NEUROFILAMENTS – THE INTERMEDIATE FILAMENTS OF NEURAL CELLS. A REVIEW

Alice ŠONSKÁ[1,*], Tomáš KUČERA[2] and Gustav ENTLICHER[3]

Department of Biochemistry, Faculty of Science, Charles University in Prague, Albertov 2030, 128 43 Prague 2, Czech Republic; e-mail: [1] alalice@natur.cuni.cz, [2] arecuk@natur.cuni.cz, [3] entg@seznam.cz

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Dedicated to the 50th anniversary of the foundation of the Department of Biochemistry, the first biochemical department in Czechoslovakia.

Cytoskeleton is one of the basic structures of eukaryotic cells. It is a system of fibrillary or tubular proteins of three classes: microtubules, microfilaments and intermediate filaments. Neurofilaments, a member of the last class, occur in neural cells, where they are necessary for the cell to function properly. They are important in supporting and partly controlling the axon diameter and axonal transport. Neurofilaments are probably involved also in regulatory mechanisms, mainly through their extremely rich phosphorylation potential. This article introduces briefly the cytoskeleton in general and focuses on the structure and function of neurofilaments. A review with 189 references.

Keywords: Cytoskeleton; Intermediate filaments; Neurofilaments; Neurons; Proteins; Microtubules; Nervous system.

1. CYTOSKELETON

During the last two decades, increasing interest of biological research has been focused on cytoskeleton. It has become evident that understanding the cell biology would never be complete without knowing how the cell moves, organizes its internal compartments or sustains its shape against various mechanical influences.

The cytoplasm of all eukaryotic cells contains a dense, more or less oriented network of tubular or filamentous structures, composed of a large amount of protein subunits. They mainly play the role of inner mechanical support and provide for cell movement. It is a dynamic network of mutually interacting fibrillary proteins, participating in a number of important processes, such as the cell division, intracellular transport, organization of the membrane structures, morphogenesis and interactions of the cell with the environment. This network is one of the basic structures of all eukaryotic cells and it is called cytoskeleton. As the name suggests, it is a kind of skeleton or supporting construction of cells. Apparently it also determinates the distribution of organelles in the cell. An extremely rich cytoskeleton has evolved in protists, where it often forms very dense networks and bunches, strengthens pseudopodia and supports other structures. It is present also in complex contractile structures.

Cytoskeletal proteins make about 20% of the bulk of cellular proteins. The cytoskeleton is mostly composed of three types of filaments differing in size, composition, function and localization in the cell. The smallest outer diameter is that of microfilaments (5–9 nm). These are built of globular actin monomers and play an important role in cell movement by interactions with other molecules (like myosin). Another of the main cytoskeleton components forms tubular structures called microtubules. Microtubules are the thickest filaments with a diameter of approximately 25 nm. They are important for intracellular transport of vesicles and chromosomes. They contribute, together with other molecules, to the cell motility, forming particular and stable arrangements in specialized structures like cilia and flagellae. Their functional differentiation and specific interactions are mediated by microtubule associated proteins (MAP). Size and cellular localization of filaments of the third class are more heterogeneous. Their usual diameter is 10–15 nm and, therefore, they are called intermediate filaments (IF). Compared with evolutionarily very conservative actins and tubulins, they are more variable in primary structure. Intermediate filaments together with microtubules are considered to be a support system, while microfilaments with microtubules play the role of motile system. They are

capable of rapid restructuralization as a reaction to the movements and shape changes of the cell. However, in the surface layer of cytoplasm, they are able to maintain the cell shape, if different from spherical, implied by the surface tension in isolated cells. Hence, they participate in controlling the shape of the cell. Their regular longitudinal organization can be observed for instance (together with neurofilaments) in the projections of neural cells. They also pass through the cell body.

The cytoskeleton is a dynamic structure, capable of rapid reorganization according to the current requirements. This allows, for example, cell movements or rapid shape changes of protists. Microtubules and microfilaments are known to have both passive and active function. Intermediate filaments were generally considered to play only structural role for a long time. This exclusively static function of intermediate filaments had to be re-evaluated relatively recently[1–5]. The contemporary concept of IF function and interactions with auxiliary proteins is reviewed in literature[6,7].

2. MICROTUBULES

One of the main components of the cytoskeleton is microtubules – tubular structures occurring in nearly all eukaryotic cells. They are localized mainly near the cell membrane, where they contribute, e.g., to the cytoplasmic streaming. Microtubules also affect orientation of cellulose microfibrils of cell wall in plants and play an important role in cellular movements, transport of membrane organelles in the cytoplasm, axonal transport, secretion, endocytosis and transcytosis (e.g. literature[8]). They are the main structural component of the mitotic spindle, a supramolecular structure responsible for the chromosome separation during mitotic cell division. The cores of cilia and flagellae are also built of microtubules.

Microtubules resemble hollow tubes of variable length. The wall of these "tubes" is formed by 13 protofilaments, composed of linearly arranged heterodimers of α- and β-tubulin. The structurally similar α- and β-tubulin polypeptides have a very homologous primary structure and molecular weight about 50 000. Since the monomers are mutually bound in "head to tail" mode, the ends of the resulting protofilament are not structurally equivalent and the protofilament exhibits certain polarity[9] that causes, for instance, different polymerization rate of both ends. The more rapidly growing terminus has conventionally been called "plus end", the other "minus end". Microtubules are usually oriented with their minus terminus towards the centrosome – a central structure from which microtubules diverge with the plus end towards the cell surface[10,11]. The interaction of

microtubules with the centrosome seems to be mediated by another member of tubulin family, the γ-tubulin. It is mostly located in the centrosome and it is able to bind to the minus end of microtubules with high affinity[12]. α- and β-tubulins are in dynamic equilibrium with microtubules, which may assemble and disassemble due to minor changes in the environment. Tubulin heterodimers polymerize into protofilaments if the free tubulin concentration reaches a certain limit – critical concentration. Its value is affected by a number of factors. Polyanions, Ca^{2+} and various microtubule inhibitors increase the critical concentration. On the other hand, the presence of polycations or MAPs, elevated temperature or acid pH leads to the stimulation of polymerization, i.e. a decrease in a critical concentration. Tubulin polymerization requires energy in the form of GTP. GTP is bound to tubulin subunits and it is hydrolyzed to GDP during the microtubule polymerization.

The critical concentration is different for the minus end and the plus end. If the actual concentration is between these values, the minus end disassembles and the plus end grows. In this way, the microtubule is translocated through the cytoplasm. This process is called treadmilling[13,14]. It has been suggested that it is a mechanism, additional to interactions with the centrosome, contributing to the radial polarity of microtubules in the cell[15].

In addition to the tubulines mentioned above, there are other members of this family, relatively newly discovered tubulines δ, ε, ζ and η. They show much less homology to each other and to the tubulins α, β and γ and they are not ubiquitous in eucaryotic organisms. The knowledge of their biological function is scarce. They probably play a role in centriole and basal-body formation. For a review see literature[16,17].

It is well known that microtubules play a key role in the nuclear division, chromosome segregation and intracellular transport, maintenance of the cell shape and other activities. To enable such a wide variety of functions, it is necessary that the uniform microtubules be structurally differentiated. Such differentiation is achieved in several ways: tubulin isogenes coding for different types of tubulin, posttranslational modification of tubulin molecules and interactions with associated proteins. Proteins associating and interacting with microtubules are considered to be an important factor in the regulation of microtubule stability, since MAPs stimulate tubulin polymerization[18]. Considering the structural homogeneity of microtubules, it is well assumable that various proteins associating with microtubules mediate the complex and coordinated changes of cytoskeleton during mitosis, meiosis and conjugation. Hence, MAP proteins differentiate microtubules,

which are otherwise very uniform. Besides, MAPs serve as cytoskeletal "crosslinkers", mediating mutual interactions among microtubules and interactions of microtubules with actin filaments, intermediate filaments and membrane organelles.

3. MICROFILAMENTS

Microfilaments are other fibrillary structures of the cytoskeleton. They occur, for instance, in the surface layer of cytoplasm, function as the axes of microvilli (finger-like projections of cell surfaces) and compose, together with microtubules, contractile ring in mitotic cells. During the animal cell cytokinesis, in the final phases of mitosis, they are ordered circularly at the site of the future division. The proceeding division is then accompanied by a contraction of the circle, which is dispersed when the cell division is complete. Actin filaments accumulate and orient themselves in pseudopodia during amoeba-like movements, they take part in the elongation and contraction of various cellular projections, e.g. neuronal axons. Thus, microfilaments, as well as microtubules, are responsible for changes of the cell shape and play a role of a mechanical support. Besides, in cooperation with myosin, they form the contractile apparatus driving many types of intracellular movements like cytoplasmic streaming or formation of various projections and invaginations. On a higher level of organization, actin and myosin are the main components of muscle cells.

Microfilaments are polymers composed of a globular protein of relative molecular weight about 42 000, called G-actin. This protein occurs in three isoforms: α, β and γ. The α-actin is present only in cells of skeletal muscles, β- and γ-actins in all eukaryotic cells[19]. Actin of neural cells is a mixture of β- and γ-actins. Actin, as well as tubulin, is evolutionarily very conservative.

4. INTERMEDIATE FILAMENTS

By size, intermediate filaments fall between microtubules and microfilaments. Together with them they form a dynamic core of eukaryotic cells and are present in both nucleus (nuclear lamina) and cytoplasm (10–15 nm diameter filaments).

IF form, together with microtubules and microfilaments, a dynamic scaffolding of most eukaryotic cells. They usually build a network, extending from the nucleus to the cytoplasmic membrane, often with higher density in the subcortical cytoplasm and near the nuclear envelope. Compared with tubulin and actin, rather less is known about the structure, function

and assembly dynamics of IF. Individual types of IF are morphologically very similar to each other. However, they differ significantly in size and composition of the polypeptide subunits[20], both between various types of cells[21–23] and between cells of corresponding types in various organisms. For example, in epithelial cells, there are cytokeratin filaments, fixed to desmosomes and passing through all the cytoplasm (so called tonofilaments). In the cells of connective tissues, cartilage and differentiating muscles, IF consist of vimentin, in smooth muscles of desmin, called also skeletin. In the cell nucleus, we encounter lamins. In the cytoplasm of some cells, more than one IF type occur: several types of polypeptides, different from glial and astrocytal filaments, contribute to neurofilament composition.

4.1. System and Nomenclature of Intermediate Filaments

Around 1980, it was usual to sort IF into five classes according to the type of the cells they occurred[24]: keratins in epithelial cells, vimentin in mesenchymatic cells, desmin in the muscles, glial fibrillary acidic protein in astroglia and neurofilaments in neural cells. However, this system soon proved to be insufficient, as some cells contained more than one IF class. A new system was proposed, based on the primary structure of these proteins, coming from their sequencing and sequencing of the corresponding genes. Such data show and confirm that the polypeptide chains of all IF proteins contain a central domain, rich in α-helical structure, with conservative secondary structure, but with clearly distinct differences, allowing their assignment to different sequential types[25,26]. The same data suggest that all IF proteins have terminal domains of very variable lengths and primary structure, consisting of subdomains. The subdomain organization is characteristic of each sequential type of the central domain[27]. The arrangement of introns and exons in the genes coding for these proteins has been shown to be also typical of each sequential type. Hence, these three criteria provide us with the rules to sort IF proteins and to file newly discovered ones into the system.

According to this system, IF are divided into six classes: I. acidic keratins, II. basic and neutral keratins, III. cytoplasmic IF proteins vimentin, desmin, glial fibrillary acidic protein and a neuronal IF protein periferin[28–30], IV. neurofilaments (NF) and α-internexin[31], V. lamins[32–34] and finally VI. nestin[35]. These six classes can be further divided into three groups: Group A contains IF that are formed *in vivo* by two different polypeptide chains, i.e. keratins. All the other cytoplasmic IF are homodimeric and

belong to group B. Group C contains IF of the nucleus – lamins. Table I shows the present system of IF proteins.

4.2. Structure of Intermediate Filaments

The IF proteins are very heterogeneous. A mammal species may contain over forty types. Each type of cell, however, expresses only several of them. Molecular weight of IF monomers is between 40 000 and 200 000. It is common to all IF that they are composed of two polypeptide chains which contain, in the conservative central α-helical domain, repeated heptapeptides[21,32,37,40–43]. The first and fourth amino acid of every heptapeptide is usually non-polar[44]. This leads to formation of a non-polar coiled belt along the helix. This belt is the interaction site of two polypeptide chains forming the double-strand superhelix – the central rod domain of the resulting dimer, which is the basic building unit of IF[26,37,45–48]. The central domain of IF polypeptide has relatively constant size (molecular weight

TABLE I

Groups and classes of intermediate filament proteins. Adapted from literature[36–38]. The column of molecular weights (MW) contains the molecular weights of the appropriate human proteins inferred from their amino acid sequences. The values are taken from the online UniProt Knowledgebase[39] and rounded to thousands

Group	Class	Protein	MW	Location
A	I	acidic keratins	45 000–61 000	epithelia
	II	neutral/basic keratins	51 000–66 000	epithelia
B	III	desmin	53 000	muscle
		vimentin	53 000	mesenchyme
		glial fibrillary acidic protein	50 000	glia and astrocytes
		peripherin	54 000	peripheral neurons
	IV	neurofilaments:		neurons
		NF-L	62 000	
		NF-M	102 000	
		NF-H	112 000	
		α-internexin	55 000	neurons
	VI	nestin	177 000	multipotent CNS stem cells
C	V	lamins	66 000–74 000	all nucleated cells

40 000–45 000) and secondary structure. It contains 308–315 amino acid residues. It is lengthened at both ends by non-helical amino- and carboxy-terminal domains (called N-terminal head domain and C-terminal tail domain)[25,49–53]. Compared with the central domain, these are very variable in length and primary structure, which leads to very different molecular sizes of IF proteins. The C-terminal variable domains are usually substantially longer than N-terminal ones. The C-terminal domain may contain up to several hundreds of amino acid residues, e.g. in the largest protein of the neurofilament triplet[54]. On the other hand, in some cases this C-terminal domain may be even absent, e.g. in the smallest keratin[55]. The length of the central rod domain of the dimer is about 50 nm. The continuity of the helix of this domain is interrupted three times, dividing it into two major parts called helices 1 and 2, which themselves are further divided into parts A and B (Fig. 1). Therefore, four parts can be distinguished in the central rod domain: 1A (35 amino acid residues), 1B (101 amino acid residues), 2A (19 amino acid residues) and 2B (121 amino acid residues). Each of these segments is a left-handed coiled coil. The segments are separated by "linkers" L_1 (8–14 amino acid residues), L_{12} (16–17 amino acid residues) and L_2 (8 amino acid residues) of non-helical structure, the above mentioned interruptions of the helix continuity. Approximately in the middle of segment 2B, another minor discontinuity can be found.

The IF dimers may bind together both longitudinally and laterally. This fact makes them interesting objects of structural biology research. It has been found that the monomers are arranged in a parallel way in the coiled coil and the dimers are, therefore, polar. The polarity is maintained only when IF dimers are bound longitudinally (as they bind head to tail), while the lateral arrangement seems to be always antiparallel[56,57].

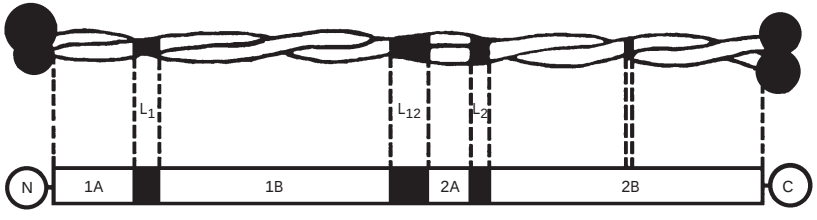

FIG. 1
Simplified model of intermediate filament dimer, according to literature[49]. 1A, 1B, 2A, 2B – two-stranded helical domains; L_1, L_{12}, L_2 – non-helical "linker" or "loop" domains; C, N – C- and N-terminal domains

The assembly of IF proteins and formation of their ordered 3D-structures has been studied for quite a long time[38,58–60]. The probable IF arrangement on different levels of complexity is shown in Fig. 2. The exact mechanism according to which the different interaction modes of dimers determine the final arrangement of a filament or even paracrystal (formed very regularly *in vitro*) is not yet fully resolved.

Regulation of IF organization is complex and in some cases it includes posttranslational modifications of IF proteins. It has been found that IF proteins are phosphorylated and that changes in their phosphorylation correlate in some cases with changes of IF organization[63,64]. Subsequently, attention has been paid to the influence of these posttranslational modifications on IF proteins assembly into filaments. It seems that disassembly of cytoplasmic IF filaments may be induced *in vitro* by phosphorylation with various kinases, including the protein kinase C and cAMP-dependent protein kinase[65,66]. This assumption was tested and, though IF disassembly was not observed during the experiments, notable changes took place in its organization, e.g. formation of bundles and their aggregation.

The mechanism of IF filaments assembly regulation and reorganization of IF network is dependent on the cell type and phosphorylation/dephosphorylation by different kinases/phosphatases at various specific sites. The number and location of phosphorylation sites show that IF phosphorylation probably has some other functions than just the possible regulation of IF filaments assembly and organization. This has led some authors[67] to propose a hypothesis that IF phosphorylation may be, in some cases, a part of a signal transduction cascade, enabling regulation of activity or cellular compartmentalization of regulatory molecules associated with IF. Deeper insight into IF cellular function might be achieved through understanding

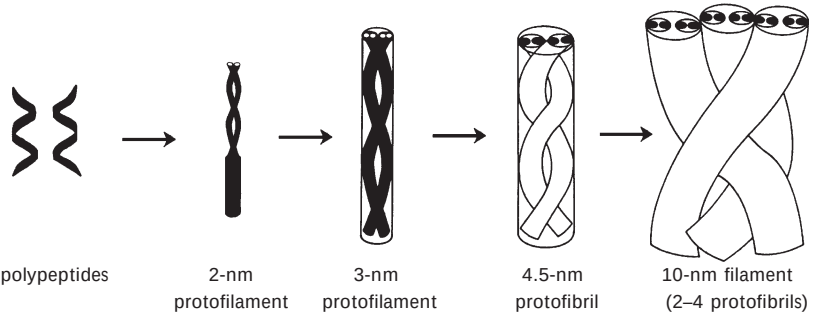

FIG. 2
Hierarchical arrangement of intermediate filaments, adapted from literature[61,62]

better their dynamic properties. Therefore, many interesting studies from the last years deal with IF expression, regulation and in particular phosphorylation[68–73].

Though IF are present in the great majority of mammal cells (besides the cytoplasm, they have been also found in the nucleus, where they form the elementary structure of the fibrous lamina, located below the inner nuclear membrane), many of their functions are still a matter of speculation. Now, a lot of information is available (e.g. sequence data) for an acceptable structural model to be suggested. However, no IF protein has been successfully crystallized and, therefore, no X-ray crystallographic model has been proposed. Also the assembly patterns of individual IF proteins are known only roughly and the exact structure of the network, in which the proteins are arranged in filaments, is still to be resolved. The most accented research subjects in the field are the structure of IF and the dynamics of its polymerization and assembly[74,75]. Recently, the molecular interactions between IF proteins are also extensively examined.

5. CYTOSKELETON OF THE NEURAL CELLS

Mammalian nervous system is a highly complex three-dimensional network composed of millions of neurons and auxiliary cells. By means of an intricate array of neuronal connections called synapses, it is able to receive and integrate information from many sources, to process these inputs and initiate a proper response. To achieve the required degree of complexity in this arrangement, neurons exhibit a unique morphology consisting in many neural projections, called axons and dendrites. Axons are long thin structures with constant diameter, while dendrites are usually shorter and tapered. Generally, dendrites receive the input and axons transmit the output. Other differences in the molecular and organelle components of these projections exist, but are not mentioned here.

Radical changes in the structure and interconnection of neurons take place during the development of the nervous system. Immature neurons are subject to chemical and mechanical influences, which cause their migration to various parts of the nervous system, elongation of axons and dendrites towards other cells, establishment and break of synaptic connections with these cells, until the definitive branching and interconnection are achieved. The site of branching or elongation of a neural projection is called growth cone. The growth process is the first step of neuronal network formation. Initiation and elongation of axons and dendrites take place in precise spatial and time sequences, dependent on internal and ex-

ternal factors. The internal factors determine the basic polarity of the cell and organize the cytoskeleton of elongating axons, while the external ones modulate the movements of growth cone. Formation of the neural projections, their length, direction of their growth as well as their branching are dependent on cytoskeleton, which mediates also organelle movement and transport of metabolites along axons, the so-called axonal transport.

5.1. Microtubules of the Neural Cells

The arrangement of neuronal microtubules (neurotubules) suggests their important role in establishing and maintaining the shape of the cell[76]. It has been shown by electron microscopy that they are particularly abundant in axons and dendrites, where they form typical longitudinal bunches. In axons, they are always oriented with the minus end towards the cell body, having thus the same polarity[77–79], while in dendrites there is no unified polarity[80]. This different orientation of microtubules may contribute to the differences between axons and dendrites in their cytoplasmic organelles, since the transport of the organelles along microtubules is oriented[81–83]. Thus, the neuron compartmentalization and polarity are probably dependent on the arrangement of microtubules.

5.2. Microfilaments of the Neural Cells

The neural cell microfilaments contain only β- and γ-actins. Their molecules are in a permanent exchange between the monomeric form and the actin filaments[84]. A great portion of F-actin is associated with the plasma membrane[85], where it may play various roles, for instance release of vesicles with neurotransmitters or adhesion to other cells. The growth ends of axons and dendrites are very mobile formations due to the actin network, since the cytoskeleton of the growth cone is composed predominantly of actin microfilaments. On the growth cone surface, there are protruding tiny finger-like projections, called filopodia, and large flat projections, called lamellipodia, the latter built of a non-oriented network of actin filaments, the former of their oriented bundles. These structures emerge from the cortical cytoskeleton of the growth cone and mediate the first contact with the environment during the elongation.

5.3. Neurofilaments

The intermediate filaments located specifically in neurons are called neurofilaments. They constitute one of the six classes of intermediate filaments (see Table I). As basic cytoskeletal components of neurons, they take part in many neural processes.

Most IF are built of only one predominant protein[86]. The unique feature of NF is that they are composed, in their native state, of three different proteins, differing from each other mainly in the molecular weight. Hence, NF are protein filaments formed by a triplet of polypeptides.

For the first time, NF were described more than a hundred years ago. The discovery of NF in a cell was made possible by the histochemical staining that had been used for a long time in neuroanatomy to visualize neurons. Later on, it was found that these fibrillary structures are constituted by 10 nm thick filaments, abundantly occurring in neurons. It has also been shown that their function may probably overlap with that of other cytoskeletal elements and that biochemical modifications may influence their roles.

NF were investigated more in detail only when electron microscopic techniques with high degree of resolution appeared[87]. At present, we know that NF are the main component of the neuronal cytoskeleton. Their quantity and arrangement are dependent on the position inside the cell. They generally occur in smaller quantities in the dendrites, less ordered than in axons[88], where they may even constitute the main structural element. They form circles, classic synaptic buttons in the synapses[89]. The NF amount in growing nerves increases during the development, probably stabilizing the whole cytoskeleton of the neural cell[90,91]. NF have been observed in the central bundle of the elongating axon and in the growth cone, suggesting their function of an inner strengthening skeleton[92,93]. Unlike microtubules[94], their arrangement and, first of all, quantity, affect the axon diameter[95]. The NF-to-microtubules ratio in axon changes during its development. In immature axons, microtubules are the predominant component of the cytoskeleton while, with increasing axon diameter, the NF amount increases[90] until their regular network fills up the whole axon. Hence, NF determine, to some extent, the diameter of the axon. However, the NF content in an axon is likely to be correlated with the degree of axon myelination[96]. Besides, scanning transmission electron microscopy studies with native and *in vitro* reconstituted NF reveal a high number of NF polypeptides per filament cross-section. Moreover, this number varies along the filament. This feature makes NF (and probably IF in general), very distinct from the other cytoskeletal structures, providing them with a poten-

tial to exchange subunits not only on the two ends, but all along their length, suggesting that their spatial and time turnover may be very different from that of microtubules and microfilaments[97]. In large motoneurons, the low-molecular-weight subunit of NF has been found to be an important factor of dendritic growth and branching[98].

The initial identification of NF subunits was problematic, since neurofilaments are hardly soluble and no NF ligands were available. The NF subunits were discovered accidentally about 1975. Neurofilaments are usually considered stable structures, due to their low solubility *in vitro* under physiological conditions. The apparent relative molecular weight of the largest protein of NF triplet is 200 000. It is called NF-H (high-molecular-weight or heavy subunit). Another protein of the triplet, NF-M (middle-molecular-weight or medium subunit), has the apparent relative molecular weight 160 000 and the last triplet member, NF-L (low-molecular-weight or light subunit), 68 000[99,100]. These figures correspond to apparent molecular weights, as established by denaturating polyacrylamide gel electrophoresis. The molecular weights found using analytical ultracentrifugation and amino acid sequences are lower[56,101].

As already stated, NF are rather stable structures under physiological conditions. Their disassembly to free subunits requires denaturating conditions like concentrated urea or guanidine hydrochloride. Separated free NF-L spontaneously aggregate into long filaments of approximately 10 nm diameter under proper conditions[49,102,103]. On the other hand, NF-H and NF-M, under typical reconstitution conditions, assemble into relatively short filaments, characteristic "crumbled" forms[104]. It has not been proved yet whether NF-H and NF-M are also incorporated into the NF-L filament structure during the reconstitution experiments. It is not clear as well whether the dephosphorylated and phosphorylated subunits may assemble together into one filament[105,106]. However, it has been shown that NF-M and NF-H incorporation is necessary to maintain the interfilament distance. The C-terminal domains of NF-H and NF-M (called sidearms) then serve as simple spacers between the filaments, perhaps enhanced by the "entropic brush" mechanism[107–109]. (Generally, the entropic brush is formed by polymer chains protruding from a surface. They cover the surface in a high number per area unit, so that they are forced to stretch out perpendicular to the surface, resembling a brush. The termal movements of the chains then inhibit other large molecules from approaching the surface[110].) Moreover, the abilities of NF proteins to assemble differ among animal species[111]. NF assembly may also be influenced by special regulatory proteins, such as presenilin-1[112].

As already mentioned, NF contain a conservative α-helical central region and two hypervariable terminal domains[25,101,113–115]. The N-terminal region, the "head", is evolutionarily less conserved. The C-terminal region, the "tail", is of variable length, causing the differences in molecular weight of mammalian NF. In the case of NF-L, the central region contains 308 amino acid residues. It consists of α-helices 1A, 1B and 2 separated by two short non-helical "linkers". The central domain is flanked by 93 residues of the N-terminal domain and 142 residues of the C-terminal domain[101]. The basic model of NF structure was worked out using a combination of biochemical approaches and electron microscopic techniques. The length of the central region has been estimated to be about 48 nm. It was found that in assembled filaments, there is an overlap of the neighbouring central regions[116] (about half the central region length). NF constitute the main component of the neuronal cytoskeleton. However, the details of their arrangement and contribution of the individual subunits to the final structure are still unknown. NF do not form paracrystals (unlike e.g. actin) and no diffraction crystallographic data are available. Nevertheless, experiments with antibodies specific to individual NF subunits[117–119] have provided certain knowledge on the NF polypeptides localization in the filaments. It has been unequivocally shown that *in vivo*, the neurofilaments always contain all three types of subunits[118,119]. As can be seen in immunologically labelled preparations, NF-L antibodies are deposited homogeneously along the filament, while NF-H and NF-M antibodies are bound very irregularly[118,119]. It can be deduced from these findings that the "backbone" of the filament is primarily built of NF-L, while NF-H and NF-M are localized rather peripherally. On the other hand, proposals exist suggesting that the NF-M [114] and NF-H [54] central regions are firmly anchored in the filament backbone as an integral part of the protofilament and their C-terminal domains radially protrude outside the filament. Now, the generally more accepted concepts are derived from the fact that the most abundant subunit of native NF is NF-L, that is assumed to build-up the basic filament – a core or backbone of the whole structure, to which NF-H and NF-M are connected[113,120]. And it is only NF-L that is able, *in vitro*, to assemble into the structures observed in IF, while NF-H and NF-M form only very short filaments[97,121]. In preparations obtained by the negative staining method, reconstituted NF-L are observed to form 10 nm thick filaments typical of IF, although they have been found also in protofilament structures[58,122–125]. Using the method of rotary metal-shadowing, the filaments exhibit characteristic segments, repeating with 22 nm periodicity[49,58,97,121,125]. All triplet proteins are contained in the native filaments, the C-terminal domains of NF-H and NF-M projecting

from the core filaments again with the periodicity of 22 nm [113] to the average distance of 63 and 55 nm, respectively[120,125].

Axonal neurofilaments are arranged in bundles, where the NF-H and NF-M C-terminal domain projections connect the individual adjacent filaments increasing their resistance to deformation[120,126,127]. Interactions of microtubules with the NF projections have also been observed, both *in vitro* and during the slow axonal transport of NF subunits and tubulin[128].

Neurofilaments, as well as other IF, are more or less phosphorylated. The NF-H subunit contains more phosphate groups (100 mol per mol of protein) than NF-M (25 mol/mol)[129]. The lowest PO_4 content has been observed in NF-L, about 0.4 mol/mol [130,131]. It is possible, by means of immunological techniques, to visualize *in vivo* the distribution of NF phosphorylation degree at the level of a cell[132,133] as well as at the level of a tissue[134]. It was often assumed that the degree of phosphorylation, similarly to other IF [66,135], could affect the NF polypeptide stability[106]. The phosphorylated NF are mainly localized in axons, less in the cell body and dendrites (except the brain tissue from Alzheimer patients, where NF are hyperphosphorylated and concentrated in the cell body[136].

Highly phosphorylated are, in most cases, the C-terminal domains of NF-H and NF-M, most abundantly on the multiple sequential repeats KSP, KXSP or KXXSP (depending on the source organism). Other phosphorylation sites are in the glutamate-rich E-segment[137]. Complete phosphorylation of the C-terminal domain is a relatively slow process, in many neurons occurring only in axons[138]).

The highly phosphorylated tail ends of NF-H and NF-M seem to be responsible for the direct NF–NF contact or interactions with other cytoskeletal proteins. NF phosphorylation increases with increasing axon diameter and with stable NF network formation in the course of axon maturation[139]. During their transport along the axon, NF interact with other axonal structures. As they are gradually post-translationally modified passing through the axon, the character of these interactions may be changed by phosphorylation, leading to a different NF transport and possibly to their incorporation into the stable networks, contributing to the axon radial growth. Moreover, the phosphorylation status may be different not only quantitatively but also qualitatively, depending on variability of the kinases, phosphatases and substrates available in different cell compartments, leading to different NF interactions with other cell components[140].

The NF phosphorylation affects the rate of its axonal transport also via changing its affinity to the appropriate motor complexes. It has been found recently that the C-terminal phosphorylation of NF-H slows down the NF

axonal transport[141,142], probably by weakening the NF interaction with the fast axonal transport motor kinesin[143–145]. NF were believed for a long time to be transported by a slow-transport system. However, the observed slow rate of their transport is the result of repeated cycles of NF attachment to and detachment from the above fast motor complex. There are observations showing that another fast axonal-transport motor dynein participates in NF transport[146]. However, these mechanisms may contribute only partially, the question of other undiscovered motors is still open[144].

As to the effect of phosphorylation on NF-L filament assembly, controversial results have been published. Some authors[147–151] show that NF-L protein phosphorylation not only impedes the NF-L filament assembly, but also causes disassembly of already assembled filaments. It has even been shown that phosphorylation of serine in position 55 is responsible for this phenomenon[152]. Nevertheless, according to other authors, phosphorylation of NF-L has only very limited (or none at all) influence on NF-L filament assembly[153–156]. It has been shown that dephosphorylation of NF-L proteins by phosphatases does not exhibit any influence, either[105,121].

Lowered phosphorylation of NF proteins seems to be related to the decreased axon diameter in the Ranvier nodes. Phosphorylated NF proteins are a main component in regenerating axons[157]. Microtubule destabilization and neurofilament phosphorylation precede dendritic sprouting after axotomy of lamprey central neurons[157]. Phosphorylation of NF proteins also stabilizes neurofilaments[158] and protects them against proteolysis in some organisms[74]. Phosphorylation of NF-H tails, required for the above mentioned “entropic brush” function, has been shown to be regulated through a signalling cascade involving myelin-associated glycoprotein[159–161], controlling in this way the neurofilament spacing. On the other hand, results have been obtained, showing that NF-H can be truncated of the tail domain without any effect on axon diameter or the rate of NF axonal transport[162].

Besides its ability to be phosphorylated, NF-L has been recently suggested to function as a regulatory (targeting) subunit of a phosphatase[163]. According to this finding, it may play a role in cellular distribution of PP1 phosphatase activity.

It seems that NF phosphorylation is a complex problem and its functional role remains to be elucidated.

6. DISEASES OF THE NERVOUS SYSTEM RELATED TO MALFUNCTION OF INTERMEDIATE FILAMENTS

The nervous system diseases have been studied by microscopic methods for a long time. They are often accompanied by changes in fibrillary elements of neural cells, the most common of which being known as the neurofibrillary degeneration. This symptom, consisting in increase in the number and thickness of some fibrillary structures in neural cell cytoplasm, occurring e.g. in Alzheimer disease, was described for the first time by a German neuropathologist Alois Alzheimer[164] in patients who died of a rapid progressive presenile dementia. In silver-stained preparations, Alzheimer observed intraneuronal inclusions or clusters in the cell bodies and the proximal parts of axons and dendrites. These clusters were also found in degenerating nerve terminations, where they are a component of the senile plaque, also typical of the Alzheimer disease. Later it was found that neurofibrillary degeneration is the main morphological feature of many nervous system diseases[165], neurodegenerative diseases[166,167], diseases of motor neurons[168], for example the most common form of human motor neuron disease, the amyotrophic lateral sclerosis[169] and a number of neural disorders caused by intoxications. With establishment of electron microscopy, it was found that the neurofibrillary degeneration can be divided into two main groups. The first group contains all the above diseases except Alzheimer syndrome. They are characterized by formation of clusters composed of bundles of approximately 10 nm thick filaments, morphologically indistinguishable from normal neurofilaments. The second group includes Alzheimer disease, some types of dementia and Down syndrome. The clusters are built of morphologically distinct filaments. These structures, commonly called paired helical filaments, consist of two helically coiled filaments of approximately 9–11 nm diameters. In some Alzheimer disease types, also 15 nm thick filaments have been observed.

In transgenic mice, increased expression of one of the neurofilament triplet proteins, NF-L, is related to morphological changes in central nervous system, resembling the human motor neuron disease[170]. Similarly, it has been found that the mice with over-expressed NF-H showed symptoms of amyotrophic lateral sclerosis (a progressive muscle dystrophy, caused by a loss of spinal and cortical motor neurons) accompanied, besides the progressive neurological defects of the motoric system, by an abnormal increase in the amount of neurofilaments, especially in the cell body and the proximal parts of axons of the motor nerves. The over-accumulation of NF alone appears very early in the degenerative process. The neurofilament

over-expression[171] or NF mutations disrupting NF assembly[172–174] probably lead to a damage to axonal transport, thus causing neuron enlargement with consecutive axonopathy and muscular atrophy. On the other hand, there is a growing number of observations suggesting that the primary cause of these diseases may not be directly related to NF, the NF hyperaccumulation being a secondary phenomenon, as it has been shown, e.g., in the case of amyotrophic lateral sclerosis[62,71,73,175]. A factor leading to the hyperaccumulation and malphosphorylation of NF may be, e.g., an alteration of the axonal transport[176–178] or the oxidative stress[179]. The number of diseases demonstrating NF inclusions is still not definitive, as new syndromes are being described[180].

An abnormal neurofilament aggregation can also be found *in vivo* and *in vitro* for instance after intoxication by Al^{3+} ions[181], 3,3′-iminodipropanonitrile[182], acrylamide[183,184], formaldehyde[185], colchicine, vinblastine or vincristine[186,187]. Changed levels and some properties of NF have been observed upon intoxication with some frequently abused drugs[188,189].

The common clinical symptoms of most motor nerve diseases are progressive muscle weakness together with denervation-induced atrophy of the skeletal muscles, paralysis, loss of the motor neurons and finally death. Neither the origin nor the mechanism of these pathological processes are known yet. There are just well-founded suspicions that NF play a key role there. Hence, studying the NF damage can also lead to suitable diagnostic tools for many of the neuropathies above, in particular the motor nerves diseases, neurodegenerative disorders or toxin-induced neuropathies. The accumulated data from a number of studies show that the NF overaccumulation is an integral part of these pathogenic processes in motor nerves degeneration (e.g. literature[170]).

It remains a question, what influence could NF post-translational modifications have and what role these modifications, in particular phosphorylation, play in such pathological states.

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