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Chapter 10

Neuronal Intermediate Filaments in Amyotrophic Lateral Sclerosis

Philippe Codron, Julien Cassereau, Joël Eyer and Franck Letournel

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Abstract

Neuronal intermediate filaments (NIFs) are the most abundant cytoskeletal element in mature neurons. They are composed of different protein subunits encoded by separate genes such as neurofilament light chain (NFL), neurofilament medium chain (NFM), neurofilament heavy chain (NFH), α-internexin and peripherin. NIFs are dynamic structures playing important functions in cell architecture and differentiation, interactions between proteins or subcellular organelles, and in axonal calibre determination and myelination. Consequently, their presence modulates electrophysiological properties of axons. NIFs have long been assigned a role in the pathogenesis of amyotrophic lateral sclerosis (ALS). Indeed, accumulation and abnormal phosphorylation of NIF subunits in motor neuron are one of the major pathological features in both sporadic and familial forms of the disease. Moreover, mutations in the NFH and peripherin genes and elevated cerebrospinal fluid NIF levels reported in ALS cases, associated with studies in transgenic mice, provided the evidence that primary defects in NIFs could be causative for motor neuron disease. However, the processes leading to the NIF abnormalities and the links to the pathogenesis of ALS remain unclear, leaving a challenging open field for further investigations in this highly disabilitating disease. Here, we review the main characteristics of these NIFs and their involvement in the pathomechanisms of ALS.

Keywords: Intermediate filaments, Neurofilaments, cytoskeleton, amyotrophic lateral sclerosis, tubulin, microtubules, axonal transport

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1. Neuronal intermediate filaments

1.1. Characteristics

Intermediate filaments (IFs) are components of the cytoskeleton, together with microtubules (MTs) and microfilaments. IFs are defined by their diameter when examined by transmission electronic microscopy (10 nm), which is intermediate between microtubules (15 nm) and microfilaments (6 nm). They also differ from these two structures by the various sizes and primary organisation of their constitutive proteins, their non-polar architecture and their relative insolubility. Intermediate filaments form a large family of proteins; they are classified into five types according to their gene organisation, size, structure and cell-type expression (**Table 1**). IFs expressed in neurons of the central and peripheral nervous systems are called neuronal intermediate filaments (NIFs) and include nestin, synemin, vimentin, α-internexin, peripherin and neurofilaments (NFs) that are composed of three subunits, neurofilament light chain (NFL), neurofilament medium chain (NFM) and neurofilament heavy chain (NFH) (for low-, medium-, and high-molecular-weight NFs) [1–5].

Neurons express differentially IF proteins depending on their developing stage and their localisation in the nervous system. While nestin, synemin and vimentin are mainly expressed during the neuronal development, NFs, peripherin and α-internexin are the main intermediate filament subunits in mature neurons from the central and peripheral nervous system [6]. In this chapter, we focus on those three subtypes of NIFs.

Type	Name	Cell/tissue
I	Acid keratins	Epithelia
II	Basic keratins	Epithelia
III	Desmin	Muscle
	GFAP	Astroglia
	Peripherin	PNS neurons
	Vimentin	Mesenchyme
IV	Neurofilaments (NFL, NFM, NFH)	PNS and CNS neurons
	α-Internexin	CNS neurons
	Nestin	CNS stem cells
V	Nuclear lamins	Nucleus

IFs found in mature neurons are NFL, NFM, NFH, peripherin and α-internexin. Abbreviations: GFAP, glial fibrillary acidic protein; CNS, central nervous system; PNS, peripheral nervous system [7].

Table 1. Classification of intermediate filaments.

1.2. Expression and post-translational modifications

Genes coding for NFL and NFM (*NEFL* and *NEFM*) are closely linked on chromosome 8 (8p21), while NFH gene (*NEFH*) is located on chromosome 22 (22q12.2) [8–10]. Peripherin is encoded

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by *PRPH* located on chromosome 12 (q12–q13) [11], and α-internexin is encoded by *INA* located on chromosome 10 (10q24.33) [3]. As for other IFs, NFs, peripherin and α-internexin share a common tripartite structure, with non-helical amino- and carboxy-terminal regions (head and tail domains) flanking a 46-nm-long central *α*-helical rod domain composed of approximately 310 highly conserved amino acids [9, 10, 12] (**Figure 1**). These segments are joined by short non-helical linker sequences, aligning the individual IF subunits prior to filament assembly. While peripherin and NFL have a short-tail domain, those of NFM and NFH are longer and contain numerous KSP (Lys-Ser-Pro) repeats that can be phosphorylated on serine (S) residues. These sites are frequently modified by phosphorylation, glycosylation, nitration, oxidation and ubiquitination, which can impact NIF interactions and dynamics [6].

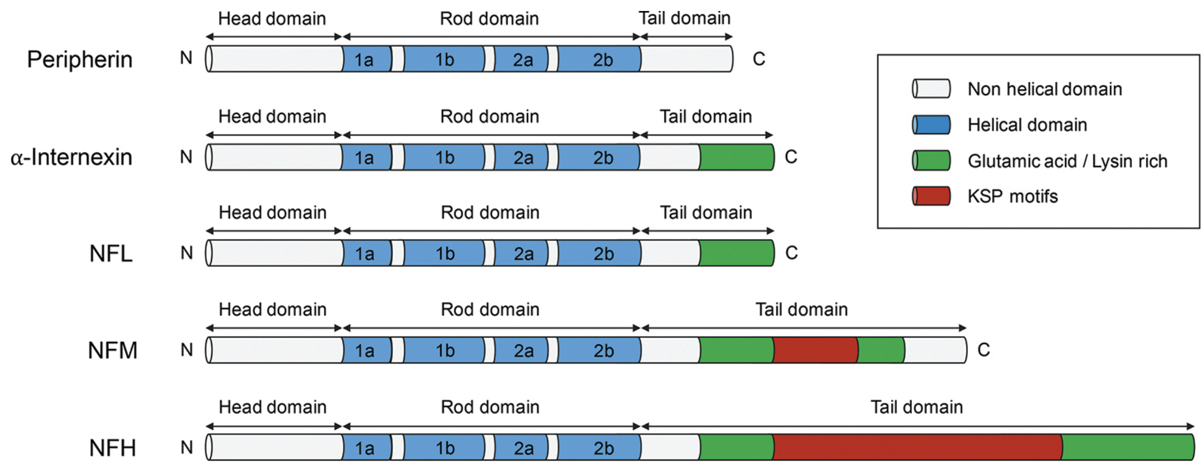

Figure 1. Schematic representation of adult neuronal IF subunits. All NIF subunits share a highly conserved central helical domain of 310 amino acid residues involved in the formation of coiled-coil structures. Flanking this central rod domain are the amino- and the carboxy-terminal domains conferring functional specificity to the different types of NIF proteins. The NFM and NFH carboxy-terminal regions contain Lys-Ser-Pro (KSP) repeats, which can be phosphorylated. Abbreviations: NF, neurofilament; NFL, NF-light; NFM, NF-medium; NFH, NF-heavy; C, carboxy-terminal; N, amino-terminal.

Multiple aspects of IF biology are regulated by their post-translational modifications. The phosphorylation state of NIF proteins depends on a dynamic balance between the activities of kinases and phosphatases. Phosphorylation of the head domain by secondary-messenger-dependent protein kinase A (PKA) and protein kinase C (PKC) prevents NIF subunits assembly or leads to the disassembly of pre-existing filaments [13, 14]. Phosphorylation of the KSP motifs on NFM and NFH tail domains by cyclin-kinase Cdk5 and microtubule-associated protein (MAP) kinase promotes the formation of cross-bridges with MTs and slows NF axonal transport [15, 16]. Phosphorylation of the head and tail domains is closely related; indeed, phosphorylation of NFM head domain by PKA reduces the phosphorylation of tail domain by MAP kinases [17]. This mechanism could be a way to protect neurons from abnormal accumulation of phosphorylated NIFs in perikarya. NIF dephosphorylation is mainly catalysed by phosphatase 2A; dephosphorylation of the head domain is necessary to allow NIF polymerization and transport into the axon, while dephosphorylation of the tail domain facilitates their interaction with other cytoskeletal proteins and their degradation [18, 19].

NIFs are also post-translationally modified by glycosylation and nitration. Glycosylation resides on attachment of *O*-linked *N*-acetyl glucosamine (O-GlcNAc) to S and threonine (T) residues; the precise function of glycosylation is still unknown, but several clues suggest a role in the NIF assembly [20]. NIF nitration is catalysed by superoxide dismutase 1 (SOD1) on tyrosine residues; the nitration of NIFs changes hydrophobic residues into negatively charged hydrophilic residues, thereby disrupting their assembly and stability.

1.3. Transport, assembly and degradation

Following their synthesis in the cell body, NIF proteins are assembled into filamentous structures and transported into the axons. They are transported bidirectionally in the axon along microtubules using kinesin (anterograde) or dynein (retrograde) motor proteins [21, 22]. Studies analysing the transport of green-fluorescent protein (GFP)-tagged NIF subunits have shown that NIFs are transported intermittently in axons, their movements being interrupted by prolonged pauses. Only a small fraction of NIFs moves at any given time and direction, and approximately 97% of NIFs spent their time pausing [23–25]. The direction of NIF transport is modulated by their phosphorylation status, since phosphorylation promotes their release from kinesin and increases their affinity for dynein [22, 26].

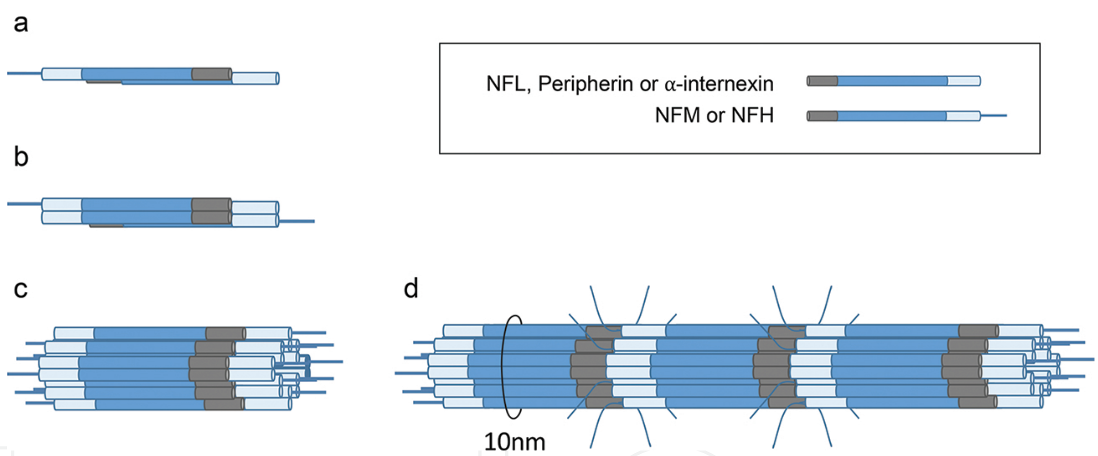

Figure 2. Schematic model of IF assembly in mature neurons. Two NIF subunits (NFL and either NFH or NFM) form head-to-tail coiled-coil dimers (a), anti-parallel half-staggered tetramers (b), protofilaments (c) and 10-nm NF (d). C-terminal domains of NFM and NFH form lateral projections and participate in the stabilisation of the filament network [33].

NIF subunits can assemble into filaments as soon as they are expressed in neurons, depending on their post-translational modifications. Subunits can also disassemble and reassemble during their transport. NIF assembly does not require nucleotide binding or hydrolysis. The first step of the filament formation is the dimerisation of an NFL subunit with either an NFM or an NFH subunit, via the association of their 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. Tetramers combine to form protofilaments, which finally assemble to constitute the final 10-nm filament [27, 28] (**Figure 2**). The C-terminal domains of NFM and NFH form lateral projections extending from the filament core [29]. Those projections participate to the stabilisation of the filament network and interact with other filament structures and subcellular organelles. Peripherin and α-internexin can co-assemble with NFL, NFM and NFH to form NIFs in mature neurons, respectively, in the peripheral and in the central nervous system [30–32]. Thus, NIFs are heteropolymers composed of different subunits, with a ratio changing during neuronal development and activity. This stoichiometry is particularly important and can lead to severe NF disorganisation when unbalanced.

In normal neurons, non-phosphorylated NIFs are found primarily in the soma and proximal axons, while phosphorylated NIFs are located more distal in axons and in terminals [34]. Inside the axon, NIFs are organised into a three-dimensional array interconnected with the other components of the cytoskeleton by several cross-bridges. NIFs, microtubules and actin filaments are interlinked by proteins of the plakin family including, among others, plectin, bullous pemphigoid antigen-1 protein (BPAG1), actin cross-linking factor 7 (ACF7), desmoplakin, envoplakin and periplakin [35–38]. Lateral projections of NFH and NFM tails also fasten adjacent structures (**Figure 3**).

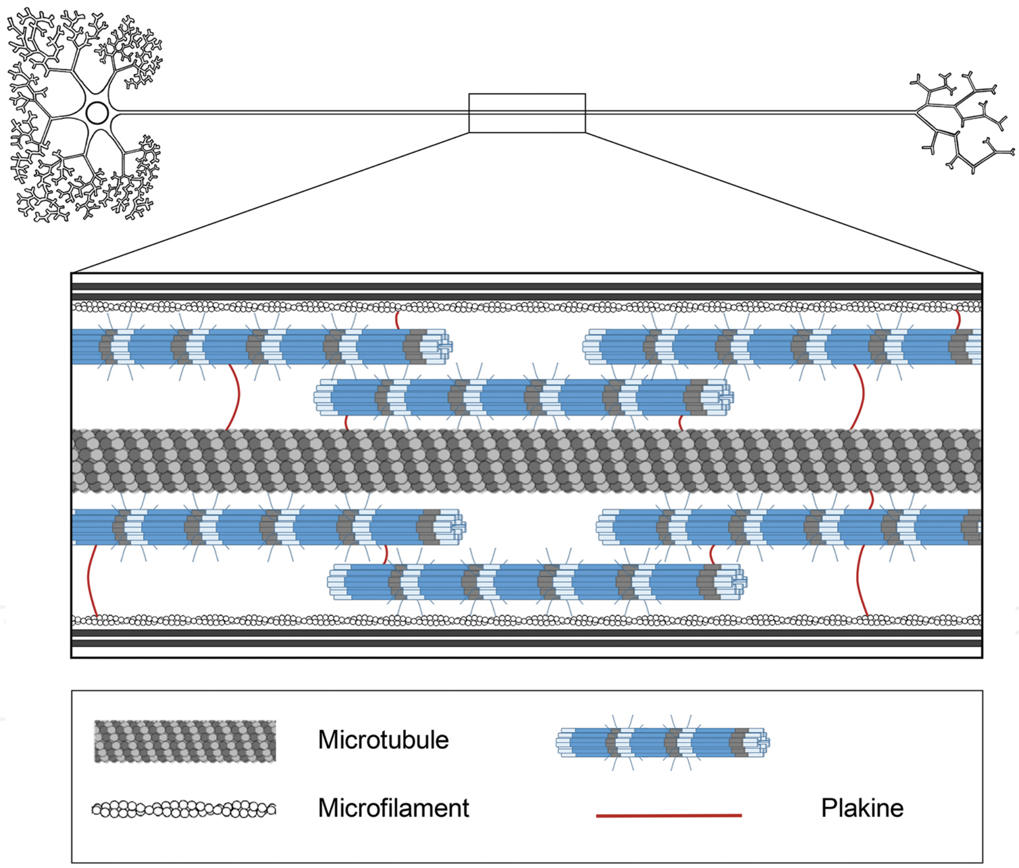

Figure 3. Schematic representation of the cytoskeleton organisation in axons. The components of the axoplasm are organised into a three-dimensional array interconnected by NFM and NFH tails and plakin-family proteins [39].

Following their synthesis, assembly and disassembly, NIFs are slowly transported towards the nerve terminal where they are degraded by specific calcium-activated proteases, such as calpain I, and neutral proteases. NIFs are also degraded by non-specific proteases like cathepsin D, trypsin and α-chymotrypsin. As mentioned above, post-translational modifications regulate NIF degradation: for example, phosphorylation protects NIFs from proteolysis, while ubiquitination facilitates their degradation [40, 41].

1.4. Roles

As members of the cytoskeletal system, NIFs work together with microtubules and microfilaments to enhance structural integrity and cell shape [42]. In the last decades, it has become increasingly apparent that IFs, instead of being inert, are in fact highly dynamic structures [43] relaying signals from the plasma membrane to the nucleus [44], orchestrating the position and function of cellular organelles [45] and regulating protein synthesis [46]. These interactions are principally mediated through NIF-associated proteins that can modulate NIF structure and function. Linker proteins such as Fodrin, Hamartin or MAP2 are responsible for NIF interactions with filaments and organelles [29, 47, 48], whereas enzymes (principally kinases and phosphatases) modulate their architecture, assembly and spacing.

Another major role recognised for NIFs is to modulate the calibre of axons, with a direct repercussion on the axonal conduction velocity, myelin thickness and inter-nodal length. Indeed, NIF density is correlated with axonal calibre in sciatic nerve fibres of rats and mice [49]. Moreover, the axonal radial growth during axonal development or regeneration coincides with the entry of NFs into axons [50]. In the same way, triple heterozygous knockout mice (NFL±, NFM± and NFH±), with a reduction of NF content but with a normal structure and stoichiometry of the NIF network, exhibit a 50% decrease of the axonal diameter in L5 ventral root [51]. Finally, the disruption of the NFM gene expression or the deletion of its carboxy-terminal domain in mice reduces the inter-filament spacing and axonal calibre, illustrating the preponderant role of NFM in determining axonal diameter [52, 53]. The phosphorylation state of NFM and NFH carboxy-terminal domains might be linked to axon calibre control by regulating NF transport and inter-filament spacing, but the exact mechanisms remain unknown.

Thus, NIFs have a central role in cell architecture, dynamics of the organelles, axon structure and calibre. Therefore, defects in their metabolism could lead to neurodegenerative processes.

2. Implication in amyotrophic lateral sclerosis

2.1. Clinical features

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterised by the loss of motor neurons of the spinal cord, brain stem and motor cortex. Common clinical symptoms of the disease are progressive paralysis, muscle atrophy and death within 2–5 years usually from respiratory failure [54]. Although most cases are sporadic (sALS), approximately 10% of ALS patients have a positive family history (fALS). To date, there is no curative treatment of the disease.

Primary evidence for a contribution of NIFs in ALS pathogenesis came from neuropathological observations. Most of all, ALS is characterised by the loss and degeneration of upper motor neurons in the motor cortex (Betz cells), and lower motor neurons in the brainstem (cranial motor nuclei) and spinal cord (anterior horn) [55]. One of the hallmarks of both sporadic and familial ALS is the presence of inclusion bodies in the perikarya of degenerating motor neurons, described as Lewy body-like inclusions (LBLIs), Skein-like inclusions (SLIs) or hyaline conglomerate inclusions (HCIs). Other typical images observed in the disease are motor neurons with swollen argyrophilic perikarya, and large swellings of the proximal part of the axons called spheroids. In immunocytochemical studies, these abnormalities have been shown to contain several proteins, such as ubiquitin or stable tubule-only polypeptide (STOP) [56], but they are particularly reactive for neurofilament subunits [57, 58] and peripherin [59, 60] (**Figure 4**). Interestingly, NIF inclusions in the cell body and the proximal axon are hyperphosphorylated, while as mentioned above in normal neurons NIFs are dephosphorylated in those sites and only phosphorylated in more distal part of the axon.

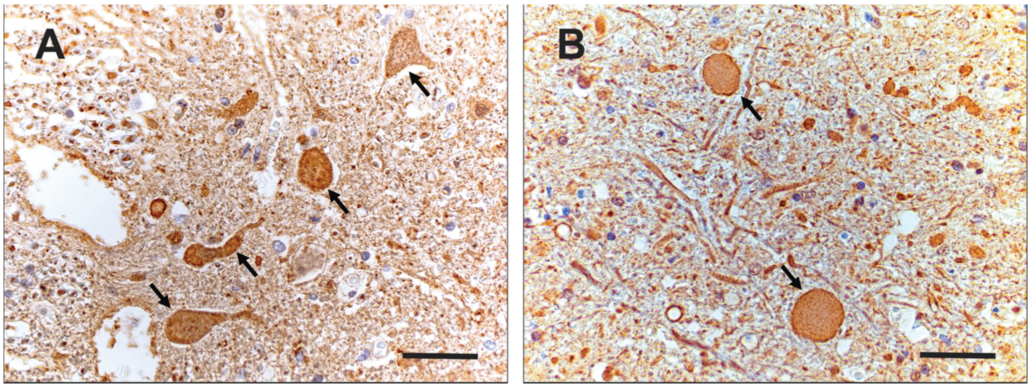

Figure 4. Neuropathological features in ALS. Immunohistochemistry for neurofilaments subunit (phosphorylated form): diffuse labelling in neuronal swelling perikarya (a) and axonal spheroids (b) in ventral horn of cervical spine. Scale bars, 20 μm.

Evidence for the involvement of NIFs in the pathogenesis of ALS has been reinforced in the last 20 years by the discovery of NIF gene mutations linked to the disease. Indeed, codon deletions and insertions in *PRPH* and *NEFH* genes have been identified in several sporadic ALS patients [61–64]. Although these mutations are not considered as a cause of familial ALS, they could be a risk factor for sporadic ALS occurrence.

Other evidences came from several studies showing that cerebrospinal fluid NIF levels are significantly higher in ALS patients than in patients with other neurodegenerative diseases, especially for those with rapidly progressive disease [65, 66]. Although their contributions to ALS pathogenesis remain unclear, all these clinical and neuropathological features suggest that NIFs represent a component of the pathological mechanisms of the disease.

2.2. Animal model contributions

On the basis of these findings, several animal models have been developed, including mice knockout for NIF genes, and mice expressing mouse, human and modified NIF subunits. While deletions of NIF genes have limited phenotype and thus are not extensively used to study ALS pathogenesis, the axonal calibre reduction seen in knockout mice for NFL, NFM and NFH genes demonstrated that neurofilaments play an important role in the radial growth of axons (**Table 2**). Interestingly, transgenic mice overexpressing either NFL, NFM, NFH, human NFH, peripherin or a mutated NFL show clinical and/or neuropathological alterations similar to those found in ALS (**Table 3**). Finally, in order to investigate NF dynamics, NFH-LacZ and NFH-GFP mice have been generated; while NFs are retained in cell bodies and deficient in axons in NFH-LacZ mice, the fluorescent fusion protein is normally transported along axons in NFH-GFP mice, suggesting that β-galactosidase reporter alters the fusion protein dynamics whereas GFP does not [67, 68]. All these animal models are therefore very useful to study the processes underlying NIF accumulation and their role in motor neuron death.

Mice	Motor dysfunction	Axonal calibre reduction	References
NFL -/-	No	>50%	[69]
NFM -/-	No	>50%	[70]
NFH -/-	No	10%	[71]
α-Internexin -/-	No	No	[72]
Peripherin -/-	No	No	[73]

Table 2. Knockout mice for NIF genes.

Mice	Motor dysfunction	NF inclusions	References
Mouse NFL	Yes	Spinal motor neurons and DRG	[74]
Mouse NFM	No	Spinal motor neurons and DRG	[75]
Mouse NFH	No	Spinal motor neurons and DRG	[76]
Human NFL	No	Thalamus and cortex	[77]
Human NFM	No	Cortex and forebrain	[78]
Human NFH	Yes	Spinal motor neurons and DRG	[79]
Mutated NFL (tail)	Yes	Spinal motor neurons and DRG	[80]
α-Internexin	No	Purkinje cells	[81]
Peripherin	Yes	Spinal motor neurons	[82]

Table 3. Mice overexpressing neuronal IF genes or expressing mutated neuronal IF proteins.

2.3. Pathophysiological hypotheses

Accumulation of neurofilaments in motor neurons undeniably participates in the pathogenesis of ALS, breaking perikarya and axonal structures, disrupting organelles dynamics and interactions, and affecting axonal transport. However, it is still difficult to determine whether

NIF aggregations are the cause or consequence of the disease. For example, the motor neuron loss caused by SOD1G85R mutation is still present despite the absence of NFL in transgenic mice [83, 84], but the animal's lifespan is prolonged by approximately 15%, suggesting an increased neuron toxicity when NFs are present in SOD1-mediated disease.

The mechanisms governing the formation of IF aggregates in ALS remain unclear because multiple factors can potentially induce the accumulation of NIFs. Firstly, these accumulations could result from perturbations of NIF transport through their abnormal phosphorylation, leading to accumulation in cell bodies and in proximal axons. Glutamate excitotoxicity could be involved in this process by activating mitogen-activated protein kinases and protein kinase N1 [85, 86]. Direct disruption of the transport motors themselves could also result in NIF accumulation, as it has been demonstrated in transgenic mice harbouring mutations or modified expression in kinesin and dynein genes [87]. Finally, one of the emerging hypotheses is that the aggregation of NIFs in ALS could result from their altered stoichiometry. Indeed, overexpression of NFL, NFM or NFH in mice provokes NF aggregations and morphological alterations similar to those found in ALS [74–76]. Remarkably, the motor neuron disease caused by excess of human NFH in transgenic mice can be rescued by a correct stoichiometry with the co-expression of human NFL transgene in a dosage-dependent fashion [88]. In a similar way, the onset of peripherin-mediated disease in transgenic mice overexpressing *PRPH* is accelerated by the deficiency of *NEFL* [82], peripherin interacting with NFM and NFH to form disorganised NIF structures. Another interesting point supporting this hypothesis is that NFL mRNA level is 70% decreased in degenerating motor neurons from ALS patients [89]. This could be due to reduced transcript stability, with a possible involvement of mutated SOD1 and TAR DNA-binding protein (TDP-43) that can bind and destabilise NFL mRNA [90, 91].

2.4. The paradox concerning perikaryal versus axonal aggregation of NIF, and the protective effect of perycarial NFH accumulation

Transgenic mice carrying mutant SOD1 transgenes develop neuronal, clinical and pathological features similar to those observed in ALS [92]. Surprisingly, the removal of axonal NIF by crossing the SOD1 transgenic mice with the NFH-LacZ transgenic mice does not affect the pathogenesis induced by SOD1 suggesting that axonal neurofilament aggregation is not the cause of ALS [93]. On the other side, overexpression of mouse NFL and NFH in SOD1G93A mice and overexpression of human NFH in SOD1G37R mice increase their lifespan by, respectively, 15 and 65%, associated with an increase of perycarial NF inclusions and a decrease of axonal spheroids (**Table 4**). Taken together, these last results suggest a protective effect of perikaryal accumulation of NFH proteins in motor neuron disease caused by mutant SOD1. Several hypotheses have been proposed to explain this protective effect. One possibility is that NF proteins may act as calcium chelators thanks to their multiple calcium-binding sites [94]. It also cannot be excluded that the accumulation of NFs could interfere with glutamate receptors and prevent glutamate excitotoxicity [95]. Finally, NF inclusions may act as a phosphorylation sink for cyclin-dependent kinase 5 or for toxic oxygen radical species induced by mutant SOD1, thereby reducing damage to other essential cellular components [96].

Mice	Lifespan	Perycarial NF inclusions	References
SODG85R – NFL -/-	Increased by 15%	No change	[84]
SODG93A – NFL overexpression	Increased by 15%	Increased	[97]
SODG93A – NFH overexpression	Increased by 15%	Increased	[97]
SODG37R – human NFH overexpression	Increased by 65%	Increased	[98]

Table 4. Effects of NF changes in SOD1-mediated disease.

3. Future directions

Implications of NIF abnormalities in the pathogenesis of ALS remain unclear. Despite extensive studies over the past 20 years, it is still unknown how these abnormalities occur and what are their exact contributions to the disease pathogenesis. Understanding how they are formed remains an important objective in the study of both sporadic and familial forms of the disease. Perhaps, the analysis of future generation of mouse models with new familial ALS mutations or conditional control of abnormal NIF proteins will help to address this issue.

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