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Tubulin polyglutamylation, a regulator of microtubule functions, can cause neurodegeneration

Satish Bodakuntla, Carsten Janke* and Maria M. Magiera*

¹Institut Curie, PSL Research University, CNRS UMR3348, F-91401 Orsay, France

²Université Paris Sud, Université Paris-Saclay, CNRS UMR3348, F-91401 Orsay, France

*corresponding authors:

Maria M. Magiera, Carsten Janke, Institut Curie, rue Henri Becquerel, Centre Universitaire, Bâtiment 110, F-91401 Orsay, France

Telephone: +33 1 69863088; +33 1 69863127; Email: Maria.Magiera@curie.fr;

Carsten.Janke@curie.fr

Abstract

Neurodegenerative diseases lead to a progressive demise of neuronal functions that ultimately results in neuronal death. Besides a large variety of molecular pathways that have been linked to the degeneration of neurons, dysfunctions of the microtubule cytoskeleton are common features of many human neurodegenerative disorders. Yet, it is unclear whether microtubule dysfunctions are causative, or mere bystanders in the disease progression. A so-far little explored regulatory mechanism of the microtubule cytoskeleton, the posttranslational modifications of tubulin, emerge as candidate mechanisms involved in neuronal dysfunction, and thus, degeneration. Here we review the role of tubulin polyglutamylation, a prominent modification of neuronal microtubules. We discuss the current understanding of how polyglutamylation controls microtubule functions in healthy neurons, and how deregulation of this modification leads to neurodegeneration in mice and humans.

Introduction

Microtubules are key structural components of the neuronal cytoskeleton. They are involved in the establishment and maintenance of neuronal polarity, axon growth and branching, and serve as tracks for trafficking of cargoes in neurons [23, 52, 54]. A growing body of evidence points toward dysfunctions of the microtubule cytoskeleton in the pathogenesis of several neurodegenerative diseases [21, 79, 98]. Numerous genes mutated in the most common age-related neurodegenerative disorders (Parkinson's, Huntington's and Alzheimer's diseases) are related either to microtubule stability (tau protein [37]), microtubule severing (spastin [101]), or microtubule-based transport (huntingtin, kinesin, dynein [79]). However, the role of microtubules themselves in the pathogenesis of neurodegenerative disorders has only recently begun to emerge.

Microtubules are subject to a wide range of posttranslational modifications [72]. The tubulin code concept (Fig. 1A) [47, 97] proposes that these modifications can functionally specialise individual microtubules, thus locally and temporally adapting them to their wide variety of functions within living cells. Perturbations of tubulin modifications are expected to cause microtubule dysfunctions, which in neurons could eventually lead to neurodegeneration. Here we review recent findings that demonstrate the role of polyglutamylation, a key modification of neuronal microtubules, in neurodegeneration. We critically evaluate how cellular and molecular mechanisms that are implicated in neurodegeneration could be induced by perturbed polyglutamylation.

What is tubulin polyglutamylation?

Polyglutamylation is a posttranslational modification that adds secondary peptide chains of glutamates to its target proteins (Fig. 1A). Polyglutamylation has been first identified on brain tubulin [1, 25, 99], where it is highly enriched compared to most other tissues [128]. The first enzyme catalysing this novel modification was purified as a multi-protein complex from

mouse brains [48, 93]. Among the proteins of this complex was tubulin tyrosine ligase-like 1 (TTLL1), the catalytic subunit. This discovery bolstered the identification of additional TTLL enzymes, most of them catalysing polyglutamylation [45, 48, 124]. Strikingly, each TTLL enzyme generates a characteristic polyglutamylation pattern on tubulin (Fig. 1B) [124]. This specificity is determined by structural features within the active sites of those enzymes [73, 84]. Some glutamylases, such as TTLL4 and TTLL5, also modify other, non-tubulin substrates [92, 113, 123]. However, given that the main neuronal glutamylases TTLL1 [48] and TTLL7 [45] are tubulin-specific [71, 124], the glutamylation of non-tubulin substrates might play only minor roles in differentiated neurons.

Polyglutamylation is a reversible modification [2]. Enzymes responsible for deglutamylation belong to the family of cytosolic carboxypeptidases (CCP) [56, 96, 117]. Similar to glutamylases, deglutamylases have distinct enzymatic activities [6, 96, 129], thus allowing them to control the generation of specific polyglutamylation patterns on microtubules (Fig. 1B). CCPs also remove glutamates exposed on the carboxy-terminus of several proteins [108, 115] including detyrosinated α -tubulin, thus generating another posttranslational modification of tubulin, $\Delta 2$ -tubulin [89, 96].

Perturbation of tubulin polyglutamylation causes neurodegeneration in mouse models

The first mouse model of neurodegeneration to be linked to polyglutamylation was the Purkinje-cell degeneration (*pcd*) mouse. This mouse was identified in the 1970ies, and since then studied as a model of neurodegeneration with a characteristic spontaneous degeneration of Purkinje cells in the cerebellum at about 1 month of age [82]. Apart from the degeneration of Purkinje cells, *pcd* mice also display retina degeneration [10, 63], degeneration of the mitral cells in the olfactory bulb [38], degeneration of thalamic neurons [85, 86] and male sterility [41]. Early electron microscopy studies showed abnormal inclusions and organelle accumulation within the dendrites of degenerating Purkinje cells [61], which were postulated

to play a role in the degenerative process. Later reports found defects in myelination [16], excessive DNA damage [4, 5, 121], increased apoptosis [33, 34], endoplasmic reticulum (ER) stress [58], autophagy [7] and defects in synaptic connectivity [11, 61]. Excessive DNA damage and apoptosis were both excluded as potential causes of neurodegeneration in *pcd* mice, as neither the inactivation of DNA repair, nor the disruption of apoptotic pathways prevented Purkinje cell degeneration [126].

The mutation causing the phenotypes of *pcd* mice was later mapped to the *Nna1* gene, coding for a protein containing a zinc carboxypeptidase domain [29]. Rescue experiments showed that only an enzymatically active Nna1 could prevent neurodegeneration in *pcd* mice, thus demonstrating that the carboxypeptidase activity of this protein is essential for its neuroprotective function. However, the substrates modified by Nna1 were not known at the time [127]. The discovery that Nna1 is a member of a larger family of cytosolic carboxypeptidases (CCPs) [51, 95], which are involved in tubulin deglutamylation [8, 56, 96, 117] (Fig. 1B) strongly suggested that perturbed tubulin polyglutamylation could be the cause of the observed neurodegeneration in *pcd* mice.

As predicted, *pcd* mice showed excessive accumulation of tubulin polyglutamylation in the cerebellum and the olfactory bulb [96], two brain regions in which massive neurodegeneration had been described [38, 82]. Assuming that excessive polyglutamylation is the primary cause of the observed degeneration implied that reducing polyglutamylation of tubulin, i.e. microtubules, in the affected neurons might prevent, or at least delay their degeneration. This could indeed be demonstrated by targeting the main neuronal polyglutamylase, TLL1 [48]. Initial experiments using electroporation of interfering RNAs into cerebella of *pcd* mice avoided the degeneration of some Purkinje cells [96]. In a more thorough genetic experiment, *Tll1* was knocked out selectively in Purkinje cells of *pcd/Tll1-flox* mice using Purkinje-cell specific expression of cre recombinase (L7-cre [94]). In these

mice Purkinje cells did not degenerate, and even survived throughout the entire life of these mice [71]. This demonstrated that (i) accumulation of polyglutamylation on microtubules, generated by the tubulin-specific glutamylase Tll1 is the cause of the degeneration observed in *pcd* mice, and that (ii) this process is cell-autonomous, as it could be rescued by lowering tubulin polyglutamylation levels only in the Purkinje cells of the *pcd* mice. These conclusions are further bolstered by earlier experiments, in which cerebellar tissue from wild-type mice was grafted into cerebella of *pcd* mice. Not only did the tissue grafts survive in the *pcd* brains; wild-type Purkinje cells also replaced the degenerated neurons and formed functional synaptic connections with the host brain [32, 112].

But why was the degeneration in *pcd* mice limited to only some regions of the brain? One possible explanation was that other deglutamylases from the CCP family prevent hyperglutamylation in brain regions that remain unaffected, such as the cerebral cortex and the hippocampus. Indeed, when *Ccp6*, a deglutamylase strongly expressed in brain except for the cerebellum and the olfactory bulb [96], was depleted in *pcd* mice, a strong accumulation of hyperglutamylation in previously unaffected brain regions was observed. Consequently, cortical pyramidal neurons were found to degenerate in the *Ccp1/Ccp6* double-knockout mice [71].

These mouse models established that increased tubulin polyglutamylation affects the survival of a large spectrum of neurons in the nervous system. It also revealed that different types of neurons are not equally sensitive to hyperglutamylation. In the cerebellum, for instance, Purkinje neurons die relatively synchronously within four weeks [82], while granule cells degenerate only partially and over a much longer time interval [118]. Mitral cells in the olfactory bulb, while morphologically similar to Purkinje cells, start to degenerate only after two months [38]. Finally, pyramidal neurons in the cerebral cortex are only reduced by about 20% after five months despite massive accumulation of hyperglutamylation in brains of

pcd/Ccp6 (*Ccp1/Ccp6*) double knockout mice [71]. The reasons for the different time courses of degeneration of different neuronal populations could either be related to differences in their overall susceptibility to physiological perturbations [31, 102], or to different levels and activities of polyglutamylating enzymes [65]. Moreover, given that CCP1 has other, non-tubulin substrates [108, 115], it cannot be excluded that deglutamylation of these proteins contributes to the degeneration of neurons in *pcd* mice.

The role of microtubule polyglutamylation in neuronal function

The discovery that accumulation of polyglutamylation on microtubules is sufficient to induce neurodegeneration univocally demonstrated the importance of this tubulin modification for neuronal function and homeostasis. Determining which microtubule functions are controlled by polyglutamylation is therefore of key importance to understand the molecular mechanisms of this novel type of neurodegeneration.

Microtubules and their many partners

Polyglutamylation takes place at the carboxy-terminal tails of tubulins that decorate the outer surface of microtubules (Fig. 1A), and is thus expected to control interactions of microtubule-associated proteins (MAPs) with microtubules (Fig. 2A). MAPs are a large, heterogenous family of proteins that interact with microtubules to control their dynamics, structure and function. The so-called ‘structural MAPs’ are thought to stabilise microtubules [12]. In neurons, predominant MAPs of this group are tau, a key player in Alzheimer’s disease [36], as well as MAP1A, MAP1B and MAP2. Another group of microtubule-interacting proteins are the microtubule-severing enzymes spastin, katanin and fidgetin, which regulate microtubule mass and dynamics in cells [77]. In neurons, these enzymes play important roles in axon outgrowth and plasticity [18, 28, 100]. Mutations in spastin are linked to the most frequent form of a neurodegenerative disorder, hereditary spastic paraplegia [27, 30, 42].

Microtubules are the ‘tracks’ for molecular motors such as kinesins and dynein. In neurons, these motor proteins play a pivotal role in transporting a wide range of cargoes to distinct subcellular locations, thus assuring neuronal function and survival [40, 70]. Given the complex architecture of many neurons, it is not surprising that defects in neuronal transport are common features of human neurodegenerative diseases [17, 22, 55, 79, 110, 122]. In fact, transport defects, and therefore microtubule dysfunctions, might act upstream of other pathogenic events in neurodegenerative diseases [9, 111].

Polyglutamylation as regulator of microtubule interactors (Fig. 2A)

A large variety of microtubule-interacting proteins play key roles in neurons, and many of them have also been linked to neurodegenerative disorders. Even more strikingly, most of these proteins have also been linked to microtubule polyglutamylation. Early studies have suggested that binding affinity of several major neuronal MAPs, such as tau, MAP1B and MAP2 to tubulin is regulated by polyglutamylation [14, 15]. These results were obtained under non-native conditions and still await validation in native states. More thorough molecular insights have been gained on the regulation of the microtubule-severing enzyme spastin by polyglutamylation. Initial experiments in cells, and reconstitution of microtubule severing in cell-free conditions showed that in particular long polyglutamate chains on tubulin strongly induce severing by spastin [59]. More detailed analyses further revealed that polyglutamylation controls the severing activity of spastin in a non-linear, biphasic way: while the initial addition of the modification activated severing, further accumulation of polyglutamylation inhibited spastin activity [120]. In vivo observations suggested that katanin can also be activated by polyglutamylation of microtubules [106].

The molecular motors kinesin and dynein were long expected to be regulated by polyglutamylation. Early studies already suggested that kinesin motor proteins bind stronger to polyglutamylated tubulin [62]. First functional evidence obtained by direct measurements

of motor protein movement on native microtubules showed that polyglutamylation can regulate the motility of several motor proteins. Strikingly, different patterns of polyglutamylation had distinct effects on velocity and processivity of the motors, and not all motor proteins were affected in the same way [64, 109].

Polyglutamylation regulates cargo traffic in neurons (Fig. 2B)

Motor proteins in differentiated neurons are heavily implicated in intracellular cargo transport, which needs to cover long distances [19, 70]. It was therefore intuitive to hypothesise that polyglutamylation of microtubules could act as a regulator of neuronal traffic. Early indications for a role of polyglutamylation in intracellular transport came from observations in mice lacking the polyglutamylase subunit 1 (PGs1) of the TLL1 complex, which show reduced polyglutamylation in the brain. In neurons of these mice, the kinesin motor KIF1A was unable to move into neurites, while two other kinesins, KIF3A and KIF5, were not affected. Given that the KIF1A motor is particularly involved in carrying synaptic cargoes [67], this study suggested that polyglutamylation could regulate the delivery of synaptic vesicles, and might therefore also affect synaptic transmission [44]. Indeed, first insight into how synaptic activity could control postsynaptic cargo transport by modulating polyglutamylation was provided by another study. Activating hippocampal neurons by either stimulating AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole-propionate)-type glutamate receptors, or by inhibiting glycine-responsive receptors resulted in strongly increased levels of tubulin polyglutamylation, and a concomitant accumulation of vesicles containing the synapse-scaffolding protein gephyrin [119] in the cell bodies. Strikingly, the vesicle transport was restored when TLL1 was downregulated, strongly suggesting that polyglutamylation directly controls the trafficking of these vesicles [69]. This was recently confirmed in a mouse model where lack of spastin leads to microtubule hyperglutamylation and reduced delivery of AMPA receptors to synapses [68].

In primary hippocampal neurons cultured from hyperglutamylation mouse models, mitochondria, lysosomes, LAMP1 endosomes and BDNF (brain-derived neurotrophic factor) vesicles consistently showed reduced motility, though mitochondria and BDNF vesicles appeared to be most affected [13, 71]. Strikingly, the impact of polyglutamylation on the transport of these cargoes appeared to be cell-type specific: while in hippocampal neurons both, mitochondria and lysosomes were affected by hyperglutamylation [13], mitochondria, but not lysosomes showed reduced transport in cerebellar granule neurons [35]. The reasons for these differences are not yet understood, but it is clear that cell-type specific microtubule properties, as well as the differential expression of enzymes involved in polyglutamylation could be involved [65].

By which mechanism does polyglutamylation induce neurodegeneration? (Fig. 2C)

To determine the molecular causes of hyperglutamylation-induced neurodegeneration, different mechanisms were tested. Given that increased polyglutamylation is known to induce microtubule severing [59, 120], the role of spastin was tested by generating a *pcd(Ccp1)/spastin* double-knockout mouse. If the increased polyglutamylation in *pcd* mice would have contributed to the early degeneration of Purkinje cells via the stimulation of spastin-mediated severing, then deletion of spastin should rescue this phenotype. This, however, was not the case [71]. Instead, the observation of organelle accumulation in axons of mice with hyperglutamylation-induced neurodegeneration pointed towards defects in axonal transport. Indeed, perturbed cargo traffic was identified in neurons from these mice [13, 35, 71], but so far, no direct evidence for a causative role of transport defects could be provided.

Another important player in neurodegeneration are MAPs, in particular tau protein. Exposing cultured neurons to toxic beta-amyloid oligomers caused an upregulation of polyglutamylation on dendritic microtubules, which was accompanied by missorting of tau

protein from its typical axonal into more dendritic localisations. This was accompanied by microtubule loss [130], a common end point in neurodegenerative diseases [49].

Polyglutamylation was suggested to directly cause these two phenotypes given its known role in regulating tau-tubulin interactions [15] and spastin-mediated microtubule severing [59, 120].

Whether the proposed mechanisms work alone or in synergy, and to which extent they differ between different neurodegenerative disorders still remains an open question. Many microtubule-based mechanisms are interconnected, and polyglutamylation might affect several of them. Some MAPs have been shown to directly influence microtubule-based traffic [43, 80, 107, 114], and MAPs themselves compete on the microtubule lattice, which might allow a coordinated traffic control in cells [81]. Loss of the microtubule-severing enzyme spastin has recently been demonstrated to increase microtubule polyglutamylation in neurons, which in turn led to a reduced efficiency of neuronal transport [68]. Finally, MAPs have been suggested to shield microtubules from severing enzymes [91], thus controlling microtubule dynamics in cells. To which extent polyglutamylation can control these processes needs to be determined.

Given that combinations of polyglutamylases and deglutamylases allow for the generation of many different modification patterns on cellular microtubules, it is possible that polyglutamylation plays the role of central controller that coordinates the behaviour of multiple microtubule interactors in a targeted manner. This would provide a paradigm by which this modification could control microtubule dynamics, stability, and microtubule-based transport in neurons. It also implies that perturbations of this process do not affect a single, but rather several interconnected microtubule-based processes, which could synergistically contribute to the degeneration of neurons.

Tubulin polyglutamylation and its links to human neurodegeneration

The first human neurodegenerative disorder that has been directly linked to deregulated tubulin polyglutamylation was an infantile-onset, progressive neurodegeneration of the central and peripheral nervous systems caused by mutations in *CCP1*. Most obvious pathological representations of this disease are cerebellar atrophy and lower motor neuron degeneration [53, 104, 105]. As in the *pcd* mouse model, the *CCP1* mutations found in these patients consistently led to enzymatically inactive variants of *CCP1*. Many of the pathological hallmarks described in those patients are recapitulated in the *pcd* mice, which makes these mice an excellent model to study the human disease. It also allows the cautious conclusion that, similar to what has been shown in *pcd* mice, the degeneration in *CCP1* patients might be prevented by interfering with the polyglutamylating enzyme *TLL1* [71, 104]. Most importantly, the high similarity between the pathologies in mice and men also point to the possibility that new insights obtained from other mouse models with perturbed polyglutamylation are relevant in humans. For instance, mice lacking *Ccp1* and *Ccp6* have been used to demonstrate that perturbed polyglutamylation in the cerebral cortex leads to the degeneration of pyramidal neurons [71]. Pyramidal neurons are often affected in human late-onset neurodegenerative disorders, such as Alzheimer's disease [74], which opens the exciting perspective that perturbation of polyglutamylation could be involved in the pathology of these disorders.

Mechanisms that lead to deregulation of polyglutamylation in neurodegenerative diseases could be diverse. Mutations in other genes involved in polyglutamylation often lead to less obvious defects in mice, but might cause yet unexplored subtle defects in human neurons, especially given the longer life span of humans as compared to mice. It is also possible that polyglutamylation is deregulated by changing expression patterns of polyglutamylation-

related enzymes, or their posttranslational regulation, fields that have so far been little explored.

Several examples for dysregulation of enzymes catalysing polyglutamylation have already been found in genome-wide analyses of patients with neurodegeneration. A clinical study of 2,237 individuals revealed a strong association of single nucleotide polymorphisms (SNPs) in the *AGBL1* gene, coding for the deglutamylase CCP4, with learning disorders [78]. Recent studies using single-nucleus RNA sequencing and transcriptomics on samples from Alzheimer's disease patients showed differential expression of *TTL7*, one of the two major brain glutamylase, in oligodendrocytes [39, 76]. Whole-exome sequencing [125] and proteomic studies [103] identified *AGBL3* (encoding the deglutamylase CCP3) as one of the genes linked to early-onset Alzheimer's disease. The *AGBL2* gene, encoding the CCP2 deglutamylase, was shown to be present at a locus associated with susceptibility to Alzheimer's disease [60]. Both, CCP2 and CCP3 are deglutamylases expressed in the brain, but at relatively low levels. Knocking out both enzymes in mice did not lead to any obvious neurological phenotypes [117]. However, this does not exclude that these enzymes have specific functions in neurons that, when perturbed, could lead to late-onset defects followed by degeneration in humans. Given the rapid increase in the landscape of studies reporting mutations in polyglutamylation enzymes, it is conceivable that alterations of this modifications directly or in combination with additional risk factors could be linked to other late-onset human neurodegenerative diseases [57].

Conclusion and perspective

The microtubule cytoskeleton plays undisputedly an important role in many neurodegenerative diseases. However, which molecular processes are perturbed by microtubule dysfunction has remained an open question, in particular in late-onset, progressive neurodegenerative disorders such as Alzheimer's or Parkinson's diseases. The

recent discovery that the deregulation of a posttranslational modification of tubulin – polyglutamylation – directly causes the degeneration of neurons opens a novel perspective in the research of causes of neurodegeneration. Modulations of the microtubules themselves have timidly emerged as possible causes of neuronal dysfunctions: A growing number of tubulin mutations lead to neurodevelopmental disorders, and occasionally are also linked to neurodegeneration [20, 116]. Deregulation of other tubulin modifications, such as detyrosination and acetylation, were linked to neurodevelopmental processes [26, 50, 66, 75], synaptic function [87, 90], and disease [24, 46, 88, 131]. This multitude of processes have in common that they potentially modulate microtubule properties and functions, thus impacting, in many cases subtly, the physiological functions of the microtubule cytoskeleton [47]. Polyglutamylation stands out as it has so far not been linked to neurodevelopment, but specifically to degeneration. This converges with the observation that polyglutamylation accumulates in differentiated neurons [2, 3], and most enzymes catalysing polyglutamylation are prominently expressed in the adult brain [13]. Hence, polyglutamylation emerges as a promising mechanism and a potential druggable target in several neurodegenerative diseases.

Figures

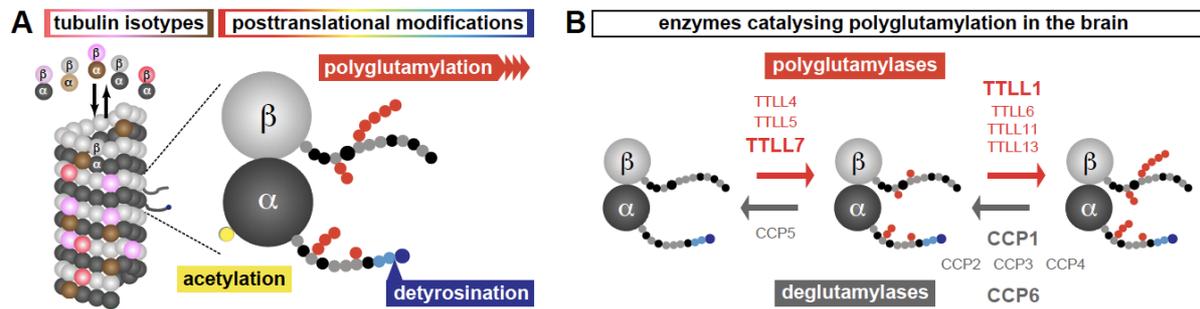


Figure 1: Tubulin polyglutamylation is part of the tubulin code that generates microtubule diversity

A) Mechanisms to generate molecular heterogeneity of microtubules: microtubules are dynamically assembled from different α - and β -tubulins. Tubulins are also subject to a variety of posttranslational modifications. In the nervous system, the key modifications are acetylation, polyglutamylation and detyrosination. **B)** Polyglutamylation is catalysed by a variety of enzymes from the tubulin-tyrosine-ligase-like (TTL) family. These enzymes have distinct specificities that allow them to generate specific modification patterns, which in turn could regulate specific functions of the microtubule cytoskeleton (Fig. 2). Polyglutamylation is removed by enzymes of the cytosolic carboxypeptidase (CCP) family. The key enzymes expressed in neurons and brain [45, 48] are shown in bold.

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Author contributions

All authors researched data for the article, contributed to discussion of the content, wrote the article, reviewed and edited the manuscript.

Competing interests

The authors declare no competing interests.

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