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Chapter II

Neurofilament Proteins in Brain Diseases

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Abstract

Neurofilaments are the main components of intermediate filaments in neurons, and are expressed under three different subunit proteins, NFL, NFM and NFH. Neurofilaments act with microtubules and microfilaments to form and maintain the neuronal structure and cell shape. Phosphorylation is the main post-translational modification of neurofilaments, which influences their polymerization and depolymerization, and is responsible for their correct assembly, transport, organization and function in the neuronal process. In particular, phosphorylation is essential for the repulsion of the neurofilament polymers in axons, which determines the axonal diameter and the velocity of electrical conduction. The phosphorylation state of neurofilaments is regulated in a complex manner, including interactions with the neighbouring glial cells.

Abnormal expression, accumulation or post-translational modifications of neurofilament proteins are found in an increasing number of described neurological diseases, such as amyotrophic lateral sclerosis, Parkinson's, Alzheimer's and Charcot-Marie-Tooth diseases, or giant axonal neuropathy. Some of these diseases are associated with mutations discovered in the neurofilament genes. Recently, altered expression and phosphorylation states of neurofilament proteins have also been shown in metabolic diseases affecting the central nervous system either during development or in adulthood, such as hepatic encephalopathy due to hyperammonemia, methylmalonic and propionic acidemias, and diabetic neuropathy. Finally, accumulation of neurofilament proteins in

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the cerebrospinal fluid has been described as discriminating marker for patients with multiple sclerosis, and as predictor of long-term outcome after cardiac arrest. This review will focus on the most recent investigations on neurofilament proteins in neurodegenerative, neurodevelopmental and metabolic diseases, as well as on the use of neurofilaments as markers of diseases.

Keywords: Neurofilaments, phosphorylation, neurodegenerative diseases, metabolic diseases, neurodevelopmental diseases, axon.

Introduction

Three types of filament proteins compose the cellular cytoskeleton: microtubules (Ø: ~25 nm), microfilaments (Ø: ~7 nm) and intermediate filaments (IFs; Ø: ~10 nm). Microtubules are essentially made of tubulin, and are involved in maintaining cell shape, in mitosis (formation of spindle fibers) and in the mouvement of organelles or vesicles. Actin is the main component of microfilaments, which are responsible for cell movements, muscular contraction, cytokinesis, mechanical strength, and, more specifically in CNS, axonal outgrowth and synaptic plasticity. Depending of the cell identity, a greater variety of proteins are found in IFs, which are prominent in cells that must withstand important mechanical stress, and are classified in five different types. The most important IFs in neurons are neurofilaments (NFs), which belong to type IV IFs and are exclusively neuronal. NFs establish an extremely stable tubular system of the neuronal cytoskeleton, having a 10 nm diameter. While NFs have been identified as structures since more than 100 years with the discovery of the silver staining technique, their precise roles in neuronal cytoskeleton have remained elusive until recently.

NFs are heteropolymers made of 3 different subunits: light (NFL), medium (NFM) and heavy (NFH) chain neurofilaments (Figure 1). These subunits assemble in a filamentous structure composing the main part of the axonal cytoskeleton. NFs interact with neighbouring cellular structures or other elements of the cytoskeleton through side arms protruding ouside of their filamentous structure. Their assembly in heteropolymers, as well as their interactions with neighbouring cellular structures, are regulated by post-translational modifications, from which the most important is phosphorylation, occuring in their head and side arms domains (Figure 1). Part of these post-translational modifications of NFs are regulated by glial cells in axonal vicinity. NFs participate to the rigidity of the axon, to its tensile strength, and to the regulation of axonal calibre. In that sense, NFs are essential to the formation and maintenance of the neuronal cell shape, and particularly of the axon, a structure with a diameter of 1 to 25 µm extending sometimes 100'000 times farther (1 m or more) than the neuronal cell body (10 to 50 µm in diameter). NFs also participate to the transport guidance of organelles and particles along the axon.

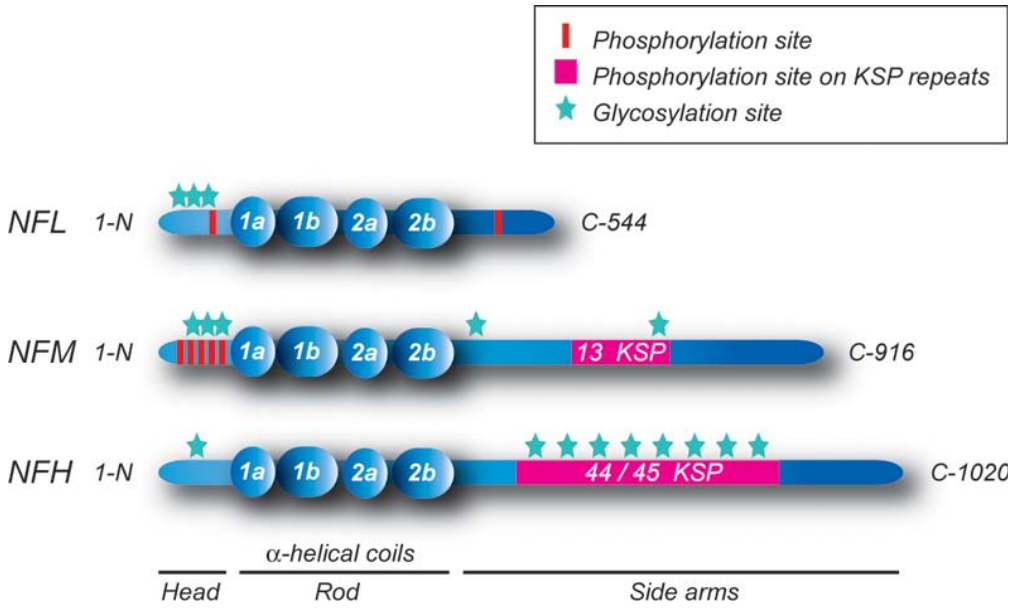

Figure 1. Schematic representation of human NFL, NFM and NFH proteins. Head, rod (α-helical coils) and side arms domains are indicated, as well as phosphorylation (including on KSP repeats) and glycosylation sites.

These last years, an increasing list of human brain diseases have been associated with NFs proteins. NFs proteins per se can be altered, either by mutations in their genes, or by alteration of their post-translational modifications, and particularly their phosphorylation state. The abnormal accumulation of neurofilaments have been observed in many neurodegenerative diseases, including Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) or Charcot-Marie-Tooth (CMT) disease. More recently, altered expression and phosphorylation states of NFs have also been shown in metabolic diseases affecting the central nervous system either during development or in adulthood, such as hepatic encephalopathy due to hyperammonemia, methylmalonic and propionic acidemias, and diabetic neuropathy. Finally, the extracellular release of NFs proteins, due to axonal mechanical break-down or damage, and their accumulation in the cerebrospinal fluid can be followed as discriminating markers for patients with multiple sclerosis, and as predictor of long-term outcome after cardiac arrest.

This review will discuss NFs proteins expression and assembly in filamentous tubular structures, as well as their post-translational modifications. Focus will be made on the most recent NFs investigations in neurodegenerative, neurodevelopmental and metabolic diseases, and on the use of NFs as markers of diseases.

Neurofilament Proteins

NFs, as peripherin, α-internexin and nestin, belong to type IV IFs, with which they share common sequence structures. Three NF subunits contribute to the assembly of neurofilaments: Light (NFL), medium (NFM) and heavy (NFH) chain NFs (Figure 1). Human

NFL is encoded by the *NEFL* gene located on chromosome 8 (8p21) and consists of 544 amino acids. Human NFM is encoded by the *NEFM* gene also located on chromosome 8 (8p21) and consists of 916 amino acids. Human NFH is encoded by the *NEFH* gene located on chromosome 22 (22q12.2) and consists of 1020 amino acids. NFL, NFM and NFH have a molecular weight of 60, 100 and 110 kDa respectively, calculated on their amino acid sequence; however, due to important posttranslational modifications (i.e. phophorylation and glycosylation), NFL, NFM and NFH exhibit higher molecular weight on SDS-PAGE: 68 kDA, 160 kDa and 205 kDa respectively (for reviews, see: Lee and Cleveland, 1996; Parry and Steinert, 1999b; Al-Chalabi and Miller, 2003; Liu et al., 2004; Lariviere and Julien, 2004).

NFs are exclusively expressed by neurons. IFs, including NFs, are expressed differentially during CNS development and maturation. Undifferentiated brain cells express the type III IF protein vimentin (Bignami et al., 1982; Cochard and Paulin, 1984), while later neuroblasts express nestin, α-internexin, and peripherin (Portier et al., 1983; Lendahl et al., 1990; Kaplan et al., 1990). The neuronal differentiation induces the expression of NFs (Shaw and Weber, 1982; Carden et al., 1987; Nixon and Shea, 1992). NFL appears first at the start of neuronal differentiation, overlapping with α-internexin and peripherin expression (Willard and Simon, 1983; Carden et al., 1987). NFM follows NFL shortly after, when neurite elongation starts, *NEFL* and *NEFM* genes being located on the same chromosome and regulated in coordination. NFH appears later during axonal maturation (Willard and Simon, 1983; Carden et al., 1987).

NFs, as all IF proteins, share a common structure. In the centre of the protein, a rod domain of approximately 310 amino acids forms highly conserved α-helical motifs (regions 1a, 1b, 2a and 2b, Figure 1). Every seventh residue in this central rod domain is hydrophobic, facilitating the formation of α-helical coiled-coil parallel homo- or heterodimers (see below). The central rod domain is flanked by less conserved aminoterminal globular head and carboxyterminal side-arm tail. Head and tail confer their functional specificities to the different IF proteins: whilst the central rod domain is mainly responsible for NF assembly, head and tail interact with the environment of NFs (e.g. protein-protein interactions or axonal diameter) (Heins et al., 1993). The head domain also contributes to NF assembly (Gill et al., 1990). NFs are obligate heteropolymers in vivo, with NFL being required to form proper heteropolymers with either NFM or NFH (Lee et al., 1993; Ching and Liem, 1993). The dimer is formed by the head to tail coiled apposition of two NF proteins (NFL and either NFM or NFH) by their central rod domain. Two NF dimers assemble then in an half-staggered antiparallel NF tetramer (Cohlberg et al., 1995). The final 10 nm filament of NFs is formed by the lateral and longitudinal helical association of eight NF tetramers (Heins and Aebi, 1994; Fuchs and Weber, 1994; Fuchs and Cleveland, 1998; Parry and Steinert, 1999a; Herrmann and Aebi, 2000). The other IF proteins α-internexin and peripherin may also co-assemble, as homodimers however, with the NF heterodimers, especially during development (α-internexin, peripherin) and in restricted sets of mature neurons (peripherin) (Kaplan et al., 1990; Fliegner et al., 1994; Beaulieu et al., 1999). During neuronal differentiation (i.e.: neurite formation, axonal growth and maturation), the nature of the NF fibers changes, starting with heterodimers NFL-NFM only, followed, once NFH starts its expression, by NF fibers constituted of NFL-NFM and NFL-NFH heterodimers (Carden et al., 1987). Along

time, a specific NF tetrameric unit can be replaced by another, explaining the differential stoichiometry observed in the NF fibers from development to mature CNS, influencing also axonal structure and functions.

NFL is essential for the precise NF assembly and for the maintenance of axonal calibre (Zhu et al., 1997). NFM participates in cross-bridges between NF fibers, stabilizes the NF filament network, participates in neurite longitudinal extension, and influences the axonal radial growth (Elder et al., 1998a; Jacomy et al., 1999; Elder et al., 1999a; Elder et al., 1999b). NFH also contributes to cross-bridges between NF fibers and may interact with microtubules, microfilaments and other cytoskeletal elements (Elder et al., 1998b; Jacomy et al., 1999; Elder et al., 1999b). In contrast to NFM, NFH does not seem to influence the axonal radial growth (Rao et al., 2002b).

NFs, after synthesis in the neuronal cell body, are then rapidly transported into the axons. Until recently, it was not clear whether NFs were transported into the axon as polymeric structures (« polymer hypothesis »), or as individual subunits (« subunit hypothesis ») (Baas, 1997; Hirokawa, 1997; Nixon, 1998). Radioisotopic pulse labeling studies argued for the polymeric hypothesis with NFs moving slowly in axons at an average rate of 0.2 to 1 mm/day, a much slower speed than any known axonal transport (Xu and Tung, 2000). On the other side, photobleaching experiments with fluorescence-tagged NFs argued for the subunit hypothesis, with the bleached axonal segment remaining stationary and slowly recovering its fluorescence (Okabe et al., 1993). The solution to this controversy came from recent works using live cell imaging and GFP-tagged NFs, that showed a fast transport of NF polymers (bursts of average speed of 1 to 2 mm/s) interrupted by prolonged pauses (Roy et al., 2000; Wang et al., 2000). As these fast bursts of NFs transport can be bidirectional, and due to the high proportion of paused NF fibers (> 90%), the resulting overall NF transport appears slow. NFs seem to use the conventional kinesin and dynein motor system (Shah et al., 2000; Yabe et al., 2000), and appear to dissociate from these motor systems after phosphorylation (Yabe et al., 1999). NFs are also translocated in dendrites of specific types of neurons, and seem required for the proper dendritic arborization of large motor neurons (Kong et al., 1998; Zhang et al., 2002).

Two major modifications are added post-translationally on NFs: phosphorylation and glycosylation. These modifications are dynamic and thought to regulate assembly, transport, structure and functions of NFs.

Various phosphorylation sites have been identified in the head (N-terminal) and tail (C-terminal) regions of NFs.

The head region of NFL and NFM can be phosphorylated at different positions (Figure 1) by protein kinases A, C and N (Sihag and Nixon, 1989; Sihag and Nixon, 1991; Hisanaga et al., 1994; Mukai et al., 1996; Cleverley et al., 1998; Nakamura et al., 2000). The phosphorylation of the NFL and NFM head region occurs rapidly after protein synthesis in the neuronal cell body, and inhibits the NF filament assembly in perikaria (Gibb et al., 1996; Gibb et al., 1998; Ching and Liem, 1999). This phosphorylation is transient, and the dephosphorylation of the NFL and NFM head region is a prerequisite for the axonal NF assembly in filaments (Gibb et al., 1998). Moreover, the transient phosphorylation of the head region of NFM also inhibits the phosphorylation of its C-terminal tail region (Zheng et al., 2003). Thus, before NF translocation in the axons, the phosphorylation of the head region

of NFL and NFM protects neurons from a pathological accumulation of NF aggregates in their cell bodies .

Upon entry of NFs into the axon, the C-terminal side-arm domain of NFM and NFH, as well as the short C-terminal region of NFL, become phosphorylated. In particular, NFM and NFH are phosphorylated on Lys-Ser-Pro (KSP) repeat domains (Figure 1). In humans, NFM has 13 KSP repeats, while NFH exists with two polymorphic forms of either 44 or 45 KSP repeats (Figlewicz et al., 1993). Most of the serine residues of the KSP repeats can be phosphorylated, meaning that each mole of NFM and NFH can contain about 10 and 50 moles of phosphate, respectively (Julien and Mushynski, 1982; Grant and Pant, 2000). In axons, more than 99% of assembled NFM and NFH proteins are phosphorylated on their KSP repeats, in particular in myelinated internodal regions, while this proportion is much weaker in cell bodies, dendrites and nodes of Ranvier (de Waegh et al., 1992; Hsieh et al., 1994). Unphosphorylated NFs represent only ~1% of total NFs in the neurons. In the axon, NFs are phosphorylated in a proximal to distal gradient (Sternberger and Sternberger, 1983; Pant and Veeranna, 1995). The C-terminal region of NFL is phosphorylated by caseine kinase II (Nakamura et al., 1999), while the kinases that phosphorylate NFM and NFH KSP repeats in their C-terminal tail domains include GSK-3α/β, cdk5/p35, ERK1/2 and JNK1/3 (Guan et al., 1991; Giasson and Mushynski, 1996; Sun et al., 1996; Li et al., 2001).

In axons, the phosphorylation of multiple KSP repeats increases the negative charge of NFM and NFH, resulting in side-arm formation of their C-terminal tail and increased inter-neurofilament spacing (Nixon et al., 1994). This allows the radial axonal growth (i.e. regulation of axonal caliber), which increases axonal conduction velocity (de Waegh et al., 1992; Yin et al., 1998). The C-terminal phosphorylation of NFs also slows down their transport rate in axons, and mediate interactions with other cytoskeleton proteins, in particular microtubules (Hisanaga et al., 1991; Yabe et al., 2001; Shea et al., 2003). The phosphorylation of NFM seems preferentially responsible for the radial axonal growth, while the phosphorylation of NFH acts on the NF transport rate and their interactions with other proteins (Lewis and Nixon, 1988; Rao et al., 1998; Rao et al., 2003). The myelination of axons, both by Schwann cells in peripheral nerves and by oligodendrocytes in CNS, promotes the phosphorylation of NFM and NFH C-terminal tail, thus promoting the radial growth of myelinated axons and increasing their conduction velocity (de Waegh et al., 1992; Sanchez et al., 1996; Yin et al., 1998; Sanchez et al., 2000).

NFL, NFM and NFH are also post-translationally glycosylated by addtion of O-linked N-acetylglucosamine moieties on serine and threonine residues located in their head regions (NFL, NFM and NFH) as well as in their KSP repeat carboxyterminal region (NFM and NFH) (Figure 1) (Dong et al., 1993; Dong et al., 1996). The proximity of the O-GlcNAcylation and phosphorylation sites in the NF head domain suggest that competition between the two modes of post-translational modifications regulates NF assembly (Gill et al., 1990; Wong and Cleveland, 1990; Chin et al., 1991; Dong et al., 1993). On the other hand, in the nodes of Ranvier where NFs are more closely packed than in the internode axonal segments, O-GlcNAcylation probably replaces phosphorylation in the carboxyterminal KSP repeat region of NFM and NFH, rendering interactions between NFs more attractive than repulsive.

Therefore, phosphorylation / dephosphorylation and glycosylation / deglycosylation of NFs (by kinase / phosphatase and O-GlcNAc transferase / *N*-acetyl-β-D-glucosaminidase respectively) contributes to the assembly, structure and functions of NFs (Dong et al., 1993; Nixon, 1993; Xu et al., 1994; Dong et al., 1996).

Many neurons extend very long axons, up to 1 m in humans. To maintain the integrity and functions of these axons, some of their structural proteins, including those of the axonal cytoskeleton, have long lifetimes. For NFs in the human sciatic nerve, this average lifetime was estimated to 1 to 2 years (Lee and Cleveland, 1996). This very high stability of NFs is thought to be due, at least in part, to their phosphorylation which protects them from protease degradation (Goldstein et al., 1987; Pant, 1988). In physiological conditions, NF degradation only occurs in the axon terminus (presynaptic compartment), where NFs are dephosphorylated by protein phosphatase 2A (PP2A) (Gong et al., 2003), and then digested by calmodulin, a Ca^{++}-dependent protease (Maxwell et al., 1997).

Apart from their major role in regulating axonal caliber in function of their state of phosphorylation, NFs have been demonstrated or are postulated to have other functions in the axon. While gene knockout experiments demonstrated that NFs are not essential for axonal elongation, they nervertheless might facilitate it by stabilization of cytoskeletal elements and inhibition of axonal retraction (Zhu et al., 1997; Elder et al., 1998a; Elder et al., 1998b; Elder et al., 1999a). NFs participate, together with microtubules and microfilaments, to the axonal structural integrity, to the neuronal shape as well as to the axonal mechanisms of transport. They do so by direct or indirect interactions with microtubules (Hisanaga et al., 1991) or motor proteins like dynein, kinesin and myosin Va (Yabe et al., 1999; Shah et al., 2000; Yabe et al., 2000; Rao et al., 2002a), or with other crosslinking proteins like dystonin (Yang et al., 1999; Chen et al., 2000). NFM has been shown to interact with the D(1) dopamine receptor in subsets of neurons (Kim et al., 2002). Finally, of peculiar importance for the neuronal and axonal long term stability, NFs seem to protect axons from toxic components, by sequestrating for example Cdk5/p25 complexes which induce apoptosis (Nguyen et al., 2001), or by coupling of carbonyl groups issued of the oxidative stress on the lysine residues of KSP repeats (Wataya et al., 2002).

Neurofilament Proteins in Brain Diseases

As discussed above, the tight regulation of NF subunits expression, post-translational modifications, stoichiometry between NFL, NFM and NFH, and NF axonal transport, allows the correct assembly of NF filaments. This in turn contributes to the normal axonal growth, maturation, and stability along time. Any dysregulation of these precise mechanisms of NF regulations is susceptible to induce severe pathological consequences on neurons. In particular, the hallmark of numerous human neurological diseases is the abnormal accumulation of NFs in neuronal perikarya (for recent reviews, see Al-Chalabi and Miller, 2003; Liu et al., 2004; Lariviere and Julien, 2004; Petzold, 2005), which alters axonal growth, mechanisms of particles and organelles transportation, stability, and dynamic of interactions between NFs and other axonal proteins (Herrmann and Griffin, 2002). For a long time, it was admitted that NF abnormalities in human neurological disorders were secondary

to neuronal dysfunctions. Recent studies demonstrate however that dysregulations of NFs themselves can be the cause of these pathologies. The second part of this review will focus on NF dysregulations in neurodegenerative, neurodevelopmental and metabolic diseases of central and peripheral nervous systems, as well as on the use of NFs as markers of specific diseases.

NFs in Neurodegenerative Diseases

Amyotrophic Lateral Sclerosis (ALS)

ALS is a progressive neurodegenerative disease affecting motor neurons in the brain and spinal cord, with a typical onset between 40 and 60 years of age. ALS patients usually die wihin 5 years after ALS diagnosis, due to motor neurons death and loss of function of the relative innervated muscles, and progressive partial or total paralysis. Most of the cognitive functions in ALS patients remain preserved. ALS is a heterogeneous syndrom, in which the neuropathological hallmark is an abnormal aggregation of NFs in the degenerating motor neurons (Manetto et al., 1988; Munoz et al., 1988). 5-10% of ALS cases are familial (autosomal dominant), while all the remaining cases are sporadic. 1-2% of all ALS cases (20-25% of familial ALS cases) are due to mutations in the Cu/Zn superoxide dismutase (SOD1) gene (Andersen, 2006), while the basis of the remaining ALS cases is still not known with precision. Mutations in SOD1 are thought to be linked to abnormal accumulation of NFs in ALS (Rouleau et al., 1996). Due to the abnormal accumulation and aggregation of hyperphosphorylated NFs in the ALS degenerating neurons, mutations in the NF genes have also been sought for a long time as good causative candidates for ALS. Indeed, different mutations have been found in NFs, in association with ALS (Figures 2,3,4). Codon deletions and insertions have been identified in the KSP regions of NFH in association with few sporadic cases of ALS (Figure 4) (Figlewicz et al., 1994; Tomkins et al., 1998; Al-Chalabi et al., 1999). More recently, missense mutations have also been found in the head and rod domains of NFH in other ALS cases (Garcia et al., 2006) (Figure 4). In association with ALS, the same group also identified recently a deletion in the tail domain of NFL (Figure 2), as well as missense mutations in the head, rod and tail domains of NFM (Figure 3) (Garcia et al., 2006). However, none of the mutations found in NF genes have been clearly identified as causative agent of ALS, nor linked to the familial dominantly inherited ALS (Al-Chalabi and Miller, 2003; Garcia et al., 2006), and it is thought now that these mutations in NF genes have to be considered as risk factors for sporadic ALS. However, the alteration of NF homeostasis seems to be an important part of the pathogenesis of ALS (Figures 2,3,4). As shown with mutant SOD1 transgenic models of ALS (Nguyen et al., 2001), the deregulation of specific NF kinase pathways (e.g: cdk5/p35) might cause the aberrant hyperphosphorylation of NFH and NFM side arms. This in turn might slow the axonal transport of NFs, which accumulate in neuronal perikarya (Williamson and Cleveland, 1999). The abnormal accumulation of NFs in the ALS degenerating neurons has also been associated with a significative decrease of NFL mRNA, which could increase the imbalance between NF subunits and precipitate further the neuronal degeneration (Bergeron et al., 1994;

Wong et al., 2000). This decrease in NFL mRNA seems due to the direct binding of mutant SOD1 to NFL mRNA, which destabilizes it (Ge et al., 2005). Interestingly, the two main posttranslational modifications of NFs, i.e. phosphorylation and glycosylation, might be conversely deregulated in ALS, as O-glycosylation of the C-terminal tail domain of NFM is decreased, while its phosphorylation is increased, in a transgenic rat model of ALS (Ludemann et al., 2005).

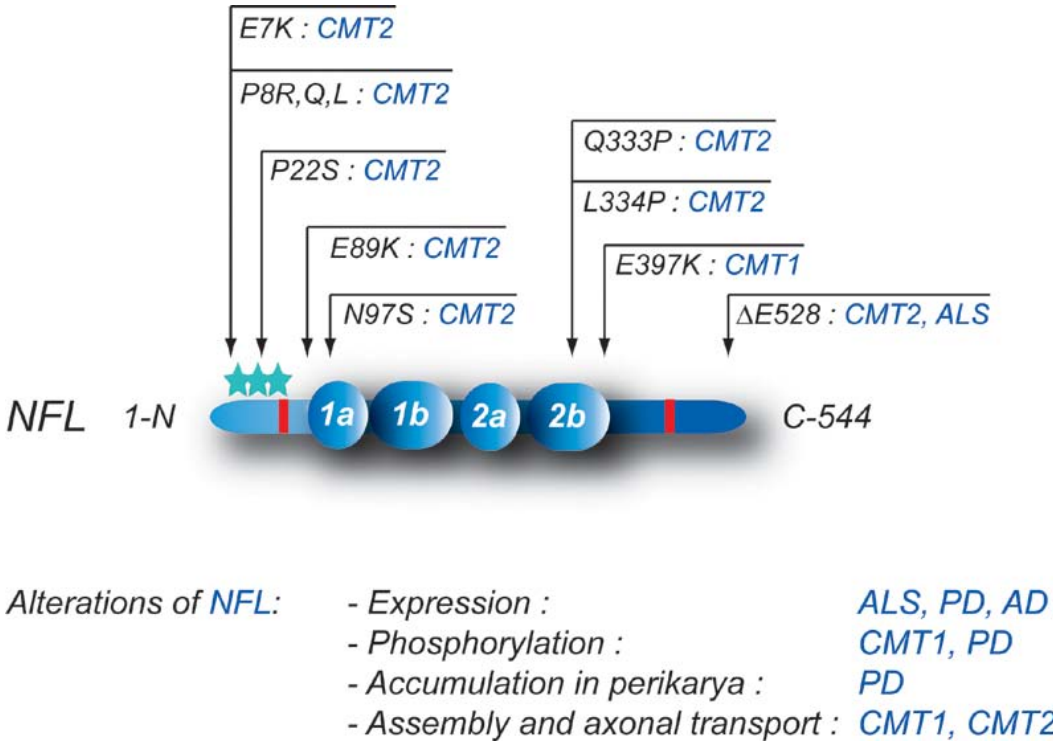

Figure 2. Schematic representation of NFL alterations in various brain diseases. Mutations identified in association with diseases are indicated above the NFL scheme, while the disease effects on NFL are indicated below the NFL scheme. AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; CMT1, CMT2: Charcot-Marie-Tooth disease; PD: Parkinson's disease; Δ: deletion.

Charcot-Marie-Tooth Disease (CMT)

CMT is the most common inherited neurological disorder of the peripheral nervous system, affecting 1-4:10'000 individuals. CMT clinical phenotype is characterized by the progressive degeneration of motor and sensory neurons in the distal part of the limbs, leading to the slow loss of normal use of feet, legs, arms and hands (Skre, 1974; Reilly, 2000). CMT neuropathies are heterogeneous in the genes involved and, based on electrophysiological criteria, are classified in CMT1, a primary demyelinating form with reduced nerve conduction velocities, and CMT2, a primary axonal loss form. Some forms of CMT with overlapping characteristics between CMT1 and CMT2 have been classified as intermediate CMT. CMT is generally inherited with an autosomal dominant pattern. Recently, different

missense mutations and one amino acid deletion have been identified in the *NEFL* gene (coding NFL) in several families in association with CMT (Figure 2) (Mersiyanova et al., 2000; De et al., 2001; Georgiou et al., 2002; Yoshihara et al., 2002; Jordanova et al., 2003; Choi et al., 2004; Zuchner et al., 2004). All these mutations are associated with the primary axonal loss form CMT2, with the exception of Glu397Lys being associated with the demyelinating form CMT1. These mutations in NFL are thought to disrupt NF assembly and axonal transport, as well as to alter NFL post-translational modifications. Other forms of CMT (CMT1) are caused by mutations in genes primarily expressed in Schwann cells and involved in myelin formation. These mutations lead to alterations in myelination, which in turn alter NFL, NFM and NFH phosphorylation states (Watson et al., 1994). The disruption of NF assembly and the alteration of NF phosphorylation states are thought to contribute, at least in part, to the CMT disease mechanisms leading to axonal degeneration.

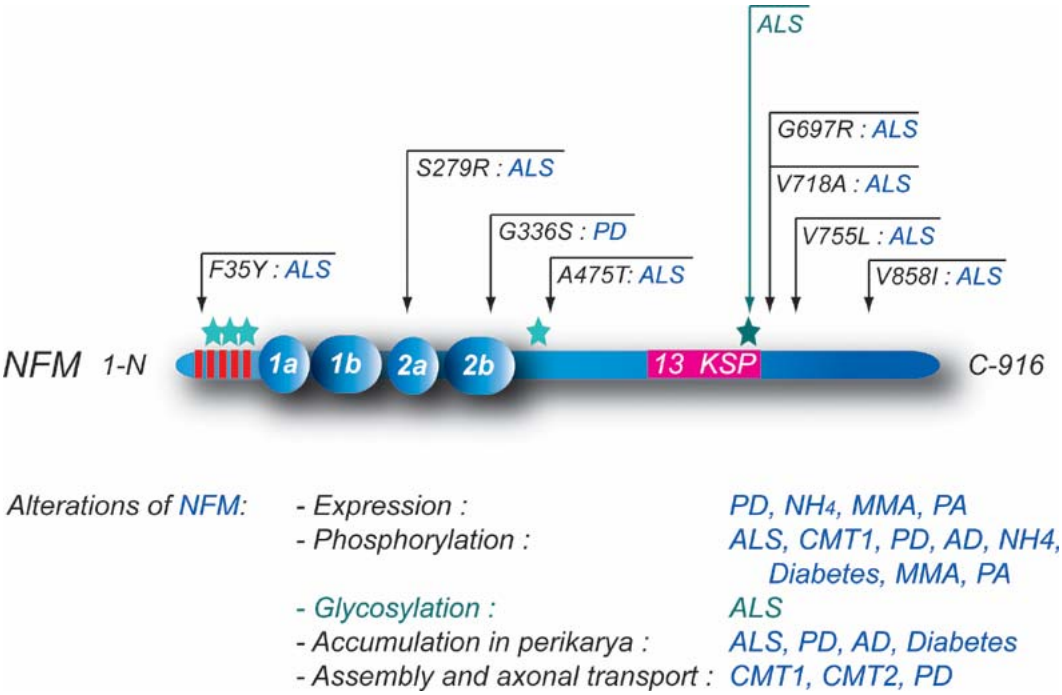

Figure 3. Schematic representation of NFM alterations in various brain diseases. Mutations identified in association with diseases are indicated above the NFM scheme, while the disease effects on NFM are indicated below the NFM scheme. AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; CMT1, CMT2: Charcot-Marie-Tooth disease; MMA: methylmalonic aciduria; NH_4: hyperammonemia; PA: propionic aciduria; PD: Parkinson's disease.

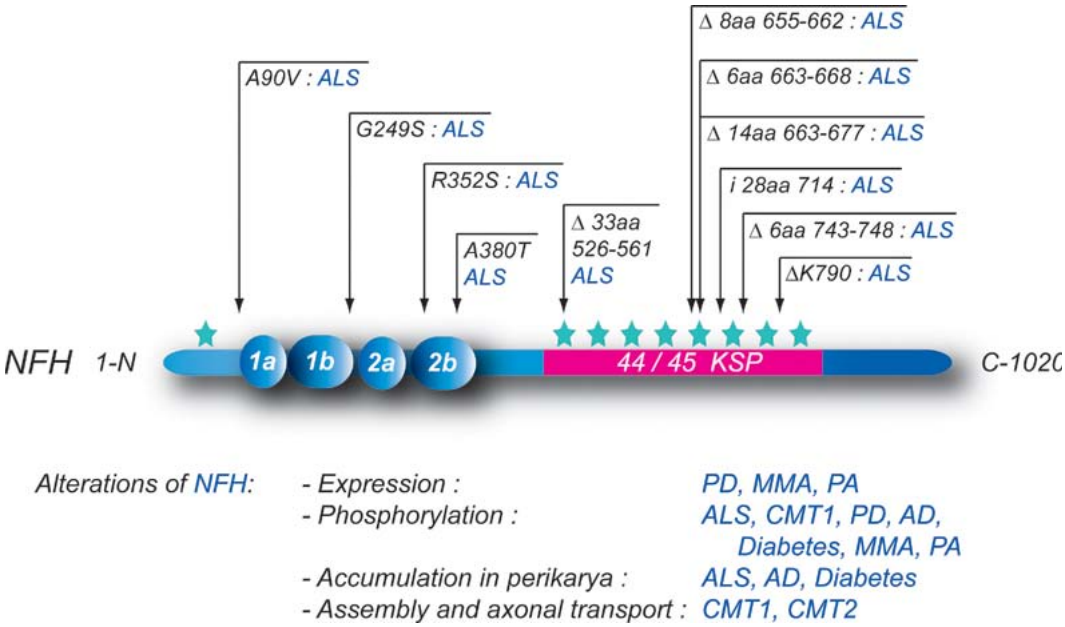

Figure 4: Schematic representation of NFH alterations in various brain diseases. Mutations identified in association with diseases are indicated above the NFH scheme, while the disease effects on NFH are indicated below the NFH scheme. AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; CMT1, CMT2: Charcot-Marie-Tooth disease; i: insertion; MMA: methylmalonic aciduria; PA: propionic aciduria; PD: Parkinson's disease; Δ: deletion.

Parkinson Disease (PD)

PD is a progressive neurodegenerative CNS disorder affecting dopaminergic neurons of substantia nigra and leading to decreased dopamine availability. The principal pathological modifications in PD affected neurons are the so-called Lewy bodies, which are inclusions of accumulated proteins in neuronal perikariya and are made of numerous proteins, including NFL, NFM and NFH, α-synuclein, ubiquitin and subunits of the proteasome (Galloway et al., 1992; Trimmer et al., 2004). In particular, abnormally phosphorylated NFs have been identified in PD associated Lewy bodies (Hill et al., 1991; Trojanowski et al., 1993), but the reasons for this alteration of NF phosphorylation have not been precisely identified so far (Figures 2, 3, 4). Familial forms of PD have also been identified, in which the principal mutations found are located in the parkin, α-synuclein and ubiquitin C-terminal hydrolase L1, all three related to cellular ubiquitin proteasomal system (Lim et al., 2003). A significative decrease of NF mRNAs and proteins has also been observed in the PD affected neurons of substantia nigra (Hill et al., 1993; Basso et al., 2004) (Figures 2, 3, 4). Recently, a point mutation in the *NEFM* gene, located in the rod domain 2b of NFM and changing Gly to Ser (Gly336Ser) (Figure 3), has been identified in a patient that developed PD very early, at the age of 16 (Lavedan et al., 2002). Due to the position of this mutation in the very highly conserved region of IFs (rod, α-helical coils) involved in their assembly mechanism, it was speculated that this mutation could alter NFM assembly into NF filaments (Lavedan et al., 2002). As this mutation has been found in only one PD patient which moreover had three

unaffected siblings (Lavedan et al., 2002; Han et al., 2005), it is not sure so far that this mutation is really causative of PD. If yes however, the NFM G336S mutation does not seem to interfere with either assembly nor cellular distribution of NFs (Perez-Olle et al., 2004), but could rather alter interactions of NFM with other PD susceptibility proteins (Al-Chalabi and Miller, 2003).

Alzheimer's Disease (AD)

Among neurodegenerative diseases, AD is the leading cause of dementia, with risks over 65 years of age varying from 6-10% for men to 12-19% for women (Seshadri et al., 1997). CNS regions involved in memory and thinking skills are the first affected, followed by neuronal death in other brain regions as disease progresses, which eventually causes the death of the patient. Despite intensive work on AD, its precise cause is still unknown. One of the important secondary features of AD is the neuronal cytoskeleton disruption, due to the inappropriate hyperphosphorylation of cytoskeletal proteins such as tau or NFs (Sternberger et al., 1985; Gong et al., 2000) (Figures 3, 4). In particular, hyperphosphorylated NFH accumulates in neuronal perikaryon and proximal axon (Sternberger et al., 1985), due most probably to an imbalance between kinase and phosphatase activities (Trojanowski et al., 1993; Maccioni et al., 2001; Veeranna et al., 2004). After accumulation in neuronal perikarya, these cytoskeletal proteins aggregate in abnormally modified filaments, and progressively form the neurofibrillary tangles and AD senile plaques, which are the hallmarks of AD. Recently, hyperphosphorylated NFM has also been identified in AD amyloid plaques (Liao et al., 2004). NFL mRNA is also significatively decreased in AD degenerating neurons (McLachlan et al., 1988) (Figure 2).

NFs in Other Neurodegenerative Diseases

The expression and post-translational modifications of NFs have been found altered in a number of other neurodegenerative conditions (summarized in figures 2, 3).

Giant axonal neuropathy (GAN) is a rare autosomal recessive neurodegenerative disorder progressively affecting both peripheral and central nervous system. GAN is due to mutations of the gene encoding gigaxonin, a protein suggested to be associated to IFs (Bomont et al., 2000; Herrmann and Griffin, 2002). GAN, due to the gigaxonin disruption, is thus characterized by the presence of giant axons filled with massive segmental accumulations of disorganized NFs (Asbury et al., 1972; Herguner et al., 2005).

A recent work has shown that leprous nerve atrophy, characterized by a diminution of axonal calibre and paranodal demyelination, might be due to dephosphorylation of NFM and NFH (Save et al., 2004).

NFH have been shown to be dephosphorylated in an experimental model of glaucoma, a neurodegenerative condition affecting the optic nerve in association with high intraoccular pressure (Kashiwagi et al., 2003).

Glutamate excitotoxicity induces a rapid degradation of the neuronal cytoskeleton. It was shown recently that glutamate toxicity, primarily mediated by NMDA receptor, initiates a rapid loss of NFs in the affected axons, while other axonal markers remain intact for a longer period (Chung et al., 2005).

Distal hereditary motor neuronopathies (dHMNs) are a heterogeneous group of disorders in which motor neurons selectively undergo age-dependent degeneration. Mutations in the small heat-shock protein HSPB1 (also called HSP27) are responsible for one form of dHMN. The mutant forms of HSPB1 seem to disrupt NF assembly, to alter axonal transport system, and lead to the accumulation and aggregation, in neuronal perikarya, of cellular components, including NFM (Ackerley et al., 2006).

Huntington's disease (HD) is caused by a polyglutamine repeat expansion in the N-terminal domain of the huntingtin protein. Huntingtin is localized in the cytoplasm where it may interact with cytoskeletal and synaptic proteins. The mechanism of HD pathogenesis remains unknown but recent investigations suggest that the mutant huntingtin found in HD might interact aberrantly with cytoskeletal proteins, including NFs, and thus affect the axonal cytoskeletal integrity (DiProspero et al., 2004).

Neuronal intermediate filament inclusion disease (NIFID) is a recently described novel neurological disease of early onset, presenting considerable variability in clinical phenotypes, including frontotemporal dementia, as well as pyramidal and extrapyramidal signs. The pathological hallmark of NIFID is the presence of abnormal aggregates of α-internexin, NFL, NFM and NFH in the affected neurons (Cairns et al., 2004). α-internexin, a class IV IF protein, has not been identified in any pathological protein aggregates of any other neurodegenerative disease.

NFs in Neurodevelopmental and Metabolic Diseases

Diabetes Neuropathy

Diabetes is associated with a symmetrical distal axonal neuropathy predominantly affecting sensory nerves and neurons of dorsal root ganglia. Diabetic neuropathy is characterized by a reduced conduction velocity, and axonal atrophy. Both in human diabetic patients and in streptozotocin-induced diabetic rats, abnormal aggregations of NFs and other cytoskeletal proteins have been observed in the affected neurons, together with an abnormal increase of NFM and NFH phosphorylation (Figures 3, 4) (Schmidt et al., 1997; Fernyhough et al., 1999). These alterations of NF phosphorylation seem to occur through the activation of the NF kinase c-Jun N-terminal kinase (JNK) (Fernyhough et al., 1999; Middlemas et al., 2006). NFs mRNAs are reduced. The affected neurons present defects of axonal transport mechanisms, a reduction in axon calibre, and a diminished capacity of nerve regeneration, all characteristics relying on the integrity of axonal cytoskeleton. It appears thus that NF abnormalities seem to be a primary cause of diabetic neuropathy, and not only a marker of the pathology (McLean, 1997). A recent work has shown that diabetic neuropathy in an experimental model, the insulin KO mouse, does not alter only peripheral axons, but also

affects central neurons, where hyperphosphorylation of NFs together with alteration of different NF kinases activities have been demonstrated (Schechter et al., 2005).

Hyperammonemia during CNS Development

Poorly understood irreversible damages to CNS development occur in neonates and infants with hepatic deficiency or inherited defects of ammonium (NH_4^+) metabolism, manifesting on the long term as mental retardation (Bachmann, 2002; Bachmann, 2003). We have shown, in brain cell 3D primary cultures exposed to NH_4^+ as experimental model of hyperammonemia during CNS development, that NH_4^+ impairs axonal growth (Braissant et al., 1999; Braissant et al., 2002). NFs appear to be affected in this process, as both NFM expression and phosphorylation are decrease by NH_4^+ exposure (Figure 2) (Braissant et al., 2002). The correct expression and phosphorylation of NFM seem to depend on levels of creatine (Braissant et al., 2002), which can be synthesized by brain cells including during development (Braissant et al., 2001; Braissant et al., 2005). Axonal growth, as well as NFM expression and phosphorylation, are protected under NH_4^+ exposure by co-treatment with creatine in a glial cell dependent manner (Braissant et al., 2002). Our results are consistent with clinical findings in hyperammonemic neonates or infants presenting irreversible brain lesions compatible with neuronal fiber loss or defects of neurite outgrowth. The alteration of NF phosphorylation under NH_4^+ exposure might occur through the dysregulation of MAPK, which are NF kinases and present altered levels of phosphorylation and activity in brain cells exposed to NH_4^+ (Schliess et al., 2002; Jayakumar et al., 2006; Cagnon et al., 2006).

Methylmalonic (MMA) and Propionic (PA) Acidemias

Among the most frequent organic acidemias, PA and MMA are due to deficiencies in propionyl-CoA carboxylase and L-methylmalonyl-CoA mutase, respectively, and lead to the increase of free propionic acid in blood and its accumulation in tissues (PA), and to the tissular accumulation of L-methylmalonic acid and secondarily of propionic acid (MMA). The levels of these metabolites in blood and cerebrospinal fluid can rise as high as 5 mM and may be even higher in neuronal cells. PA and MMA lead to chronic neurologic disabilities, seizures and developmental delay. Damages to basal ganglia, a general hypomyelination, cerebral atrophy and white matter edema are frequently encountered. So far, the exact underlying mechanisms of brain damage in PA and MMA remain to be elucidated. However, NFs might be implicated in the neuropathological aspects of MMA and PA (Figures 2, 3 4). Indeed, MMA and PA experimental models have provided evidence that neuronal NFL and NFM expression and phosphorylation are reduced under L-methylmalonic acid and propionic acid exposures (de Mattos-Dutra et al., 1997a; de Mattos-Dutra et al., 1997b; de Mattos-Dutra et al., 1998), while they are increased for NFH (Vivian et al., 2002).

NFs in other Neurodevelopomental and Metabolic Diseases

Phenylketonuria (PKU) is one of the most frequent inborn errors of metabolism, is due to the deficiency of the hepatic enzyme phenylalanine hydroxylase and results in hyperphenylalaninemia. Among other pathological characteristics, untreated PKU leads to mental retardation. Untreated PKU patients show a severe hypomyelination of their CNS. Experimental evidence has been shown that hyperphenylalaninemia delays axonal maturation and myelination during critical period of CNS development, probably through a deficit of NFH as well as myelin basic protein expression (Reynolds et al., 1993).

Progressive encephalopathy syndrome with edema, hypsarrhythmia and optic atrophy (PEHO syndrome) is a form of infantile progressive encephalopathy showing severe hypotonia, convulsions, profound mental retardation, hyperreflexia, optic atrophy and brain atrophy, in particular in cerebellum and brainstem. PEHO seems to occur in the postnatal period, without exclusion of potential prenatal onset. Interestingly, PEHO patients presented an aberrant expression of NFH in the perikarya of their cerebellar Purkinje cells, demonstrating an important disorganization of their cytoskeleton (Haltia and Somer, 1993).

NFs as Markers of Diseases

NFs, as the principal components of the axonal cytoskeleton, are released in the interstitial fluid after axonal injury or degeneration, and diffuse into cerebrospinal fluid (CSF), where they can be quantified to monitor axonal degeneration, as well as disease activity and progression. Increasing studies are published making use of NFs as markers of neuronal injury. A lot of work has been done on the measure of NFL and NFH released in CSF, as markers of axonal degeneration, to help the prediction and monitoring of the neurological decline in people with multiple sclerosis (MS). Different studies have shown that NFL CSF concentration is higher in patients with MS than in controls, making of NFL a promising marker to discriminate MS patients from patients with other neurological diseases. On the other hand, CSF NFH seems interesting for the follow up of the progression of the disease in MS patients, as it is increased during the progressive phase of MS. For more specific informations on the use of NFs as markers of MS, the reader is invited to read two detailed and recent reviews (Petzold, 2005; Teunissen et al., 2005).

As new but non-exhaustive examples, the use of NFs as markers of three other neuropathological conditions will be briefly discussed here: ALS, subarachnoid hemorrhage (SAH), and brain damages as consequence of cardiac arrest.

As discussed in a previous chapter, ALS is the most common form of motor neuron disease, presenting as neuropathological hallmark an abnormal aggregation of NFs in the degenerating motor neurons. A recent work proposes that phosphorylated NFH might be a valuable marker of axonal damage in ALS, discriminate between different categories of ALS, and be used as marker for therapeutic trials (Brettschneider et al., 2006).

Axonal degeneration is thought to be an underestimated complication of SAH, which can continue for days after the primary injury, and extend into the period of delayed cerebral ischemia. A recent study shows that phosphorylated NFH, measured daily in CSF during 14

days after the SAH episode, is significatively increased in SAH patients with bad outcome (measured at 3 months) (Petzold et al., 2005). This work demonstrates the secondary axonal degeneration following SAH, and show that the levels of phosphorylated NFH in CSF are highly predictive of a bad outcome for SAH patients.

The majority of patients surviving resuscitation after an out of hospital cardiac arrest present neurological complications due to global anoxia. Outcome prediction for these patients mainly rely on clinical observations, and on the recent measure of biochemical markers of brain damage in serum, such as brain specific proteins S-100 or NSE (Rosen et al., 2001). A recent study has shown that the levels of NFL in CSF give a reliable measure of brain damage, and are highly predictive of poor outcome for these patients (Rosen et al., 2004).

Conclusion

NFs are essential cytoskeletal proteins of the neuron, which participate in axonal rigidity, tensile strength, stability along time, regulation of calibre, and transport guidance of organelles and particles. NFs alterations have been identified in many different brain pathologies, ranging from neurodegenerative, neurodevelopmental to metabolic diseases. This list of diseases showing abnormalities in NFs will certainly increase in the near future. The identified NFs alterations range from genetic mutations, to abnormal expression, post-translational modifications and aberrant localization or accumulation in neuronal perikaryion. From this diversity of NF dysregulation in so many brain diseases, the future experimental work on NFs may unravel common mechanisms of IF accumulation and aggregation, and hopefully allow the design of better treatments for the patients suffering of these neurodegenerative diseases.

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