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Polina Belyaeva

Tubulin post-translational modifications and microtubule associated proteins in neural development and disease
Post-translační modifikace tubulinu a proteiny vázající mikrotubuly ve vývoji a onemocněních nervové soustavy

Bachelor´s thesis

Supervisor:
Mgr. Martin Balaštík, Ph.D.

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Declaration

I honestly declare that I wrote this thesis on my own and that I stated all used literature and other information sources. This work or its significant part was not used to previously acquire any other or same academic title.

In Prague, 4.5.2023 Signature

Abstract

Microtubules (MTs) are essential components of the cytoskeleton in all eukaryotic cells. Their function is particularly important in neurons where MTs stabilize their long processes and are responsible for the precisely regulated anterograde and retrograde, intra-axonal and intra-dendritic transport over long distances. MTs are essential also during development of the vertebrate brain and all its major steps: neurogenesis, neuronal migration and neuronal differentiation. MTs are regulated at multiple levels, but two seem to be particularly important: 1. posttranslational modifications of tubulin (PTMs) have been shown to control several MT properties as stability or MT-based transport. 2. microtubule-associated proteins (MAPs) that bind soluble MT subunits, MT lattice as well as MT ends and control MT-based transport and MT dynamics by either stabilizing, destabilizing or severing MTs. Consequently, deregulation of either tubulin PTMs or MAPs may induce severe changes in neuronal cytoskeleton. Bachelor´s thesis summarizes current knowledge on how PTMs (especially polyglutamylation) and MAPs (especially microtubule cleaving proteins such as spastin) regulate MT and neuronal development and degeneration.

Keywords: Microtubules, tubulin post-translational modifications, polyglutamylation, microtubule-associated proteins, spastin, neurodevelopment

Abstrakt

Mikrotubuly (MT) jsou základními složkami cytoskeletu všech eukaryotických buněk. Jejich funkce je zvláště důležitá v neuronech, kde MT stabilizují jejich dlouhé procesy a jsou zodpovědné za přesně regulovaný anterográdní a retrográdní, intra-axonální a intra-dendritický transport na dlouhé vzdálenosti. MT jsou nezbytné také během vývoje mozku obratlovců a všech jeho hlavních kroků: neurogeneze, migrace neuronů a diferenciace neuronů. MT jsou regulovány na mnoha úrovních, ale dvě se zdají být obzvláště důležité: 1. bylo prokázáno, že posttranslační modifikace tubulinu (PTM) řídí vlastnosti MT, jako je stabilita nebo transport na bázi MT. 2. proteiny asociované s mikrotubuly (MAP), které vážou rozpustné podjednotky MT, mřížku MT i konce MT a řídí transport na bázi MT a dynamiku MT buď stabilizací, destabilizací nebo přetrháváním MT. Deregulace tubulinových PTM nebo MAP může proto vyvolat závažné změny v neuronálním cytoskeletu. Bakalářská práce shrnuje současné znalostí o tom, jak PTM (zejména polyglutamylace) a MAP (zejména proteiny štěpící mikrotubuly jako spastin) regulují MT a vývoji a degeneraci neuronů.

Klíčová slova: Mikrotubuly, tubulin post-translační modifikace, polyglutamylace, proteiny asociované s mikrotubuly, spastin, vývoj nervové soustavy

List of abbreviations

MT(s)	Microtubule(s)
PTM	Post-translation modification
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
MAP	Microtubule-associated protein
TUBα	Alpha-tubulin
SVBP	Small vasohibin-binding protein
VASH	Vasohibin
CCP	Cytosolic carboxypeptidase
TTL	Tubulin tyrosine ligase
αTAT	α-tubulin acetyltransferase
HDAC	Histone deacetylase
TTLL	Tubulin-tyrosine ligase-like
ATP	Adenosine triphosphate
TIP	Tracking protein
CRMP	Collapsin response mediator protein
HSP	Hereditary spastic paraplegia
mRNA	Messenger ribonucleic acid
HD	Hydrophobic domain
MIT	MTs-interacting domain
MTBD	MT-binding domain
NBD	Nucleotide-binding domain
HBD	Four-helix bundle domain
NSC	Neuronal stem cell
hiPSC	Human induced pluripotent stem cell
SCI	Spinal cord injury
HeLa cells	Human epithelial cell line

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1. Introduction

The cytoskeleton with its dynamic cycles of growth and retraction plays a fundamental role in both the development and function of organisms. Microtubules (MTs) are key component of eukaryotic cytoskeleton. In neurons, their dynamic, tightly regulated remodeling is essential for neuronal polarization, axon growth and guidance, synaptic pruning and neuronal differentiation. Abnormalities in these processes, inflicted by dysregulation of MTs, have been associated with many human neurodevelopmental disorders as autism spectrum disorder, epilepsy, schizophrenia, or major depressive disorder. The function of microtubules is controlled on multiple levels. First by specific α- and β-tubulin isotype-composition and their posttranslational modifications (PTMs), such as detyrosination, acetylation, polyglutamylation, phosphorylation, and polyamination. Second, by interaction with microtubule-associated proteins (MAPs) such as microtubule motors, MT stabilizers, and MT-separating enzymes etc. Tubulin isotypes and PTMs are together referred to as tubulin code and have a critical effect on MT dynamics, stability and interaction with various MAPs. Important role in both development and function of MTs is played by MT severing protein spastin, which binds to and cleaves microtubules into shorter ones increasesing the availability of the plus-ends of MTs for polymerization. Spastin interacts primarily with polyglutamylated tubulin in the early stages of brain development, which is crucial for precise axon growth and guidance. Abnormal spastin function can lead to hereditary spastic paraplegia and other neurodevelopmental diseases.

The aim of this thesis is to review current knowledge of tubulin PTMs and MAPs in the context of neurodevelopment. In particular, I will focus on tubulin polyglutamylation and spastin as a connected and mutually regulated PTM - MAP system, that is essential for normal MT regulation and its defects have been linked to variety of neurological conditions.

2. Microtubules

Every cell of any organism, including bacteria and archaea, needs to have structures that allow for movement, intracellular transport, mechanical resistance and cell shaping. Such a structure is a three-dimensional cytoskeletal network of interconnected protein filaments.

Microtubules (MT) are components of the eukaryotic cytoskeleton together with actin filaments (Herman, 1993) and intermediate filaments (Herrmann and Aebi, 2016). Each of these elements has different structure and functions that is tightly controlled on multiple levels. In this thesis I am going to discuss mainly the MTs, their role in neuronal cells and their regulation by specific tubulin modifications, and by microtubule associated proteins. MTs main functions are maintaining cellular shape and trafficking of cellular components. In the nervous system, they are involved in virtually all steps of neural development, but their effect is particularly important for cell differentiation and determination of neuronal polarity as well as in process of neuronal migration (Baas et al., 2005; Lambert de Rouvroit and Goffinet, 2001; Witte et al., 2008).

2.1 Microtubule structure

MTs are composed of protofilaments that form a hollow tube with an outer diameter of 25 nm (Simic-Krstic et al., 1989). In eukaryotic cells MTs are formed mainly by 13 protofilaments (Tilney et al., 1973), but also MT with 12, 15 or even 16 protofilaments, for example axonal MTs of the ventral nerve cord of crustaceans and lobsters, which have 12 PTs (Burton et al., 1975).

The protofilaments consist of α- and β-tubulin heterodimers (Banerjee et al., 1988; Sullivan and Cleveland, 1986). The part of the protofilament that ends with α-tubulin is called the minus-end. While the part that ends with β-tubulin is called the plus-end or the fast-growing end. Minus-ends differ from plus-ends not only in that they end with alpha-tubulin but also in that they are closed by another type of tubulin – γ-tubulin. γ-tubulin acts as a nucleator and blocks the addition of tubulin dimers, thereby reducing the dynamic instability of minus end (Wiese and Zheng, 2000). The polarization of MTs is reflected also in the directionality of MT-based transport of vesicles and organelles, which can be either retrograde (to the minus-end by the dynein motor protein) and anterograde (to the plus-end by the kinesin motor protein) (Barlan and Gelfand, 2017; Vale et al., 1985).

2.2 Microtubules in neurons

In neurons, MTs are differentially oriented in different cellular compartments. In the dendrites of vertebrate neurons, MTs do not follow a particular direction of polarization (Baas et al., 1989; Yau et al., 2016). This may facilitate both the centripetal signal transmission from the axodendric synapses and the centrifugal transport of necessary substances to the postsynaptic part of the junction occur. In axons, MTs are specifically oriented: the plus-end always points towards the growth cone and the minus-end towards the body of the neuron (Chalfie and Thomson, 1979). Since the MTs overlap there, the organelles and vesicles must stop at the end of one polymer and reattach to another MT (Yogev et al., 2016). This could cause the cargo to change routes, make stops and slow down the transport. Surprisingly, it has been shown that there is an increase in axonal transport with increasing MT density, as closely spaced MTs allow cargo to associate with multiple molecular motors simultaneously and thereby enhance the axonal transport (Wortman et al., 2014).

2.3 Tubulin code

Different α- and β-tubulins isotypes have been identified (Banerjee et al., 1988) that change MTs dynamics in distinct ways due to the differences in their structure (Lu and Luduena, 1994; Panda et al., 1994). Their primary structure differs particularly at the C-terminus and in the intermediate domain (Cleveland et al., 1980). Importantly the C-termini of various tubulin isotypes are the place where various PTM occur. The specific combination of tubulin isotypes together with their particular PTMs (which will be discussed in detail later) are referred to as tubulin code (Fig.1). It is considered to give different MTs unique properties even within the same cellular compartment and specifically alter MT functions (Gadadhar et al., 2017; Verhey and Gaertig, 2007).

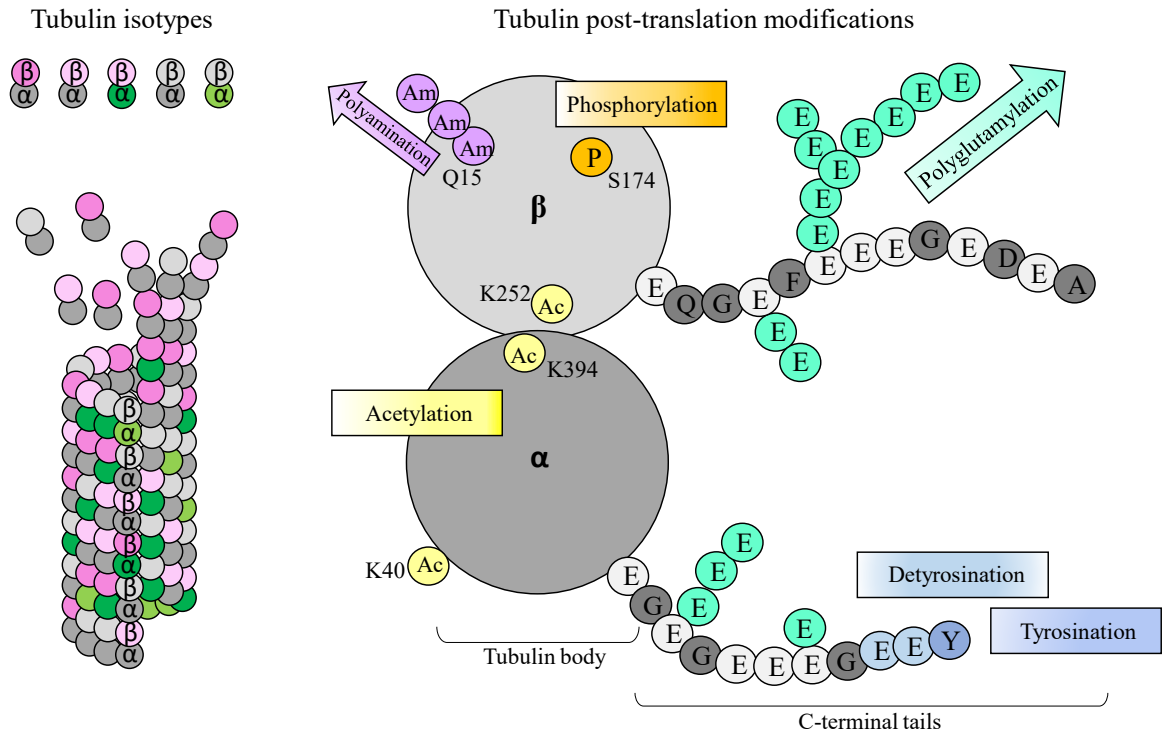

Figure 1: Individual elements of the tubulin code. The left part of the diagram shows how different isoforms of tubulin (α-tubulins: dark grey, green and dark green; β-tubulins: light grey pink and dark pink) assemble the MT lattice. The right part shows the contralateral sites with post-translational modifications of tubulins as the second element of the tubulin code. Some of these modifications occur directly at the globular core of the proteins (for example, acetylation (Ac) (yellow), polyamination (Am) (purple) and phosphorylation (P) (orange)), while others are located at the C-terminal ends of the tubulins (for example, detyrosination (light blue) and polyglutamylation (blue-green)). Adapted by (Janke and Magiera, 2020)

2.4 Microtubules in neurodevelopment

In neurodevelopment precise remodeling of MTs thorough cycles of polymerization and depolymerization of their plus-ends is critical to form the unique brain connectivity. Tubulin polymerization starts with binding of the free tubulin subunits to GTP, GTP-bound tubulin thereafter incorporates into the MT lattice on β-tubulin and is hydrolyzed to GDP. The, so called, GTP-cap at the MT end is a rapidly changing structure. It occurs at both MT ends but predominates at the growing plus-end. Regardless of its dynamic change, it holds the MT structure together to prevent its disintegration. MT ends randomly alternate periods of growth

(rescue) and shortening (catastrophe) over time. This process is called dynamic instability (Flyvbjerg et al., 1994).

The term neuronal development refers to a set of processes that play a fundamental role in the development of the nervous system from the earliest stages of embryogenesis to the adulthood. The list of these processes includes neuronal differentiation and polarization, neuronal migration, growth and axonal guidance, synaptic formation and synaptic and axonal pruning.

Neuronal differentiation is the stage in the development of neurons, during which they increase in size, produce more dendrites, extend their axons further away from the cell body, make new connections with other cells, and become specific types of neurons. Microtubule-associated proteins are mainly involved in this developmental process by directing (Kanai et al., 2000), regulating dynamics by cleavage (Hartman et al., 1998; Hazan et al., 1999; Tao et al., 2016), stabilizing of MTs (Fukata et al., 2002; Shafit-Zagardo and Kalcheva, 1998) and performing differential transport (Wortman et al., 2014). Originally, a model has been proposed suggesting that MTs assemble in the centrosome, are subsequently cleaved by severing enzymes, and moved by motor proteins to axons and dendrites to allow their growth and branching (Baas et al., 2005). Later, local cleavage of MTs by katanin proved to be a more likely model, allowing differentiation in axons and dendrites to be regulated in a faster way, which is crucial for the development of the nervous system at early stages (Lee et al., 2009).

Another key process in neurodevelopment is the polarization of neurons resulting in formation of dendrites and one axon. Neuronal precursors create neurites, which are structurally and functionally different and later transform into dendrites and an axon. A marker of a newly forming axon is the accumulation of tyrosinated and acetylated MTs in one of the cell's protrusions (Witte et al., 2008). The exact mechanism of this occurrence is still being studied, but MTs and their PTMs play a significant role in it. However, post-translationally modified MTs are not the only marker for the development of neuronal polarity. Localization of the MAP protein tau to the same neurite is also a marker of the future axon (Caceres and Kosik, 1990). The microtubule-organizing center called the centrosome plays a major role in this process (Doxsey et al., 2005), where MTs assemble and nucleate asymmetrically with respect to the whole cell . It has been shown that a future axon is established at the site of colocalization of the centrosome and Golgi apparatus in cultured hippocampal neurons (de Anda et al., 2005). However, it is not a universal marker for axon formation in all types of neurons. It remains to be elucidated whether centrosome localization is a cause of polarization or a consequence of it.

During development of the brain, newly generated neurons move from the place of origin to the place where they will remain throughout the life of the organism is called neuronal migration. Neuron migration has been studied in detail during cortical development. Here, neurons proliferate in the ventricular zone and migrate along radial fibers into the different cortical layers (Rakic, 1990). MTs are involved in several parts of this process. They participate in leading edge extension by contributing, along with actin filaments, to the formation of the growth cone at the end of the axon and dendritic tips. MTs also play a role in nucleokinesis, the process of positioning the nuclei of nerve cells in a major process that directly defines cell migration (Book and Morest, 1990). Neuronal migration is more critically dependent on MTs than leading edge extension (Lambert de Rouvroit and Goffinet, 2001).

Another important step in neural development is formation of brain connectivity through regulated axon growth and guidance. The movement of the axon is controlled by the surrounding environment, more specifically by the gradient of guidance cues in the surrounding environment. The presence of a single molecule can attract or repel an axon from that location (Low et al., 2008). Semaphorins and ephrins repel axons from a specific area (Benson et al., 2005; Reza et al., 1999). Recent studies suggest that axon guidance cues fine-tune also the processes that occur after the initial connectivity is established, e.g. synaptic pruning (Dent et al., 2004; Purro et al., 2008).

Synaptic pruning is the process during which unnecessary synapses are eliminated. The result is an increased efficiency of transferring neuronal excitation. In the neurites, which must be removed, there is a local loss of MT-stabilizing post-translational modifications, such as polyglutamylation, and localization of MTs severing proteins, such as spastin (Brill et al., 2016) finally leading to the loss of the MTs themselves (Rumpf et al., 2019).

Deregulation of neurodevelopmental processes has been linked to the pathogenesis of various neurodevelopmental disorders such as attention deficit hyperactivity disorder (Ribases et al., 2009), autism (Boeckers et al., 2002), schizophrenia (Weinberger, 1987) and depression (Fukada et al., 2012).

3. PTMs

Various post-translational modifications (PTMs) edit functional groups of an amino acid or add a new functional group to already fully synthesized protein. Those modifications occur in the course of cell life through enzymatic activities.

PTMs of tubulin have been detected in the intermediate domains (LeDizet and Piperno, 1987) and on the C-terminal tail of tubulin monomers (Hallak et al., 1977). The most common MT modifications in neurons detyrosination (Hallak et al., 1977), acetylation (LeDizet and Piperno, 1987), phosphorylation (Diaz-Nido et al., 1990; Ori-McKenney et al., 2016), polyglutamylation (Edde et al., 1990) and polyamination (Song et al., 2013). Some of these modifications will be discussed below.

Modifications of MTs affect numerous neuro-developmental processes as differentiation, migration, axonal growth and synaptic formation. Stability and dynamics of neuronal MTs is indispensable for maintaining „correct“ neuronal development and its damage can lead to neurodevelopmental diseases (Fukada et al., 2012; Ribases et al., 2009; Weinberger, 1987).

For example, in his study, Ori-McKenney demonstrated that phosphorylation of serine 172 on β-tubulin is mediated by MNB kinase, that inhibits MT polymerization. Which eventually leads to disruption of the morphology of dendrites and the electrophysiological activity of neurons (Ori-McKenney et al., 2016). MNB kinase is associated with Down syndrome and autism spectrum disorders.

Other post-translational modifications, on the contrary, stabilize MTs. Thus transglutaminase-catalyzed polyamination prevents the depolymerization of MTs. Inhibition of transglutaminase leads to the almost complete elimination of long neurites during the growth and development of neurons (Song et al., 2013).

3.1 Detyrosination

The first tubulin modification to be discussed is detyrosination. The C-terminal end of most isoforms of α-tubulin after completion of proteosynthesis is decorated with tyrosine (Nieuwenhuis and Brummelkamp, 2019). The only isoforms that are not tyrosinated are TUBα8 with phenylalanine at the C-terminus and TUBα4A, which ends with glutamic acid (Gadadhar et al., 2017).

PTM itself is a detyrosination – process in which the carboxy-terminal tyrosine is removed by the enzyme TCP, tubulin carboxypeptidase (Hallak et al., 1977). The modification is carried out on already formed MTs and causes an increase in their stability (Gundersen et al., 1987). The MTs of neurons are long-lived and highly stable, because they contain a lot of detyrosinated tubulins.

The enzyme responsible for detyrosination was discovered only recently. In 2017, two independent studies identified the carboxypeptidases vasohibins 1 and 2 as detyrosinating enzymes, which bind to the small vasohibin-binding protein (SVBP) and form a complex capable of MT detyrosination (Aillaud et al., 2017; Nieuwenhuis et al., 2017).

Deficiency or chemical inhibition of VASH1–SVBP and VASH2–SVBP in cultured neurons delayed the differentiation of axonal protrusions demonstrating the importance of detyrosination for polarization of neurons (Nieuwenhuis et al., 2017).

Faxiang Li and colleagues showed how vasohibins (or VASH1) recognize the C-terminal tyrosine of α-tubulin. The tyrosine aromatic ring interacts non-covalently with arginine at position 222, while its carboxyl group forms hydrogen bridges with the hydroxyl group of serine 221 and the amide group of arginine 222 (Li et al., 2020).

The C-terminal ends of α-tubulin isoforms that have a terminal tyrosine or phenylalanine are acidic, therefore negatively charged, because they contain many glutamates. In contrast, electrostatic maps of the surface of the VASH1 enzyme have shown the presence of highly positively charged groups in the substrate binding cleft, which may help binding of tubulin carboxypeptidase to MTs (Li et al., 2020).

After the initial detyrosination, additional modification by cytosolic carboxypeptidases (CCPs) for more stable forms Δ2 (Paturle-Lafanechere et al., 1991) and Δ3 (Aillaud et al., 2016) may occur. Rethyrosination catalyzed by tubulin tyrosine ligases (TTLs) cannot occur on such isoforms (Ersfeld et al., 1993). Loss of this enzyme in mice leads to perinatal death due to lethal neuronal defects that are manifested throughout the brain, e.g. disruption of the cortico-thalamic loop (Erck et al., 2005). At the cellular level, in cultured TTL knockout neurons, such phenomena as enlarged growth cones with abnormal involvement of detyrosinated MTs and a higher percentage of neurons with two or more axons, which may even have been excessively branched, were observed (Erck et al., 2005; Marcos et al., 2009). This finding is consistent with the findings of the previously mentioned study. Knockout of TTL and lack of VASH1-SVBP

and VASH2-SVBP affect polarization, and therefore we can assume that proper regulation of tyrosination/detyrosination cycle is necessary for determination of neuronal polarity.

3.2 Acetylation

There are many acetylation sites on tubulin monomers and a large number of enzymes involved in tubulin acetylation and deacetylation (Chu et al., 2011; LeDizet and Piperno, 1987; Saunders et al., 2022). New enzymes catalyzing this PTM are still being continuously discovered. For example, in 2009, the enzyme SAN acetyltransferase was identified as a tubulin acetyltransferase catalyzing the acetylation of β-tubulin at the lysine in position 252 on free heterodimers (Chu et al., 2011). However, this modification has a negative effect on the regulation of the rate of MT regrowth after a catastrophe. Incorporation of acetylated tubulins is slowed, suggesting that acetylation by SAN acetyltransferase prevents polymerization of MTs.

One of the most abundant α-tubulin acetylation site is lysine 40 (Lys40) in the MT lumen (Shida et al., 2010), which is acetylated by α-tubulin acetyltransferase 1 (αATAT1). Lys40 acetylation of α-tubulin was not detected in Atat1 knockout mice, suggesting that αTAT1 is one of the major mammalian acetyltransferase (Kalebic et al., 2013). However, these mice are viable and showed no major developmental defects, only minor behavioral changes, such as higher anxiety and nonlethal brain abnormalities, specifically hyperplasia of cells in the gyrus dentatus (Kim et al., 2013). Nevertheless, it was shown that α-tubulin acetyltransferase 1 is essential for standard hippocampal development (Kim et al., 2013), therefore it may play a role in higher brain functions such as memory formation and learning. How exactly αTAT1 contributes to the development or disruption of these functions remains to be explored.

For the recognition of Lys40 and its subsequent acetylation by the αTAT1 enzyme, Asp39, Asp46 and Ser38 are essential (Yuzawa et al., 2015). αTAT1 binds to α-tubulin in already formed MTs, as indicated by the fact that the rate of enzyme turnover for MTs (k_{cat}) is six times higher than for free tubulin. The value of Michaelis constant K_M of αTAT1 for free α-tubulin is almost identical to the K_M of αTAT1 for MTs, could suggest that αTAT1 has no preference and acetylates both free and polymerized tubulin (Shida et al., 2010). Alternatively, it has been hypothesized, that the enzyme reaches the acetylation site through continuously appearing holes in the MT lattice, so-called MT breathing. The formation of such holes could be due to weak lateral interactions between protofilaments (Diaz et al., 2003). However, another hypothesis has

been proposed suggesting that acetylation enzyme TAT enters the interior of MTs because of the high degree of hydration at the site of Lys40 (Szyk et al., 2014).

In addition to catalyzing the acetylation of Lys40 of tubulin, αTAT1 also colocalizes with microtubule-associated proteins such as doublecortin, responsible for MT stabilization and bundling (Francis et al., 1999; Horesh et al., 1999), and through this interaction participates in the regulation of the structure and function of MTs (Kim et al., 2013).

Acetylation is reversible, the reversible reaction being carried out by histone deacetylase 6 (HDAC6) (Hubbert et al., 2002). Inactivation of the gene for HDAC6 in knockout mice led to hyperacetylation of α-tubulin (Zhang et al., 2008; Zhang et al., 2003), which was reflected in reduced anxiety and a lower tendency to depression (Fukada et al., 2012). This corresponds with findings on αTAT1 knockouts and suggests that acetylation is involved in processes associated with emotional states such as anxiety and depression. HDAC6 also negatively affects mitochondrial transport in hippocampal neurons (Chen et al., 2010). Again, inactivation of this enzyme leads to an increase in the rate of cargo movement along MTs, but the detailed mechanism has not yet been described. So far, it has been established, that acetylation promotes the interaction of tubulin with motor proteins such as kinesin-1 (Reed et al., 2006).

Other examples of deacetylases are Sirtuin 2 (SIRT2), which has been recognized as an NAD+-dependent alpha-tubulin deacetylase *in vitro* (North et al., 2003), and histone alpha-tubulin deacetylase (HDAC5), which plays a role in peripheral neuron reaction to injury which induces HDAC5 gradient and subsequent gradial deacetylation of MTs promotes axon regeneration (Cho and Cavalli, 2012).

Recent studies of the fly neuromuscular junction have revealed a novel acetylation site – Lys394 – on α-tubulin that is important for the proper development of axonal endings and normal synaptic morphogenesis. Lys394 is located at the tubulin heterodimer interface on the surface of α-tubulin suggesting that it may regulate dimer stability and/or influence dimer addition to the MT polymer. Loss of Lys394 acetylation has been shown to lead to dramatic reductions in MT stability, but not as a result of slowing MT polymerization (Saunders et al., 2022), rather, it is related to reduction in the number of MT loops, which are hallmarks of stable MTs (Pennetta et al., 2002; Roos et al., 2000) and a higher susceptibility of deacetylated Lys394 to destabilization, for example by nocodazole. Lys394 plays a role in synaptic morphogenesis because loss of acetylation or deacetylation at this site led to disruption of the presynaptic MT

cytoskeleton. The already known enzyme HDAC6 is involved in Lys394 deacetylation, but it is not known which enzyme is involved in its original acetylation (Saunders et al., 2022).

Surprisingly, the Lys394R mutation that mimics the loss of acetylation caused different phenotypes in different neuron types (Saunders et al., 2022), which may be related to different types of neurons exhibiting various functions and regulatory mechanisms or cellular responses. The regulatory mechanisms are also dependent on the specific tubulin code of each neuron type, which in turn influences the function of these cells (Aponte-Santiago and Littleton, 2020). Further studies are needed to identify the association of Lys394 acetylation with distinct neuronal phenotypes.

3.3 Polyglutamylation

It was already known in 1990 that polyglutamylation is a major post-translational modification in the brain (Edde et al., 1990). But how exactly this widespread modification affects the development of the brain was not known for long. Recently, though it has been shown that polyglutamylation is able to regulate the axonal transport of synaptic vesicles and thus affects the synaptic transmission (Ikegami et al., 2007). Not only intracellular transport is affected by this post-translational modification (Bodakuntla et al., 2020), but also axon growth and development in general, which is associated with MT cleavage by microtubule-severing enzymes: katanin and spastin (Lacroix et al., 2010; Shin et al., 2019). Polyglutamylation was also shown to modulate the binding of various molecular motors to MTs, specifically kinesins or axonemal dyneins, and microtubule-associated proteins (MAPs) and thereby change MT properties (Bonnet et al., 2001; Boucher et al., 1994; Kubo et al., 2010). For example, the processivity and rate of kinesin-1 requires higher levels of polyglutamylation in the form of ten glutamate residues, whereas for kinesin-2, intermediate levels, approximately three to ten residues, are sufficient (Sirajuddin et al., 2014).

Recent studies have shown that individual MAPs compete with each other (Monroy et al., 2018) to coordinately control traffic on the MT grid (Hooikaas et al., 2019; Siahaan et al., 2019). Polyglutamylation interferes with these processes, but by what mechanism and to what extent polyglutamylation can affect binding of MAPs to MTs is not yet clear.

The addition of glutamate to the C-terminal end is carried out in two phases: initiation and elongation (Redeker et al., 1991). Initiation glutamylation occurs on γ-carboxyl groups by creating an isopeptide bond on glutamate already incorporated to the protein sequence of tubulin. This reaction is carried out by the glutamylase enzymes TTLL4, 5 and 7 of the tubulin-

tyrosine ligase-like family (Mahalingan et al., 2020). The individual enzymes differ in their ability to initiate the glutamate side chain and, also in their preference for either α or β-tubulin (Ikegami et al., 2006; Janke et al., 2005). For example, TTLL7, which is highly expressed in the hypothalamus, hippocampus, thalamus, cerebellum, and spinal cord of the mouse brain, prefers β-tubulin to α-tubulin *in vitro* (Ikegami et al., 2006), due to the tripartite interaction with tubulin, the enzyme is able to direct the flexible β-tubulin C-terminus to the active site (Garnham et al., 2015). TTLL7 is a side-chain initiating polyglutamylase, so it adds one glutamate to the γ-carboxyl group of internal glutamate in a polypeptide chain (Ikegami et al., 2006; Redeker et al., 1991). Whereas TTLL5 introduces monoglutamates into multiple sites of the C-terminal end of β-tubulin (Redeker et al., 1991; van Dijk et al., 2007). When overexpressed, most TTLLs will lose preference and will modify both α- and β-tubulin indiscriminately, first the preferred subunit will be modified, after its saturation the second tubulin isoform will be modified (van Dijk et al., 2007).

Elongation glutamylation and subsequent polyglutamylation are carried out by enzymes of the same family: TTLL1, 6, 11 and 13. In the same way, they add Glu to previously added Glu residues, which sets a gradient in the number of residues that can then influence the interaction of specific microtubule-associated proteins. This time the glutamate residues can be linked to both the γ-carboxyl group and the α-carboxyl group. Such specificity depends on structural features in the active sites of the enzymes (Natarajan et al., 2017). Elongating glutamylases again have preferences for specific tubulin isoforms, creating specific PTM patterns on MTs.

In the mammalian nervous system, TTLL1 and TTLL7 are the most abundant, whereby TTLL7 plays a key role in neurite outgrowth and is responsible for the intense glutamylation of β-tubulins during neuronal maturation and neuronal network formation *in vitro* and *in vivo* (Janke et al., 2005). This glutamylase is more highly expressed in adult neurons, suggesting that polyglutamylated β-tubulin plays a role in postnatal nervous system development (Audebert et al., 1994). During neuronal differentiation the main brain polyglutamylase is TTLL1, which preferentially modifies α-tubulins (Audebert et al., 1994; Bodakuntla et al., 2021; Janke et al., 2005).

Like acetylation, polyglutamylation is a reversible reaction Moreover, deglutamylation of elongating and initiating glutamates is again mediated by different enzymes of the deglutamylase family of cytosolic carboxypeptidases (CCPs): CCP1, 4, 5 and 6 (Kimura et al., 2010; Rogowski et al., 2010). Their distinct enzymatic activities provide regulation of the removal of glutamate residues from MTs (Berezniuk et al., 2013; Rogowski et al., 2010). Thus,

CCP1 and CCP6 catalyze the deglutamylation of long side chains, while CCP5 removes glutamates added primarily to the C-terminus. These cytosolic carboxypeptidases are also able to remove the genetically encoded C-terminal ends of α-tubulin to form two isotypes: Δ2- and Δ3-tubulin (Aillaud et al., 2016; Paturle-Lafanechere et al., 1991). The malfunction of the enzyme caused by mutations in the CCP genes induces the accumulation of polyglutamylation, which can subsequently lead to neurodegeneration (Magiera et al., 2018; Rogowski et al., 2010). Thus, the latter study demonstrated that Purkinje cell degeneration in the mouse cerebellum of PCD (Purkinje cell degeneration) mutant mice is caused by a mutation in the CCP1 gene leading to excess of TTLL1-catalyzed polyglutamylation and neurodegeneration. In addition, upregulation of this modification inhibits axonal transport (Bodakuntla et al., 2021), which may be related to neurodegenerative disorders characterized by transport defects in neurons: Parkinson's, Huntington's and Alzheimer's diseases (Millecamps and Julien, 2013).

CCP1 can be found throughout the brain at the same level in the cortex and cerebellum, while CCP6 is expressed only in the cerebral cortex and hippocampus, with very low levels in the cerebellum. CCP1 knockout, as mentioned, has hyperglutamylation in the cerebellum not in the cortex. This phenomenon can be explained by compensation of absent CCP1 in this region by CCP6 (Magiera et al., 2018).

Polyglutamylation affects the function and speed of microtubule-associated proteins: for example, motor proteins kinesins bind strongly to long glutamate chains (Larcher et al., 1996), as does spastin, which cleaves MTs upon binding to polyglutamylated tubulin (Lacroix et al., 2010). Previous studies have suggested that this post-translational modification may regulate the affinity of several major neuronal MAPs: tau, MAP1B and MAP2 (Bonnet et al., 2001). Further studies are needed to understand overall effect of tubulin polyglutamylation in neurons.

3.4 Localization of PTMs in neurons

Different PTMs localize to various cellular compartments during neuronal development (Fig.2). Their distribution is considered to reflect various requirement of different parts of the neurons, for example the growth cone at the end of the axon requires highly dynamic MTs for its function of exploring space and responding to external signals. Such MTs are characterized by the presence of tyrosinated, deacetylated, and polyglutamylated tubulins in their lattice (Ahmad et al., 1993).

During the early stages of neuronal development, MTs must exhibit considerable dynamics for neuronal polarization, neuronal migration, axonal growth, and synaptic formation to occur

(Witte et al., 2008). Gradually, more and more stable long-lived MTs accumulate in the cells, especially at the site of axon formation (Witte et al., 2008). Here, detyrosinated tubulin, which is not affected by MT-destabilizing drug nocodazole, is considered an indicator of stable MTs. In contrast, nocodazole in inducing disassembly of tyrosinated MTs considered less stable (Arregui et al., 1991).

Long-lived MTs in neuronal axons contain not only detyrosinated but also acetylated tubulin (Cambray-Deakin and Burgoyne, 1987). The study on dendrites shows an interesting phenomenon of the functional distribution of MTs: in this compartment, there are two different populations of MTs that supports different types of molecule transport (Tas et al., 2017). Minus-end-out MTs are acetylated and low-tyrosinated. These low-tyrosinated MTs are preferred by plus-end directed motor molecule kinesin-1 (Konishi and Setou, 2009) which, though, does not enter dendrites. This may be due to the low-tyrosinated MTs being oriented minus-end-out and thus not accessible to kinesin-1 (Tas et al., 2017). Moreover, it seems differentially modified MTs are differentially distributed inside the axon, where less tyrosinated minus-end-out MTs are found in the center of the dendrite, while plus-end-out MTs, that are less acetylated but much more tyrosinated, are found at their periphery (Tas et al., 2017). Kinesin-3, that enters dendrites, colocalizes with peripheral MTs, which correlates with the selective interaction of this protein with tyrosinated MTs (Guardia et al., 2016).

The local distribution of glutamylated α and β tubulins also changes during neuronal development. At early stages, more glutamylated α-tubulin is found due to the preference of this tubulin isoform by the glutamylase TTLL1, which is more abundant during neuronal differentiation processes (Janke et al., 2005). At later stages of development, levels of glutamylated α- and β-tubulin nearly equilibrate and accumulate in neurons to a significant extent (Audebert et al., 1994; Przyborski and Cambray-Deakin, 1997).

Changes in localization of PTMs in development of neurons suggest that changes in tubulin code and consequently changes in tubulin stability and interaction with MAPs may be involved in regulation of neural development.

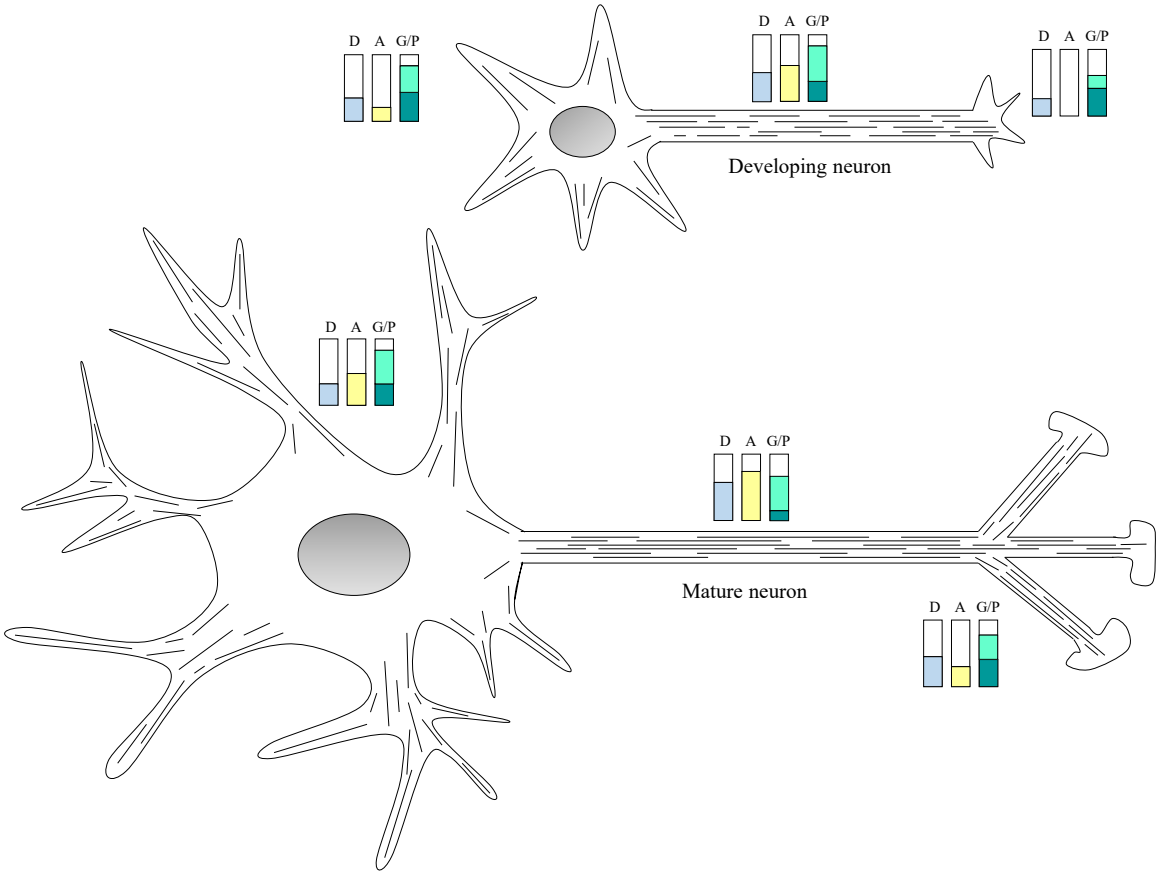

Figure 2: Localization of PTMs in various compartments of adult (bottom) and developing (top) neuronsThe developing neuron is enriched for glutamylated (G) (dark blue-green), polyglutamylated (P) (blue-green), and detyrosinated (D) (light blue) MTs in the neurites, for polyglutamylated, acetylated (A) (yellow), and detyrosinated MTs in the axon, and for glutamylated and detyrosinated MTs in the growth cone. The mature neuron contains a larger number of acetylated MTs compared to the developing neuron, which performs this modification only on axonal MTs. Modified by (Song and Brady, 2015)

4. MAPs

The term MAP refers to proteins that are associated with MTs. Originally identified as proteins that stabilize MTs, MAPs are now known to have a wide range of functions beyond stabilization. MAPs allow MTs to fold into mitotic spindles, form ciliary axonemes, and, in the nervous system, assist MTs in neuronal development. Based on their functions, MAPs are divided into several groups.

The first group includes the aforementioned microtubule motors, kinesin, and dynein, which mediate anterograde and retrograde transport along the MTs in axons and dendrites of neurons (Tas et al., 2017; Wortman et al., 2014). The second group includes proteins that uncouple MTs using energy in the form of ATP. These are the AAA proteins (ATPases Associated with diverse cellular Activities) including katanin, fidgetin, and spastin (Hartman et al., 1998; Hazan et al., 1999; Tao et al., 2016). The third group includes nucleators or stabilizing proteins. To this group belongs classical MAPs such as MAP2, MAP4, and tau, which contain an evolutionarily conserved carboxyterminal MTs-binding domain (Dehmelt and Halpain, 2005), doublecortin, which is involved in neuronal migration during brain development (Fourniol et al., 2013), as well as collapsin response mediator proteins (CRMPs) (Fukata et al., 2002). The last group includes structural MAPs, such as +tip and -tip, which preferentially bind to the plus- or minus-end of MTs (Bodakuntla et al., 2019) . The +TIPs form large interaction networks and thus enable control of growth cone (Hur et al., 2011), dendritic branching (Swiech et al., 2011), neuronal migration (Ka et al., 2014), and neurite formation (Hsieh et al., 2012), whereas functions of -TIPs are still under investigation.

4.1 MAP-2, tau

MT-associated protein families such as MAP-2 and tau are most abundant in neurons (Goedert et al., 1989; Shafit-Zagardo and Kalcheva, 1998). Three isoforms of MAP-2: MAP-2a, MAP-2b, and MAP-2c regulate neuronal development by modulating MT dynamics in the cell body and dendrites of neurons through different developmental stages (Shafit-Zagardo and Kalcheva, 1998). They regulate organization of dendritic MTs, their polarity, and dendrite growth (Dinsmore and Solomon, 1991). Specifically, MAP-2b is required for neurite outgrowth, while MAP-2c promotes greater flexibility and thus rearrangement of MTs in neurites during the early stages of development (Shafit-Zagardo and Kalcheva, 1998).

Greater attention is paid to the tau protein, which is encoded by the *Tau / MAPT* gene (Goedert, 2005). Mutations in this gene cause disruption of the native folding of the protein, which can lead to the accumulation of paired helical filaments or neurofibrillary tangles (Reed et al., 1997) associated with several neurodegenerative diseases, which are referred to as tauopathies, including Alzheimer's disease (Kosik et al., 1988), progressive supranuclear palsy (Wang and Mandelkow, 2016), Frontotemporal dementias (Ghetti et al., 2015) and tau phenotype has been identified also in Huntington's disease (Fernandez-Nogales et al., 2014). In the adult central nervous system, tau protein is represented by six isoforms, whereas only the shortest form is expressed in the fetal brain (Goedert et al., 1989). Structurally, this MT-associated protein contains 4 repeat binding domains that stabilize MTs. The N-terminal end, in contrast, protrudes from the MTs region and is involved in the linking of several MTs into bundles. It also affects dynamics, signaling cascades (Guo et al., 2017), and axonal transport (Kanaan et al., 2011). Phosphorylation of serine and threonine allows tau to relax from the MT lattice (Drewes et al., 1995). Therefore, hyperphosphorylation of tau leads to loss of tau stabilization function, destabilizes MTs and may promote neurodegeneration (Jeganathan et al., 2006).

4.2 CRMPs

Collapsin response mediator proteins (CRMPs) are MT-associated proteins important for neurodevelopment and includes five intracellular phosphoproteins: CRMP-1 to -5. These proteins are involved in neuron polarization and growth cone collapse. CRMP-2 was first discovered as an intracellular protein mediating Semaphorin 3A signaling. (Goshima et al., 1995). It was shown that CRMP-2 facilitates tubulin polymerization, thereby promoting axon growth and branching (Fukata et al., 2002). Mutation of this protein leads to abnormal termination of axons (Inagaki et al., 2001). CRMP-4 is also involved in the regulation of neurite size and growth cone (Khazaei et al., 2014). CRMP-5 regulates filopodial dynamics and growth cone development (Hotta et al., 2005), and overexpression of the protein leads to the enlargement of these structures (Ji et al., 2014). CRMP-5 expression is high in the fetal and neonatal brain, in which active neuronal differentiation and synapse formation occur; in the adult brain, expression declines to very low levels. (Inatome et al., 2000). CRMPs interact with other maps and participate in MTs rearrangement (Ji et al., 2018; Ji et al., 2021; Li et al., 2021).

4.3 Severing enzymes

Severing enzymes generate internal breaks in MTs. Mutations of the severing enzymes abundantly present in neurons also lead to various neurodegenerative diseases such as hereditary spastic paraplegia. Such enzymes include katanin, fidgetin and spastin. They are members of ATPases associated with diverse cellular activities that use energy from ATP hydrolysis to cleave MTs and share a homologous structure of a catalytic domain at the C-terminal end.

4.3.1 Katanin

Katanin targets and cleaves stable MTs with a preference for acetylated tubulins (Mao et al., 2014; Sudo and Baas, 2010). Katanin consists of two subunits: the catalytic p60 and the regulatory p80 (Hartman et al., 1998). This protein influences axon development: its expression is increased during growth and gradually decreases if the axon does not reach the desired length or its target (Karabay et al., 2004). It has been shown that a mutation in katanin that renders it inactive causes an increase in the body of the neuron and, conversely, a decrease in the number of axons and a decrease in the number of dendritic spines (Banks et al., 2018). Suggesting that catanin plays one of the key roles in the formation of the required number of neuronal protrusions.

4.3.2 Fidgetin

Fidgetin represents a functional inversion of catanin targeting non-acetylated dynamic MTs (Leo et al., 2015). Fidgetin is not as well described as the other two severing enzymes, but it is known to be involved in dendrite pruning as well as dendrite degeneration (Tao et al., 2016).

4.3.3 Spastin

Spastin was identified in 1999 as a microtubule-severing enzyme mutated in patients with hereditary spastic paraplegia (HSP) (Hazan et al., 1999). This disease is inherited in an autosomal dominant, autosomal recessive, but also X-linked recessive manner (Kenwrick et al., 1986; Novarino et al., 2014; Schule and Schols, 2011) and is associated with the progressive breakdown of the main pathways of the central nervous system running between the motor cerebral cortex and the spinal cord, the so-called corticospinal pathways (Fink, 2013; Solowska and Baas, 2015). This damage leading to rapidly developing spasticity and subsequently to the weakening of the lower limbs (Errico et al., 2002). Previously, degradation of corticospinal pathways was thought to be caused by haploinsufficiency of spastin (Qiang et al., 2019) and

subsequent lack of MT cleavage (Yip et al., 2003). Deficiency of spastin due to haploinsufficiency may cause disruption of organelle transport along MTs, which may eventually explain axon swellings of corticospinal neurons in HSP (McDermott et al., 2003). The current studies formulate a new hypothesis proposing that spastin-mediated HSPs develop through both loss of function and gain of function of the enzyme (Mohan et al., 2021). Experimental data suggest that haplosufficiency impairs the gain-of-function mechanism (Qiang et al., 2019). One of the genes underlying HSP and the most widespread is the SPG4/SPAST gene, which encodes the spastin protein (Hazan et al., 1999), (Fonknechten et al., 2000; McDermott et al., 2006).

4.3.3.1 Spastin isoforms

Open reading frame (ORF) of spastin contains two initiation codons and two major isoforms of this protein, M1 and M87, can be expressed simultaneously (Claudiani et al., 2005; Mancuso and Rugarli, 2008). The full-length M1 spastin (68 kD) contains 616 amino acids, the shorter (60 kD) form M87 consists of 86 amino acids (Claudiani et al., 2005). The M87 spastin isoform is more abundant in most tissues at all stages of development and is distributed evenly throughout the cell (Claudiani et al., 2005). The M1 isoform is absent from most of developing and adult neurons except for cytosol of neurons in spinal cord where it represent around 20 – 25% of all spastin isoforms (Solowska et al., 2008). Mutations in spastin M1 have been shown to be highly neurotoxic, causing inhibition of rapid axonal transport and impaired axon growth by binding heavily to MTs (Leo et al., 2017). Mutant M1 accumulates in motor neurons of the CNS, which may explain, at least in part, the susceptibility of motor neurons to spastin (Solowska et al., 2017). On the other hand the main function of M87 isoform is to cleave long MTs into shorter ones, which increases the availability of the plus-ends of MTs for polymerization and consequently promotes an increase in their dynamics and facilitates branching (Evans et al., 2005; Ji et al., 2021). M1 and M87 isoforms have been shown to form hexamers, important for spastin activity (Roll-Mecak and Vale, 2008). The spastin activity plays a crucial role in axonal development, neurogenesis and synapse formation.

Alternative splicing of exon 4 on mRNA can lead to the formation of two other spastin isoforms: M1Δ4 with a size of 64 kD and M87Δ4 with a size of 55 kD (Salinas et al., 2007). However, these two isoforms are not as abundant as M1 and M87 and are not yet well studied.

4.3.3.2 Spastin structure

Spastin consists of four domains that differ in their functions: the hydrophobic domain (HD), the MTs-interacting (MIT) domain, the MT-binding domain (MTBD) and the AAA domain (Fig.3).

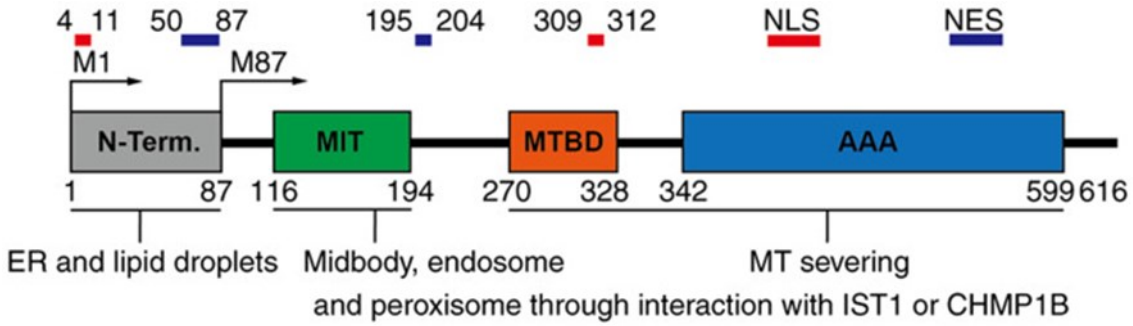

Figure 3: Structure of spastin monomers. The N-terminal domain mediates the attachment of spastin to the endoplasmic reticulum and lipid droplets. M1 and M87 isoforms differ in the presence of this domain. The MIT domain mediates interaction with MTs and other proteins such as endosomal sorting complexes. MTBD binds to MTs and the AAA domain is required for their cleavage. NLSs are nuclear localization signals (between amino acids 4-11 and 309-312). NES are nuclear export signals (between 50-87 and 195-204). These signals are required for the movement of spastin between the cytoplasm and the cell nucleus. Adopted from (Liu et al., 2021)

The hydrophobic domain (HD) forms a hairpin at the N-terminus of the protein in the region of 1 to 87 amino acid residues. This structure then helps spastin to insert into the lipid bilayer of membranes and into lipid droplets (Papadopoulos et al., 2015). Proteins with a similar hydrophobic loop enter the membrane of the endoplasmic reticulum and form its tubular structure (Park et al., 2010; Rao et al., 2016). However, it is not clear enough how and if spastin governs the shaping of ER.

Another N-terminal domain is the MTs-interacting (MIT) domain, which lies between 116 and 194 amino acid residues (Ciccarelli et al., 2003). It is assumed that this domain interacts not only with MTs, but also with proteins, such as chromatin-modifying protein or charged multivesicular body protein (CHMP1B) (Reid et al., 2005) or endosomal sorting complexes (ESCRT), which are involved in endosomal tubulation and transport, cytokinesis and other cellular processes (Allison et al., 2013). The interaction of MIT with other proteins provides

spastin with the ability to localize to specific subcellular locations, thereby regulates the spatial dynamics of MTs (Vietri et al., 2015). Interestingly, MIT domain is not required for MT cleavage (White et al., 2007).

At the position between residues 270 and 328 is the MT-binding domain (MTBD), which is necessary for the cleavage of MTs and sufficient for ATP independent interaction with the MT lattice. Unlike spastin, which included stretch 1 to 328, ΔMTBD spastin lacking region 279 to 328 was unable to bind to MTs, indicating that the protein requires amino acids 280-328 to binding with MTs (White et al., 2007). In 2018, new MTBD domain features were proposed. Namely, participation in the interaction between protein subunits and regulation of ATPase activity. It is proposed that MTBD interacts with the AAA domain of the neighbouring subunit to facilitate hexamerization of spastin, stimulate the ATPase activity of the AAA domain also of the neighbouring subunit but inhibit the ATPase activity of its own subunit (Fan et al., 2018).

The domain that directly mediates the formation of spastin hexamers in the presence of ATP and cleaves MTs is the AAA domain located in the region between amino acid residues 342 and 599 (Connell et al., 2009). This structure can be divided into two domains: a large nucleotide-binding domain (NBD) and a small four-helix bundle domain (HBD) (Roll-Mecak and Vale, 2008). The large domain contains motifs of conserved amino acids that are important for ATPase catalytic activity in specific structural motives as the Walker A or P-loop, Walker B or SwitchII, and arginine finger motifs (Ogura and Wilkinson, 2001; Putnam et al., 2001). Another amino acids contributing to ATPase activity and MT severing are leucine 470, isoleucine 473 and valine 474 in NBD domain, however mutation of these residues does not affect MT binding (Roll-Mecak and Vale, 2008).

The AAA domain represents the site with a clustered spectrum of mutations correlating with HSP disease and disruption of ATPase activity. Out of the more than 200 identified mutations, including reading frame shift mutations, large deletions, point mutations and missense mutations, around 70% are located in this domain. For example, mutations of P-loop residues suppress spastin binding to ATP or ATP hydrolysis. Arginine fingers of are also subject to undesirable changes, which in turn can lead to defects in ATP hydrolysis, but also to failures of oligomerization (Shoukier et al., 2009).

4.3.3.3 Hexamerization of spastin

As previously mentioned, active spastin consists of six monomers. In the presence of ATP, the protein weighs approximatly 250 kDa (White et al., 2007). The assembly of this structure is thought to be dependent on ATP hydrolysis and is stimulated only in the vicinity of its substrate, it implies that binding to MTs promote cooperative interactions between monomers. Hexamerization itself is essential for the catalytic activity of spastin (Eckert et al., 2012).

However, the binding of the hexamer to MTs is conditioned by three central loops of individual spastin monomers (Sandate et al., 2019), which form the so-called central pore of the protein and help to pull and push tubulin heterodimers out of the MT lattice through it (Roll-Mecak and Vale, 2008). While loops 1 and 2 contain residues that directly interact with the polyglutamylated substrate (Lys555, Tyr556, His596 and Arg601), loop 3 contains amino acids that interact with loop 2 and the ATP nucleotide (Sandate et al., 2019). The positively charged central pore of the spastin hexamer is thought to interact with the negatively charged C-terminal ends of tubulins (Roll-Mecak and Vale, 2008).

Amino acid residues at positions 415 to 417 forming the YVG motif in loop 1 are crucial for the binding of spastin to the tubulin backbone. The basic charged arginine residues and alanine residues at positions 451 and 457, respectively, are also contribute to the binding (White et al., 2007). A specific point mutation on residues Tyr415A, Arg451G, Ala457E and Glu442Q in the loops does not inhibit pore formation, but specifically prevents binding to tubulin (White et al., 2007).

4.3.3.4 Spastin and microtubule dynamics

Spastin with bound ATP interacts with MTs, this binding induces ATP hydrolysis, which subsequently leads to the extraction of tubulin from MTs. Spastin, meanwhile, exchanges ADP to ATP and can repeat the MT cutting cycle (Wen and Wang, 2013). The splitting of MTs by ATP-spasting generates repeated internal breaks (Fig.4), which can lead to complete loss of MTs (Kuo and Howard, 2021; Roll-Mecak and Vale, 2008). Sometimes nanodamages can be repaired by spontaneous incorporation of GTP-tubulin, which then produces the formation of GTP islands on the lattice of MTs (Vemu et al., 2018).

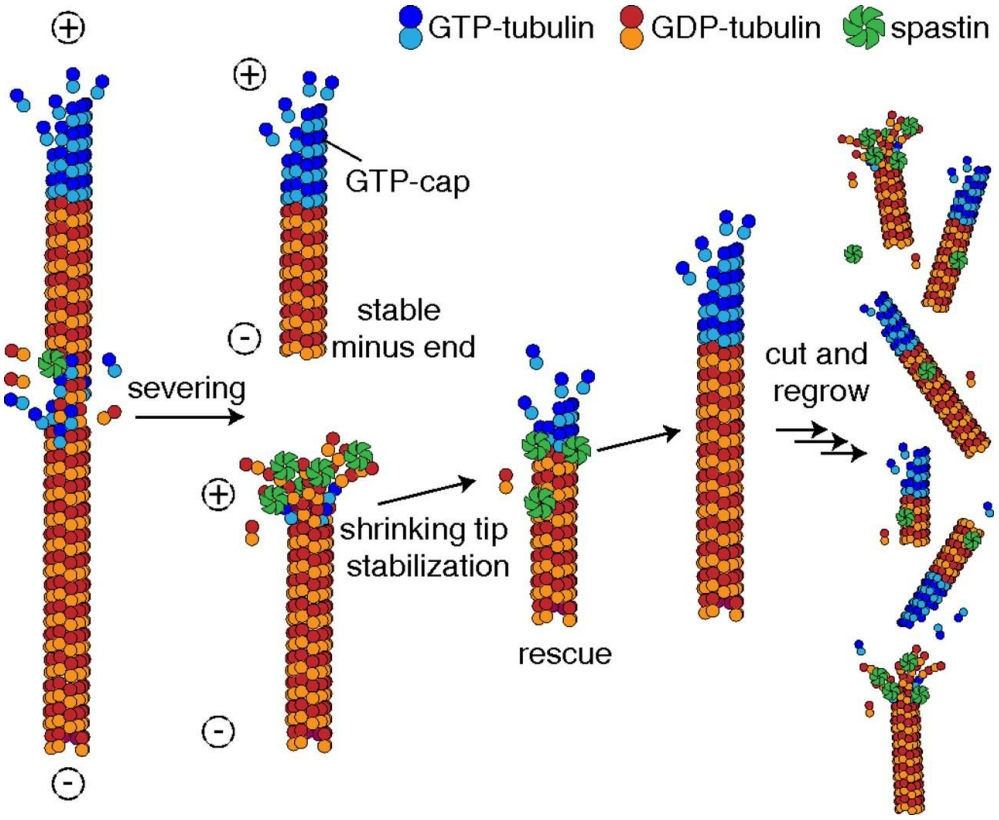

Figure 4: A model of MT mass magnification using spastin. Spastin removes individual tubulin dimers from the MT lattice, separating the individual parts of a single MT by generating nanodamages (left). The protein then binds to the GTP-cap, preventing rapid depolymerization of the entire lattice (middle). Subsequent repetition of these two steps results in an increase in MT density. Adopted from (Kuo et al., 2019)

In developing neurons MT dynamics promoted by spastin facilitates fast remodelling of structures and is important for cargo transport (Wortman et al., 2014; Yogev et al., 2016), synapses maturation and elimination (Brill et al., 2016), determination and growth of axons (de Anda et al., 2005).

Recent studies have demonstrated that spastin plays a key role in regulating embryonic neural stem cell (NSC) proliferation by showing that loss of the protein led to a reduction in the ability of NSCs to form neurons and glia and a reduction in the number and length of differentiated neurons (Jeong et al., 2019). Spastin deficiency leads also to a reduction in MT density and dynamics in neurons (Trotta et al., 2004) suggesting that formation and growth of neurons and glia require the dynamic MTs produced by this severing protein.. Cultured hippocampal neurons derived from human induced pluripotent stem cells (hiPSCs) of patients with mutations

in the *SPAST* gene exhibited an abnormal phenotype of neutrites: reduced length and less branching compared to hiPSCs without the mutation (Havlicek et al., 2014). These data support the previously mentioned idea that spastin plays a significant role in neurodevelopment.

Spastin and its tight control of the dynamics of MTs are also required in axon branching and maintenance of an active grow cone, since the grow cone is dynamic at the periphery and stable in the central part (Tanaka and Kirschner, 1991). During branching, MTs undergo destabilization, in which fragmented or also newly synthesized MTs move to the sites where the new axon branch will form (Yu et al., 1994). Severing enzymes are the main helpers in the generation of short stretches of MTs and spastin has been shown to accumulate at the growth tips and "buds" of future branches in cultured rat hippocampal neurons (Yu et al., 2008). Also, this study found an association of increased axonal branch formation with spastin overexpression.

4.3.3.5 Interaction partners of spastin

Currently, spastin-based research is focusing on the molecular mechanisms of its action, local regulation and interaction partners. Among them, CPRM protein family seems to be important. For example, in 2020, spastin was shown to co-localize in the spinal cord and interact with collapsin mediator protein 3 (CRMP3) *in vitro* and *in vivo* (Ji et al., 2021). These two proteins are involved in the neurite outgrowth regulation and branching and thus may contribute to the repair of acute traumatic spinal cord injury (SCI) (Ji et al., 2021; Yu et al., 2008). Overexpression of CRMP3 leads to an increase in spastin-mediated neurite growth and branching, indicating their joint action and putative interaction (Ji et al., 2021).

Another interaction partner is CRMP5, whose amino acids at positions 472-564 at the C-terminal end bind to residues 270-328 of the N-terminal end of spastin (Ji et al., 2018). This study also demonstrated that colocalization of spastin and CRMP5 in hippocampal neurons promoted neurite outgrowth by controlling MT dynamics.

CRMP2 has also been identified as a protein interacting with spastin *in vitro* and *in vivo* to promote neurite branching and growth, like previous collapsin mediator proteins. CRMP2 binds to spastin by its C-terminal end at the MT-binding domain (Li et al., 2021).

4.3.3.6 Spastin and polyglutamylated tubulin

Spastin prefers stable polyglutamyleted MTs, and therefore it is not surprising that the catalytic activity of this protein is potentially dependent on tubuline polyglutamylation (Lacroix et al., 2010), which could allow space- and substrate-regulated cleavage (Valenstein and Roll-Mecak, 2016). There is a negative correlation between spastin and polyglutamylated tubulin: loss of spastin in mouse hippocampal neurons induces an increase in polyglutamylated tubulin levels, leading to a reduction in the number of synapses (Lopes et al., 2020). Polyglutamylation can increase the negative charge of the C-terminal ends of α- and β-tubulins by the addition of negatively charged glutamates, so it has been proposed as a potential spastin-interacting element that regulates MT cleavage (Roll-Mecak and Vale, 2008).

An earlier study in 2010 established that spastin-dependent MT degradation in HeLa cells (a human epithelial cell line) is induced by the addition of long glutamate chains on tubulin. Thus, the presence of long glutamate chains at the C-terminal ends of tubulin is a signal to reduce the mass of MTs and thus stimulate spastin cleavage activity (Lacroix et al., 2010). A later study (from 2016) showed that it also depends on the number of glutamates added. Biphasic regulation of spastin-dependent MT cleavage and anti-cooperative behaviour of the protein at higher density of glutamylation were observed. When the number of glutamate residues at the C-terminal ends of tubulins did not exceed a threshold of eight residues, spastin activity was further enhanced by increasing number of glutamates. At the point when the threshold was crossed, the ability of spastin to cleave MTs decreased, but the so-called anti-cooperative behavior of this protein occurred. Spastin molecules showed increased binding affinity for MTs, which led to stabilization of MTs (Valenstein and Roll-Mecak, 2016). Thus, glutamylation has been shown to be able to locally regulate MT severing at different developmental stages.

5. Conclusion

A dynamic cytoskeleton with its basic component - microtubules - is essential for the proper course of virtually all neurodevelopmental processes. Microtubules and their active transformations are controlled at many levels. Critical role is played by PTMs and MAPs that can stabilize, destabilize or even sever MTs. At the same time, these levels of regulation can be interconnected, so different PTMs influence the binding and function of specific MAPs and binding of MAPs to MTs may facilitate or interfere with tubulin PTMs. Defects in the control of MTs by these components can lead to severe neurodevelopmental disorders such as schizophrenia, autism spectrum disorder or major depressive disorder. There is a need to study the roles and combinatorial effects of MTs regulators such as PTMs and MAPs in the context of neurodevelopment.

New studies in the biology of the tubulin code show that the function and rate of individual microtubule-associated proteins are controlled by post-translational modifications, creating a large spectrum of possible regulations and neuronal phenotypes.

It has been shown that detyrosination allows microtubules to interact with MAPs and promotes their stabilization. Acetylation accumulates on long-lived MTs, which are also characterized by their stability and affects motor protein localization. Polyglutamylation affects axon development, intracellular transport and interaction with various MAPs.

This bachelor thesis aimed to summarize the existing knowledge on post-translational modifications of microtubules and their associated proteins that play an important role in neurodevelopment. The focus was mainly on polyglutamylation and spastin as a mutually regulated PTM and a MAP that have been shown to generate number of different neuronal phenotypes, when deregulated.

One question that remains unanswered is how mutual regulation of microtubule dynamics by spastin and polyglutamylation specifically influence neurodevelopment. And whether these elements of regulation have any other embedded players that facilitate or, conversely, inhibit this pathway.

There is evidence that spastin cleaves specifically polyglutamylated tubulin, which is more abundant in axons. Spastin-mediated microtubule cleavage is known to increase the number of polymerization ends of MTs in the cell, thereby promoting dynamic remodelling of the neuronal cytoskeleton. This dynamic remodeling of MTs is important at early stages of

neurodevelopment because dynamic MTs are essential for neuronal differentiation and polarization, neuronal migration, growth and axonal guidance, synaptic formation and synaptic and axonal pruning. Polyglutamylation could be a signal that attracts spastin to specific cellular regions with a high requirement for microtubule cleavage. The specific localization of polyglutamylation in axons targets severing enzyme to this dynamically growing cellular compartment. Further studies of spastin and other severing enzymes together with changes of tubulin PTMs will provide new insight into the mechanisms of neuronal development and pathogenesis of neurodevelopmental disorders.

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