root

phosphorylation in their NFH-null mice, complicating the conclusions about the exact role of NFH in axonal radial growth.

It has long been suspected that the phosphorylation status of NFM and NFH carboxy-terminal domains controls axon calibre by regulating NF transport [98, 99] and/or interfilament spacing [85, 89, 246, 260]. KSP repeated motifs on NFM and NFH sidearms are variably phosphorylated, principally after the entry of NF into the axon [86–88, 94]. It has been suggested that phosphorylation of KSP repeats could increase the total negative charges on sidearms and thus causes their lateral extension by repulsive interactions [47, 87], increasing NF spacing and axonal calibre. In agreement with this assumption, Brown and Hoh [261] used atomic force microscopy to show the presence of a weak repulsive force around the core of the filament. Moreover, this repulsive force is absent in homopolymers of NFL or trypsinised native filaments which lack the sidearms present in native filaments and attenuated when the filaments are enzymatically dephosphorylated [262]. As there are more KSP repeats in NFH than in NFM (51 vs. seven in mice), it was thought that this subunit should contribute more to the axonal radial growth. This idea was reinforced by the observation that phosphorylated NFH sidearms are less pliant and larger structures than dephosphorylated sidearms [263]. Moreover, a 50% reduction in the level of axonal NFH in mice overexpressing NFM decreases axonal calibre [264] (Table 4) and modest increases in NFH slightly enhance radial growth in transgenic mice [265]. However, production of NFH-null mice [256–258], as well as mice expressing NFH deprived of its carboxy-terminal domain ($NFH^{tail\Delta}$ mice) [266] (Table 5), demonstrated no major modification in NF spacing and axonal radial growth, except a delay in the acquisition of a normal axonal calibre. However, as mentioned above, NFH-null mice are characterised by a compensatory increase for MT density and NFM phosphorylation. Similarly, the phosphorylation level of NFM is higher in $NFH^{tail\Delta}$ mice [266], making difficult to determine the exact implication of NFH in axonal calibre. In contrast, disruption of the *NFM* gene [62], or deletion of its carboxy-terminal domain in $NFM^{tail\Delta}$ mice [267, 268], reduced the interfilament spacing and axonal calibre, showing a preponderant role of NFM in determining axonal diameter. Finally, it is interesting to note that axonal calibre in double transgenic $NFM/H^{tail\Delta}$ mice from 6 months of age is similar to that of $NFM^{tail\Delta}$ mice, while NF spacing is reduced and NF network is disorganised [267]. This suggests a role for NFH tail domain in NF–NF interactions but not in the control of axonal calibre. Another possibility could be that a yet unknown molecule may be involved in the spatial spacing of NF and may bind differently to NFM and NFH.

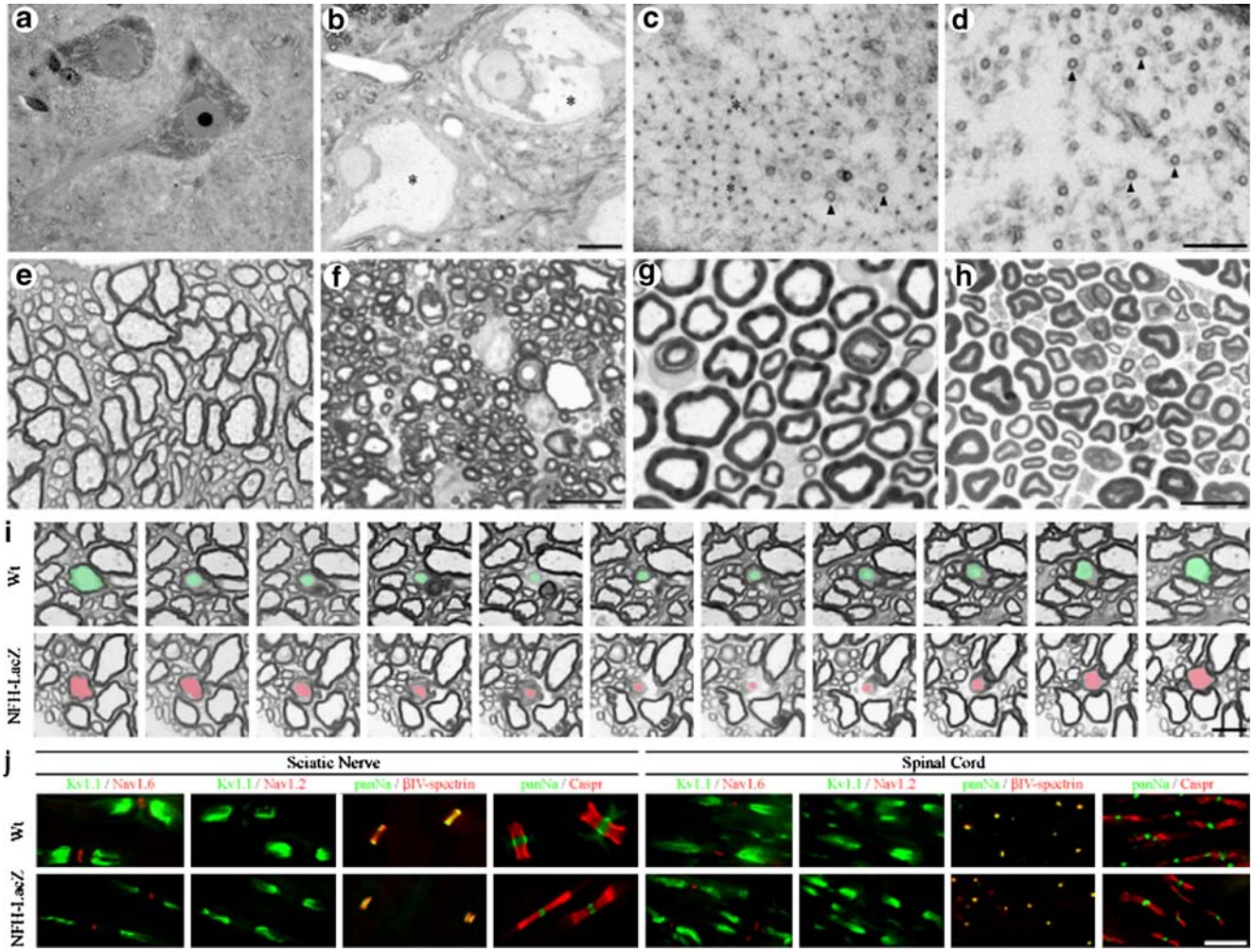

Fig. 2 The expression of the NFH-β-galactosidase fusion protein in NFH-LacZ mice causes the aggregation of NF in neuronal cell bodies, inducing their depletion from the axonal compartment and the decrease of the axonal calibre. **a**, **b** Cross sections of Wt (**a**) and NFH-LacZ (**b**) spinal cord. Note the presence of massive aggregates (*asterisks*) in transgenic motoneurons while such accumulations are absent from control neurons. *Bar*, 20 μm. **c**, **d** Electron micrographs showing the axonal cytoskeleton in axons from Wt (**c**) and NFH-LacZ (**d**) sciatic nerve. NF (*asterisks*) are the most abundant cytoskeletal component in control axons while they are absent from transgenic axons. In contrast, the density of MTs (*arrowheads*) in axons deprived of NF is strongly increased. *Bar*, 200 nm. **e**, **f** Typical cross section views of spinal cord from Wt (**e**) and NFH-LacZ (**f**) mice. Transgenic axons have smaller diameters and proportionally thinner myelin sheaths than control axons. *Bar*, 5 μm. **g**, **h** Typical cross section views of sciatic nerve from Wt (**g**) and NFH-LacZ (**h**) mice. As in spinal cord, the axonal diameter is strongly reduced in transgenic sciatic nerve. However, axons from NFH-LacZ mice recruit inappropriately thick myelin relative to their absolute reduced calibres. *Bar*, 5 μm. **i** Serial sections of spinal cord showing an axon (*green* for Wt and *red* for NFH-LacZ mice) at the level of a node of Ranvier. Despite the absence of axonal NF in transgenic axons, the extent of axonal constriction at nodes is similar between control and NFH-LacZ fibres. *Bar*, 2 μm. **j** Double immunohistochemistry showing the molecular organisation of nodes of Ranvier in sciatic nerve and in spinal cord from 4-month-old Wt and NFH-LacZ mice. The localisation of sodium channels (pan-Na, Nav1.2, Nav1.6), βIV-spectrin, Caspr and Kv1.1 potassium channels is not modified by the absence of axonal NF. Nav1.6 is co-localised with βIV-spectrin at nodes while Nav1.2 is absent from matures nodes. Caspr and Kv1.1 concentrate in paranodes and juxtaparanodes, respectively. *Bar*, 10 μm

The axonal radial growth is closely related to the myelination. In vitro myelination induces the increase of axonal calibre [269] but demyelination in vivo causes a local axonal atrophy [270, 271] and modifications in the axoskeletal organisation [245]. A severe axonal atrophy is also observed in hypomyelinated tracts from *shiverer* mutant mice [272]. A link between myelination and phosphorylation of NF has been proposed following the analysis of the dysmyelinated mouse mutant *Trembler*. This mutant revealed a decreased NF phosphorylation correlated with an increased NF density and reduced axonal calibres [108]. Moreover, NFM and NFH sidearms are highly phosphorylated in myelinated axonal segments and poorly phosphorylated in non-myelinated domains like the initial segment and the node of Ranvier where axonal calibre is reduced [108–111]. The axonal radial growth does not

require the formation of a compact myelin but only the axon ensheathment by the myelin-forming cell [246], suggesting that a molecule localised in the adaxonal membrane of the glial cell regulates the axonal calibre. Because MAG is enriched in this region and starts to be expressed when the axonal calibre expands, it has been suggested that MAG plays a crucial role for the axonal size expansion [273, 274]. A defect in the radial growth of myelinated axons was observed in MAG-deficient mice, together with a reduction of NF phosphorylation in myelinated fibres [112]. It was proposed that the binding of MAG to axonal receptors activates a signalling cascade leading to the phosphorylation of NF and consequently induces the axonal radial growth in myelinated regions [112, 113, 267, 275]. However, we recently show that the relative extent of calibre reduction at nodes of Ranvier is similar between axons containing or not NF [251], suggesting that NF and consequently their phosphorylation are not responsible for the axonal calibre difference between internodes and nodes. Thus, the axonal size reduction at nodes of Ranvier could result from a constrictive pressure exerted by paranodal loops of the myelinating cells.

Neurofilaments Contribute to the Axonal Conduction Properties

Ultrastructure of myelinated fibres is optimised for maximal conduction velocity through the axonal calibre [276–278], internodal length [279–282], myelin thickness [283–285], as well as geometry and molecular organisation of the nodes of Ranvier [286, 287]. As internodal length and myelin thickness are proportional to the axonal size [288–290], NF are key players for modulating the axonal conduction.

Several animal models with abnormal NF expression or distribution highlighted implication of NF in the conduction properties. *Quiver* quails expressing a mutated NFL display a reduced conduction velocity proportional to the decrease of the axonal calibre [291]. Similarly, consistent with axonal atrophy, lower conduction velocities are observed in NFL−/− mice and NFM−/− mice [292], in mice expressing human NFH (hNFH mice) [293], in $NFM^{tail\Delta}$ mice [267] and in NFH-LacZ transgenic mice [251, 294]. These modifications are not restricted to the conduction velocity but other electrophysiological parameters are also affected in these animals. These include altered auditory evoked potentials in *quiver* quails [295], prolongation of refractory period in NFM−/− mice [292], decreased resting membrane potential, prolonged duration of action potential and decreased inward and outward rectification in hNFH mice [293], reduced amplitude of the compound action potential and abnormalities of somaesthetic and auditive-evoked potentials in NFH-LacZ mice [251], suggesting multiple implications of NF in conduction. This is reinforced by the fact that conduction velocity, refractory period as well as correlation between the rate of rise and decay of action potential and conduction velocity are significantly modified in NFH−/− mice despite normal axonal diameter, g-ratio and internodal length [292] providing strong evidence that NF are involved in defining not only the structural but also the functional integrity of myelinated axons. Kriz et al. [292, 293] proposed that NFH may have a specific role in modulating ion channel function, but the exact molecular mechanism is still unclear even if it appeared that localisation of Na^+ and K^+ channels in, respectively, node and juxtaparanode is unaffected by the lack of axonal NF [251]. It should also be mentioned that, in contrast to NFH−/− mice, conduction velocity is not altered in $NFH^{tail\Delta}$ mice [267], indicating no implication of NFH sidearm in this parameter. Finally, it cannot be excluded that absence of axonal NF also affects the axoplasmic resistance.

The conduction velocity also depends on myelin thickness and internodal length, which are proportional to axonal diameter. Optimum conduction velocities are achieved when internodal lengths are ~100 times the axonal calibre and for g-ratio values comprised between 0.6 and 0.7 [281, 283, 285]. To determine whether changes in myelin sheath dimensions can contribute to electrophysiological defects observed in absence of axonal NF, we measured g-ratio and internodal length in NFH-LacZ transgenic mice [251]. It appeared that myelin thickness is differently regulated in CNS and PNS in response to reduced axon calibres. The calibre-reduced axons from NFH-LacZ mice are invested with proportionally thinner myelin in CNS without modifications of the g-ratio, while in PNS axons are overmyelinated compared to their reduced diameter (g-ratio of 0.52 vs. 0.63 in transgenic and control PNS). A similar disparity was reported in NFM−/− and NFH−/− mice [296]. Surprisingly, the axonal atrophy in both CNS and PNS from NFH-LacZ mice does not affect the internodal length. Consequently, internodal lengths are ~200 times the diameter of NF-deficient axons. According to Rushton [283], such a ratio increases internal resistance and reduces the capacity to activate sodium channels. These results indicate that myelin dimensions are not optimal for conduction in absence of axonal NF.

The geometry and composition of nodes of Ranvier are also crucial for the propagation of the nerve impulse. Both in PNS and CNS, nodes are characterised by an important constriction of the axon reaching approximately 30% to 15% of the internodal size [297, 298]. This constriction promotes higher conduction velocities by reducing the nodal capacity through a smaller nodal area. It also reduces

the contribution of the paranodal axolemmal membrane by restricting conductance along the periaxonal pathway [299]. Two models have been considered to explain the nodal constriction [300]. First, the contraction model suggests that myelinating cells exert a pressure via their paranodal loops sufficient to reduce the axonal diameter and/or to limit the axonal radial growth at nodes. The high content of contractile proteins (filamentous actin, myosin) and mitochondria in paranodal loops consolidates this assumption [297, 301]. Secondly, the NF model suggests that reduction of axon calibre at nodes is due to a densely packed hypophosphorylated NF network while larger internodes contain spaced hyperphosphorylated NF [108–111]. However, we recently showed that the extent of axonal constriction at nodes is similar with and without axonal NF [251], indicating that they are not required for the establishment of the nodal ultrastructure and thus arguing in favour of the contraction model.

In conclusion, NFs are crucial for the correct conduction of the nerve impulse and therefore their defects could contribute to neurodegenerative processes. They increase the conduction velocity by promoting the axonal radial growth and are essential to achieve optimal g-ratio and internodal length. On the other hand, they are not necessary for the formation and maintenance of ultrastructure and molecular organisation of nodes of Ranvier.

Axonal Transport of Neurofilaments

Neurons are highly polarised cells and their axonal length can reach more than 1 m in humans. Most proteins are synthesised in cell bodies and transported down the axon through a mechanism called axonal transport. Weiss and Hiscoe [302] demonstrated for the first time this process using ligation of sciatic nerves. This leads to an axonal swelling proximal to the ligation and to an axonal shrinkage in distal region. When ligation is removed, material accumulated in proximal region moved down the nerve at 1 to 2 mm/day. These observations emphasised the existence of a flow of material from the cell body to the nerve terminals. In the 1960s, an important step in characterising the axonal transport was achieved using radiolabelled newly synthesised proteins following injections of [^{3}H] amino acids or [^{35}S] methionine in sciatic nerve or retinal ganglion cells from living animals [303–305]. The distance travelled by labelled proteins showed that the axonal transport is divided in two major categories depending on their speed of transport [306]: the fast axonal transport (~250 to 400 mm/day in mammals) conveys mitochondria, neurotransmitters, channel proteins, lysosomes and endosomes [307–309] and the slow axonal transport (~0.1 to 4 mm/day) conveys axonal cytoskeleton and cytosolic proteins. The slow axonal transport can also be divided into two rate components: the slow component a (0.1–1 mm/day), containing NF and MTs and the slow component b (2–4 mm/day), containing actin, spectrin and other cytoplasmic proteins [154, 310].

It was first speculated that the various rates of transport were due to the association of cargoes with different molecular motors. Members of the kinesin family and cytoplasmic dynein were identified as the major motors responsible for the fast axonal transport in, respectively, the anterograde (toward the terminal end) and retrograde (toward the cell body) directions [311, 312]. However, the identity of motors responsible for the slow axonal transport of NF remained unknown. This was partially elucidated by two studies analysing the transport of green-fluorescent-protein (GFP)-tagged NF subunits transfected into cultured sympathetic neurons [313, 314]. Surprisingly, the authors observed that both GFP-NFM and GFP-NFH move at rates of up to 1 μm/s, corresponding to the rates of molecular motors ensuring the fast axonal transport. This suggests that motors used for slow and fast axonal transport are identical. However, contrary to fast axonal transport of cargoes that moved continuously, NFs are transported intermittently in axons because their fast movements are interrupted by prolonged pauses. Only a small fraction of NF is moving at any given time since it was evaluated that NF spent 97% of their time pausing [315]. Thus, the overall speed of axonal transport would not depend on the motors involved but on the duration of association between cargoes and motors. Components moving with the fast axonal transport could be attached to the motor for a long period while interaction between components moving with the slow axonal transport and their motors would be short. Another interesting discovery emerging from these studies is the description of a bi-directional transport of the NF but with a predominant anterograde prevalence. Similar results were obtained with extruded squid axoplasm [316] and along MTs in vitro [222]. Altogether, these data indicate that the overall slow axonal transport of NF is the result of a combination between fast bi-directional movements and long-lasting pauses.

The implication of kinesin and dynein in axonal transport of the NF was later confirmed by various observations. First, several evidences suggest a direct interaction between NF and kinesin or dynein [220–223, 317]. The inhibition or depletion of dynein [225, 318] affect the axonal transport of NF and lead to their proximal accumulation, while microinjection of anti-kinesin and anti-dynein antibody affect anterograde and retrograde NF transport in cultured dorsal root ganglia neurons [224]. Finally, targeted disruption of neuronal kinesin heavy chain KIF5A induces the accumulation of NF in cell bodies of peripheral sensory neurons [319], suggesting that KIF5A may be a NF motor.

Millecamps et al. [156] have provided new insights into the mechanisms of axonal transport of NF. As described above, they generated transgenic mice with doxycycline-controlled expression of hNFL, with or without endogenous mouse NFL proteins (respectively, tTA;hNFL;NFL$^{+/-}$ and tTA;hNFL;NFL$^{-/-}$ mice). They showed that the presence of the axonal NF array strongly slows down the axonal transport of NF. Indeed, when doxycycline treatment of tTA;hNFL;NFL$^{-/-}$ mice is stopped; the reappearance of hNFL occurred in synchrony along the sciatic nerve within 1 week, indicating a fast axonal transport of NF in axons deprived of stationary NF network. They estimated a rate of ~10 mm/day in axons with low NF content compared to ~1 mm/day in axons with a dense NF array. The authors suggested that these differences could be due to a decreased number of pauses during the travel of NF and/or to decreased interactions with a stationary pool.

The forms in which NF are transported (subunits or polymers) and the contribution of their phosphorylation were sources of intense investigations for many years. One model suggested that NF polymerise in cell bodies immediately after synthesis, and they are subsequently transported as filaments. On the opposite, the subunit model proposed that polymers are essentially stationary and that NF could be transported as free subunits or small oligomers that integrate stationary cytoskeletal polymers. Both hypotheses were based on various observations (for review, see [320, 321]). For example, the metabolic labelling of cytoskeletal proteins with [^{35}S] methionine shows a synchronised movement of the three NF subunits, in favour of the polymer transport model [154, 322]. Moreover, the majority of NF in axons is polymers and it is difficult to detect free monomers. Conversely, in fluorescence recovery after photobleaching experiments, no movement of the bleached area was observed and the recovery of fluorescence is gradual [323], supporting the subunit transport hypothesis. In the same way, the existence of a subunit–small oligomer transport is suggested by the finding that NFM is transported along axons after injection of a recombinant adenovirus encoding tagged NFM protein in transgenic mice deprived of axonal NF [324], even if a diffusion of this protein cannot be completely excluded [325]. An important advance was made by analysing the transport of GFP-NFM and GFP-NFH in transfected neurons [313, 314]. In these studies, intact filaments (1–15 μm of length) represent 95% of the moving structures observed, strongly supporting the polymer model. The transport of NF polymers was recently confirmed by Yan and Brown [326] who showed that NF move in the form of assembled polymers in axons of cultured neonatal mouse sympathetic neurons and, more interestingly, that moving and stationary NF are complex heteropolymers also containing peripherin and α-internexin along >85% of their length [327]. Yuan et al. [328] also demonstrated that α-internexin is a key determinant for the axonal transport of NF in CNS. They observed that NFM can be transported into axons of the optic nerve in absence of NFL and NFH, while the additional deletion of α-internexin abolishes this transport, indicating that NFM monomers alone are not efficiently transported. The finding that a small number of IFs are present in optic axons from NFL−/−;NFH−/− mice but not from α-internexin−/−;NFL−/− mice suggests that NFM is able to associate with α-internexin to form IFs. The co-localisation of α-internexin and NFM on the same filament [12] reinforced this assumption. Thus, it seems that hetero-oligomer composed of at least NFM and α-internexin is the minimal form in which NF proteins can be transported in CNS axons. It is also interesting to note that the deletion of NFH or α-internexin does not affect the NF transport [266, 328] while the loss of both proteins causes the selective acceleration of the axonal transport of NFL and NFM subunits, suggesting redundant roles of α-internexin and NFH in axonal transport of NF.

Another controversial subject concerns the role played by phosphorylation in the axonal transport of NF. Phosphorylation of NF, in particular NFH, has long been considered to decrease their transport rate. The entry of NFH into axons during development coincides with reduced axonal transport of NF [51, 329]. Moreover, the phosphorylation level of NF correlates with a slow transport of radiolabelled NF in projections of the rat L5 dorsal root ganglion [100], while hypophosphorylated NF in mouse optic axons move faster than hyperphosphorylated ones [101]. Similarly, lack of NFH accelerates NF transport [258], while increased NFH expression selectively slows NF transport [265]. Finally, NF containing NFH mutated to generate constitutively non-phosphorylated NFH move faster than those containing wild-type NFH, while NF containing NFH mutated to generate constitutively phosphorylated NFH move slower [102]. All these studies support the view that the rate of NF transport is inversely correlated to their phosphorylation state. However, the analysis of NF axonal transport in optic nerve from NFM$^{tail\Delta}$ mice and NFH$^{tail\Delta}$ mice shows no modification in the rate of NF transport [266, 268, 330] suggesting that phosphorylation of NFM and NFH sidearm is not directly involved in this process. Alternatively, a yet unknown molecule could bind differently to these mutated NF and may modulate their transport. It should also be noted that phosphorylation of both NFM and NFH subunits are tightly regulated. Indeed, phosphorylation of NFM is significantly increased in NFH−/− mice and in NFH$^{tail\Delta}$ mice [258, 266] while an increased phosphorylation of NFH is observed in NFM−/− mice and in NFM$^{tail\Delta}$ mice [62, 268]. The measure of NF transport in NFM$^{tail\Delta}$ mice and NFH$^{tail\Delta}$ mice could thus be biased by this compen-

satory phosphorylation. It would be interesting to study the NF transport in double transgenic $NFM^{tail\Delta}/NFH^{tail\Delta}$ mice in order to answer this question.

Several possibilities could explain how phosphorylation regulates the axonal transport of NF. One main observation is that phosphorylation of NF controls their association with molecular motors. In particular, phosphorylation of NF promotes their release from kinesin [221, 317] and increase their affinity for dynein [225]. Consequently, the phosphorylation of NF would cause their slowing by reducing their anterograde transport by the kinesin and by supporting their retrograde transport by the dynein. Thus, the long pauses during their travel would correspond to the moments when NFs are dissociated from their motors. This model is consistent with the intermittent movement of NF into axons [313, 314] and with mathematical modelling of NF axonal transport [331]. Future investigations should focus on determining the precise nature of the motors that drive the movements of NF and the exact function of phosphorylation as well as signals and pathways regulating it. It is particularly important to determine which carboxy-terminal phosphorylation sites are involved in this process and whether the phosphorylation sites located in NF head domains, which are involved in NF assembly, also play a role in their transport.

Neurofilaments and Pathologies

The discovery of mutations in NF proteins associated with several neurodegenerative diseases argues for the participation of NF in these pathologies. Moreover, abnormal accumulations of NF are a pathological hallmark of many human neurodegenerative disorders, including ALS, AD, PD, CMT, giant axonal neuropathy (GAN), dementia with Lewy bodies, spinal muscular atrophy, progressive supranuclear palsy and diabetic neuropathy. Multiple factors can potentially induce the accumulation of NF, including dysregulation of NF gene expression, NF mutations, defective axonal transport, abnormal post-translational modifications and proteolysis. Here, we will review some neurodegenerative disorders that involve NF abnormalities and toxic agents responsible for NF accumulations.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis, also called Lou Gehrig's disease, is a late-onset progressive motor neuron disease characterised by intraneuronal aggregates of NF, termed spheroids, in affected neurons [332–337]. About 90% of ALS cases are sporadic while approximately 10% are inherited in a dominant manner. Although the mechanisms leading to the accumulation of NF in ALS remain unclear, the overexpression of NFL [338], NFM [264] or hNFH [250] provokes NF aggregations and morphological alterations similar to those found in ALS. Remarkably, the motor neuron disease caused by excess hNFH proteins can be rescued by overexpression of human NFL in a dosage-dependent fashion [255], suggesting the importance of subunit stoichiometry in ALS pathogenesis. The 70% reduction of NFL mRNA in degenerating neurons of ALS [339, 340] reinforces this assumption. Evidence that aberrant NF accumulation can contribute to the neuronal death came from the observation that the expression of a mutated NFL subunit causes the aggregation of NF leading to a selective degeneration of spinal motor neurons and to a severe atrophy of skeletal muscles [341]. Codon deletions or insertions in the KSP repeat motifs of NFH have been identified in unrelated patients with sporadic ALS [342–344]. However, two others studies failed to identify variants in the NF genes linked to sporadic and familial ALS [345, 346]. Together, these data suggest that mutations in the NF genes are not a systematic common cause of ALS but could be a risk factor for sporadic ALS.

Mutations in SOD1, the most abundant cytosolic enzyme, account for 20% of all the familial cases. As for ALS patients, mice expressing mutant SOD1 display NF accumulation [347, 348] and exhibit a phenotype similar to that of mice overexpressing NFL or hNFH. Moreover, axonal transport of NF is perturbed in $SOD1^{G37R}$ [348, 349], $SOD1^{G85R}$ [349] and $SOD1^{G93A}$ [350] mice. To determine whether NFs are involved in SOD1-mediated disease, mice expressing mutant SOD1 were mated with transgenic mice with altered NF protein content. The withdrawal of NF from the axonal compartment and their perikaryal accumulation induced by the expression of NFH-β-galactosidase fusion protein conferred no beneficial effect in $SOD1^{G37R}$ [232], indicating that axonal NF are not necessary for SOD1-mediated disease. This was also confirmed in $SOD1^{G85R}$ deprived of NFL but the absence of axonal NF in these mice prolongs their life span by approximately 15% [351]. Surprisingly, overexpression of mouse NFL or mouse NFH in $SOD1^{G93A}$ mice [352] and overexpression of hNFH in $SOD1^{G37R}$ mice [353] also increase their life span by, respectively, 15% and 65%. This suggests a protective effect of perikaryal accumulation of NF proteins in motor neuron disease caused by mutant SOD1. However, the mechanism of protection is still unclear, even if it seems that perikaryal accumulation of NF proteins rather than axonal NF deficiency is responsible for slowing disease in these models. Indeed, the formation of large perikaryal aggregates and the massive depletion of axonal NF due to the expression of the human NFH^{43} allele cause more positive effects than human NFH^{44} allele which induces smaller aggregates and more axonal NF [353]. Moreover, the disruption of one allele for each NF gene

induces a 40% decrease of axonal NF proteins content and an important axonal atrophy without perikaryal accumulation of NF in SOD1^{G37R} mice but does not extend their life span nor does it alleviate the loss of motor axons [254].

Several hypotheses were proposed to explain this protective effect of perikaryal aggregates in SOD1-mediated disease. Thanks to their multiple calcium-binding sites, NF proteins may act as calcium chelators. According to this, a significant neuroprotection was obtained by overexpressing the calcium-binding protein calbindin-D28k in cultured motor neurons [354]. Ehlers et al. [355] have shown that NFs are involved in localisation of *N*-methyl D-aspartate (NMDA) receptors in the neuronal plasma membrane by interacting with the NMDA NR1 subunit. It cannot thus be excluded that accumulation of NF can interfere with glutamate receptor function and prevent glutamate excitotoxicity. Finally, Nguyen et al. [356] proposed that perikaryal accumulations of NF in motor neurons may alleviate ALS pathogenesis by acting as a phosphorylation sink for cyclin-dependent kinase 5 dysregulation induced by mutant SOD1, thereby reducing the detrimental hyperphosphorylation of tau and other neuronal substrates. This was supported by the fact that NF accumulations contain hyperphosphorylated NFM and NFH subunits in ALS patients [357] and in SOD1 mutant mice [347, 358] while they are normally not phosphorylated in cell bodies. However, removal of NFM and NFH sidearms led to a delay of disease in SOD1 mutant mice rather than the acceleration predicted by a kinase dysregulation model [359], indicating that perikaryal phosphorylation of NF is not an essential mechanistic contributor to reduced toxicity of SOD1 mutants. Alternatively, NF removal from the axonal compartment contributes to a more flexible axoplasm capable of enhancing axonal transport which is impaired in SOD1 mice [349, 360].

Peripherin, a type III IF protein, is also a component of spheroids in ALS [189, 347, 361]. The overexpression of wild-type peripherin in mice led to the formation of cytoplasmic protein aggregates and induces the selective loss of motor neurons during ageing [362, 363]. Because NFL mRNA levels are reduced in cases of ALS [339, 340], Beaulieu et al. [362] generated double transgenic mice overexpressing peripherin and deficient for NFL (Per; NFL−/− mice). They observed that the onset of peripherin-mediated disease is accelerated by the deficiency of NFL. In absence of NFL, peripherin interacts with NFM and NFH to form disorganised IF structures [11]. This could explain why the number of IF inclusion bodies is increased in Per; NFL−/− mice, leading to an earlier neuronal death. In contrast, peripherin toxicity can be attenuated by co-expression of NFL [364], illustrating the importance of IF protein stoichiometry. Another example of protection came from the generation of double transgenic mice overexpressing both peripherin and NFH in which peripherin-mediated disease is completely abolished [365]. Excess of NFH shifted the intracellular localisation of inclusion bodies from the axonal to the perikaryal compartment of motor neurons, suggesting that the toxicity of peripherin inclusions may be related to their axonal localisation, possibly by blocking the axonal transport. All these results underline the crucial role of IF protein stoichiometry in the formation, localisation and toxicity of neuronal inclusion bodies in ALS.

Alzheimer Disease

AD is the most common type of dementia characterised by progressive cognitive deterioration and excessive loss of memory, together with declining activities and behavioural changes. One major neuropathological change occurring in affected neurons of AD patients is the perikaryal formation of NFTs composed of tau, NF and other cytoskeleton proteins [366, 367]. It is still controversial whether NFTs represent a primary causative factor in AD. Moreover, the mechanism responsible for NFT formation is not completely understood, even if it seems that dysregulation of tau metabolism is more involved than alterations of NF proteins [368]. NF in NFT appear as paired helical filaments and differ from normal NF in their resistance to solubilisation by detergents [369], suggesting that they form highly compacted structures. Another characteristic of NF in NFT is their extensive phosphorylation [370–372], probably caused by a down-regulation of protein phosphatase 2A [373–376]. Deng et al. [377] suggested that the hyperphosphorylation and accumulation of NF in AD brain might be caused by impaired brain glucose uptake–metabolism. Indeed, they observed in AD brain and in a rat model mimicking the decreased glucose uptake–metabolism observed in brains of patients with AD a decrease in O-GlcNAcylation and an increased phosphorylation level of KSP repeats from NFM. They also showed that O-GlcNAcylation and phosphorylation of NFM are reciprocally regulated in cultured neuroblastoma cells and in metabolically active rat brain slices, providing a new mechanism of regulation of NF phosphorylation and a possible explanation on the hyperphosphorylation and accumulation of NF in AD brain.

Parkinson Disease

PD is a progressive disorder of the CNS affecting dopaminergic neurons of the substantia nigra. A neuropathological hallmark of PD is the formation of ubiquitinated protein inclusions named Lewy bodies, composed of α-synuclein, NF proteins, ubiquitin and proteasome subunits [378–382]. NF in Lewy bodies undergo inappropriate

phosphorylation and proteolysis [383, 384] and NFL and NFH mRNAs are decreased in PD [190].

Mutations in the *parkin* gene is the major cause of familial PD [385–387] but a point mutation in the *NEFM* gene was also reported in a French–Canadian patient who developed the disease at the age of 16 [388]. This mutation consists in a substitution of Ser for Gly at residue 336, a highly conserved region in the rod domain 2B of NFM. However, three other family members also carried the G336S mutation but are unaffected, arguing against the implication of NFM mutation in pathogenesis of PD. Moreover, the G336S mutation does not affect the assembly and the distribution of NF in vitro [389]. The screening of 102 French–Canadian PD patients failed to identify this mutation, indicating that G336S mutation is not common even in a PD population of similar ethnic background and does not play a major role for the development of PD [390]. Similarly, G336S mutation was never found in 322 sporadic and familial PD patients of German origin [391]. Nevertheless, this study identified in *NEFM* gene from two patients a Pro-to-Gln substitution at residue 725 and a deletion of Val in position 829, two highly conserved sites. Finally, no mutation of the *NEFL* gene has been identified in 328 sporadic and familial PD patients [392]. These results suggest that mutations in NF genes are not a primary cause of PD even if the rare variants of the *NEFM* gene identified may act as susceptibility factors for PD.

Charcot-Marie-Tooth

CMT is the most common form of hereditary peripheral neuropathy affecting both sensory and motor nerves [393]. Patients with CMT progressively develop a weakness of muscles and become unable to walk. CMT neuropathies are classified into several categories, including CMT1, CMT2, CMT3, CMT4 and CMTX. Types 1, 3, 4 and X are demyelinating whereas type 2 is an axonal neuropathy. Accumulation of NF in CMT2 was reported for the first time by Vogel et al. [394]. Several mutations of *NEFL* gene, located throughout the three functional domains of the NFL protein (head, rod and tail), are associated with CMT type 2E or CMT type 1F. The first mutation was identified by Mersinayova et al. [395] in a large Russian family with CMT2. This missense mutation in the rod domain 2B consists by the substitution of a highly conserved Gln for Pro at position 333 (Q333P). The second reported mutation was another substitution in the non-helical head domain of NFL (Pro to Arg at residue 8) found in members of a Belgian family with a severe CMT phenotype [396]. The expression of NFL^{P8R} or NFL^{Q333P} in cultured cells disrupts both NF assembly and axonal transport of NF and induces the accumulation of mitochondria in cell bodies and proximal axons [397–399]. These mutations also affect anterograde and retrograde fast axonal transport and cause fragmentation of the Golgi apparatus and degeneration of neuritic processes in culture neurons [399], providing possible mechanisms by which these mutants could be involved in axonal degeneration and CMT pathogenesis.

The Pro at codon 22 is also the target of several mutations. A Pro-to-Ser substitution (P22S) was observed in nine members of a large Slovenian CMT2 family, with a complete co-segregation between this mutation and the dominantly inherited CMT2 phenotype [400]. P22S mutation was also detected in an Italian family. Examination of nerve biopsies in these patients revealed a primary axonopathy characterised by giant axons with swellings composed almost entirely of aggregated NF [401]. A Pro-to-Thr substitution (P22T) was detected in unrelated Japanese patients with CMT disease [402]. P22 NFL mutant proteins are unable to assemble into filaments and form aggregates in vitro [126, 399]. It was shown that P22S and P22T mutations abolish the phosphorylation of the adjacent Thr21 which normally inhibits filament assembly [126] and thus could explain the formation of NF aggregates by these mutants. It is interesting to note that phosphorylation of NFL head domain by PKA alleviates aggregates in cortical neurons, providing a potential therapeutic approach to dissociate NF aggregates in CMT [126].

Thereafter, other mutations of *NEFL* gene were reported in cases of CMT. Jordanova et al. [403] screened 323 patients with CMT or related peripheral neuropathies and identified six disease-associated missense mutations and one 3-bp in-frame deletion in the *NEFL* gene. In the same way, Fabrizi et al. [404] screened 177 patients and identified four mutations in the head and rod domains of NFL, including a novel Leu268Pro substitution and a novel del322Cys_326 Asn deletion. The majority of these mutants form aggregates, except E7K and D469N. The harmful effect of NFL mutations could produce defect in axonal transport after the formation of large accumulation of NF in cell bodies and axons. The first duplication–insertion mutation of NFL in a patient with CMT was reported by Leung et al. [405]. Unlike other NFL mutations inducing the formation of NF aggregates and a blockade of their axonal transport, this mutation appeared to provoke neuronal degeneration probably through both aggregation and de-stabilisation of the neuronal IF network.

Goryunov et al. [406] showed that mutations of myotubularin-related protein 2, which lead to the CMT4B form, can cause NFL aggregation in culture cells, indicating that mutation of NFL is not necessary to induce NF aggregations in CMT. Finally, an implication of NF in demyelinating CMT cannot be excluded. Nerves from patients expressing NFL^{L268P} or NFL^{E89K} show evidence of Schwann cell abnormalities [403, 404] and abnormalities

of NF phosphorylation occur in demyelinated axonal segments [407].

Neuronal Intermediate Filament Inclusion Disease

Neuronal intermediate filament inclusion disease (NIFID), also called neurofilament inclusion body disease or neurofilament inclusion disease, is a recently described neurological disorder of early onset with a heterogeneous clinical phenotype, including fronto-temporal dementia and pyramidal and extrapyramidal signs [408–410]. The symptoms comprise behavioural and personality changes and, less often, memory loss, cognitive impairment, language deficits and motor weakness [411]. The pathological phenotype consists in neuronal loss, gliosis, swollen neurons and presence of large IF inclusions in the cell body of many neurons. These inclusions contain neither tau nor α-synuclein [408–410] but are rich in NF triplet proteins (phosphorylated and unphosphorylated epitopes) and especially α-internexin [412, 413]. The number of IF aggregates is higher in areas with little neuronal loss and lower in sites of intense neuronal degeneration. Cairns et al. [412] proposed that the formation of these inclusions is an early event in the pathogenesis of NIFID and that these aggregates are released and degraded into the extracellular space following degeneration of the neuron. The precise mechanism leading to the formation of these aggregates is unknown, but defect in axonal transport and/or gene expression may be involved. A mutation analysis of patients with NIFID revealed no pathogenic variants for all type IV neuronal IF, SOD1 and NUDEL [414]. The role of these IF inclusions in the pathogenesis of NIFID also remains to be elucidated.

Diabetic Neuropathy

Diabetes is a disease in which low levels of insulin or abnormal resistance to insulin's effects induce perturbed glucose metabolism and inappropriately high blood sugar. Diabetic neuropathy is a peripheral nerve disorder caused by diabetes affecting principally sensory nerves and dorsal root ganglia and characterised by slowing conduction velocity, impairment of axonal transport, axonal atrophy and a reduced capacity for nerve regeneration. All these features of nerve function depend on the integrity of the axonal cytoskeleton and in particular on the NF network. Consistent with this, multiple abnormalities of NF biology have been identified in models of diabetes. Medori et al. [415, 416] observed in rats with streptozotocin-induced diabetes and in BioBreeding rats (a model of spontaneous type I diabetes) an impairment of the axonal transport of NF, actin and tubulin concomitant with a proximal increase and a distal decrease of axonal cross-sectional area. The distal axonal atrophy is accompanied by an important loss of NF in this region [417]. Accumulations of highly phosphorylated NF epitopes are present in proximal axonal segments of dorsal root ganglia sensory neurons from diabetic patients [418]. An increase of NF phosphorylation, correlated with activation of JNK, was also detected in lumbar dorsal root ganglia from rat models [419]. Finally, significantly decreased mRNA levels for the three NF subunits as well as reduced NF numbers and densities within large myelinated sensory axons were reported in long-term diabetic models [420]. All these results suggest that NF abnormalities may contribute to the development of diabetic neuropathy or may be affected by this disease.

However, slowing of conduction velocity in diabetic models occurs much earlier than loss of NF investment or axonal atrophy [420]. To further elucidate the contribution of NF in diabetic neuropathy pathogenesis, Zochodne et al. [294] analysed the effect of streptozotocin-induced diabetes in transgenic NFH-LacZ mice characterised by NF-deficient axons. An accelerated diabetic neuropathy was observed in these mice. Indeed, superimposing diabetes on axons without NF was associated with an earlier reduction of both conduction velocity and nerve action potential amplitudes and increased axonal atrophy. This indicates that changes in NF expression, transport or post-translational modifications cannot account alone for conduction slowing and atrophy in diabetic neuropathy, but their presence may help axons to resist diabetic damage.

Giant Axonal Neuropathy

GAN is a rare progressive neurodegenerative disorder affecting both PNS and CNS which generally appears in infancy or early childhood [421, 422]. First signs of GAN usually begin in the PNS but as the disorder progresses the CNS becomes involved, causing a progressive decline in mental function, loss of control of body movement and seizures. GAN is caused by mutations in the *GAN* gene, which codes for the gigaxonin [423]. The major cytopathological hallmark of GAN is the presence of masses of NF producing focal enlargements in the distal regions of axons associated with a reduced number of MTs [424]. In contrast, axonal segments proximal to the swellings exhibit a reduction in number of NF [425]. Disorganisation and accumulation of other types of IFs are also found in skin fibroblasts, Schwann cells and muscle fibres [426–430]. The minimal distance separating NF in sural nerve axons of a patient with GAN is decreased compared to controls and, more surprisingly, the mean diameter of NF is increased (12.4 nm in GAN compared with 10.1 nm in controls) [431]. The mechanism of distal axonal accumulation of NF is still unclear. An acceleration of their axonal transport was observed in optic nerve from experimentally induced GAN

rat model, concomitant with a proximal decreased content of NF and their distal accumulation [432]. The authors proposed that acceleration of NF transport in the presence of a normal rate of NF protein synthesis and insertion into transport system would lead to the formation of distal axonal swellings with packed NF.

To determine how disruption of gigaxonin's function leads to GAN, Ding et al. [433] generated gigaxonin-deficient mice. Despite the development of a progressive deterioration for motor function, fertility and life span of these mice are normal and giant axons are never seen. However, these animals display enlarged axons with densely packed NF, leading to the segregation of axonal organelles, a feature characteristic of human GAN pathology. This is accompanied by an axonal loss at the age of 9–12 months. Gigaxonin can interact with the light chain of MAP1B [434], tubulin folding cofactor B (TBCB, [435]) and with MAP8 [433] and can control their ubiquitin-mediated degradation. GAN-associated mutations of gigaxonin perturb its association with MAP1B-LC, TBCB and MAP8 while gigaxonin ablation results in the accumulation of these partners [433, 435, 436]. These data suggest a crucial role of gigaxonin in the maintenance of a normal cytoskeleton network. However, the exact implication of cytoskeleton abnormalities on neurodegeneration in GAN remains to be established.

Toxic Agents that Disorganise the Neurofilament Network

IDPN is a toxin that segregates MTs from NF and thereby causes their abnormal accumulation. Its administration results in severe reduction or blockade of NF transport [437], with NF accumulation in the proximal region of axons [192] and segregation of NF and MTs distal to this region [194]. The aggregation of NF occurs through abnormal cross-linking of hyperphosphorylated NF [438]. The susceptibility of various neurons to these effects depends on their NF content; NF-rich large-calibre axons being the most affected [439]. Unlike normal mice, NFH-null mutant mice do not develop swellings of motor axons when treated with IDPN [258], demonstrating that NFH protein is a key mediator of IDPN-induced axonopathy.

The injection of aluminium chloride in spinal cord of rabbits causes the formation of NF tangles in neuronal perikarya and proximal parts of dendrites [193]. NF transport is maintained in the distal region of the axon, resulting in lack of NF in axonal segments immediately distal to the block [179]. NFs sequestered in cell bodies are highly phosphorylated [440], which may account for their abnormal axonal transport. Moreover, aluminium inhibits NF degradation and dephosphorylation [181, 441, 442] and reduces the assembly of newly synthesised NF subunits into NF [443].

The effects of acrylamide are very similar to those of aluminium. During the development of experimental acrylamide neuropathy, NF transport is inhibited and NFs accumulate in proximal axons with formation of axonal swellings [195]. The administration of acrylamide also increases the expression of mRNA for NF proteins and the phosphorylation of NF in rats [444–446] but decreases their degradation [447]. However, acrylamide-induced neurotoxicity is not initiated exclusively through its action on axonal NF because NF-deficient mutant *quiver* quails, crayfish (a species lacking NF) and NFH-LacZ mice are sensitive to neurotoxic effects of acrylamide [448–450].

Arsenic also disrupts the organisation of NF network. Protein analysis of sciatic nerves from rats treated with arsenite (the inorganic form of arsenic) showed disappearance of NF [451]. DeFuria and Shea [452] demonstrate that arsenite decreases NF transport and induces the perikaryal accumulation of phosphorylated NF in NB2/d1 cells and in cultured dorsal root ganglion neurons. These effects were prevented by inhibiting JNK and GSK-3β.

Finally, lead exposure during the development of a chicken model of auditory temporal processing results in decreased amount and phosphorylation of NFM within the axons connecting auditory nuclei in the avian brainstem [453]. During the mouse development, lead exposure increases phosphorylation of both NFM and NFH within auditory brainstem nuclei. Moreover, neuritic beadings immuno-labelled for NF are observed following lead exposure both in vivo and in vitro, suggesting an impairment of the axonal transport [454].

Conclusion

This review attempted to summarise current knowledge about NF biology. These last years, our understanding of the regulation, structure and functions of NF in normal and pathological conditions has been considerably improved by using transgenic mice models. However, several aspects of the NF biology remain elusive and further investigations are required to solve these issues. While it is clearly established that NF play a central role in the growth and maintenance of the axonal calibre, the exact mechanism employed is still unclear. In particular, it remains to elucidate why sidearms from NFM contribute more than those of NFH in the axonal radial growth, how the axonal calibre is modulated along its length, and how myelinating cells regulate the axonal architecture.

The recent development of techniques such as time-lapse imaging coupled with the use of NF proteins tagged with fluorescent probes allowed important advances in characterising the axonal transport of NF. It is now well accepted that kinesin and dynein convey NF, but the mechanisms

regulating the interaction of NF with these motor proteins or with the stationary pool of NF should help to clarify how the axonal transport of NF is regulated. It also appears that α-internexin is an integral component of NF and participates in their axonal transport in CNS. Whether peripherin plays an analogous role in PNS remains to be determined. Finally, it will be crucial to understand how the normal or disorganised NF networks interact and possibly affect the fast axonal transport in order to evaluate to which extent abnormal accumulations of NF contribute to the neurodegenerative processing observed in human neuropathologies.

Discoveries of NF mutations associated with several neurodegenerative diseases as well as the production of various mouse models provide promising avenues to understand the possible implication of NF in these pathologies. However, it appeared that NF aggregation can play both detrimental and beneficial functions. While perikaryal accumulations are generally well tolerated, axonal aggregates are often toxic. It will be important to elucidate the reasons of this discrepancy and the pathways involved in the toxic effects of axonal NF aggregates. A possibility would be that axonal neurofilamentous aggregations affect more profoundly the fast axonal transport than perikaryal aggregations by altering the microtubular assembly together with their associated proteins as observed with STOP proteins aggregated with NF in axonal spheroids in ALS patients. Finally, recent findings highlighted that perturbations in post-transcriptional regulation of NF expression could also play a role in neurodegeneration by affecting the NF homeostasis in neurons. The identification and characterisation of new NF RNA binding proteins and their functions and regulation represent challenging issues. Alternatively, the presence of NF fragments resulting from abnormal degradation of NF following their aggregation could also affect the axoskeleton dynamics. These possibilities represent promising research areas to explore the implication of NF accumulations routinely observed in neurodegenerative diseases.

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