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Tubulin modifying enzymes as target for the treatment of tau-related diseases

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Abstract

In the brain of patients with Alzheimer's disease (AD), the number and length of microtubules (MTs) are significantly and selectively reduced. MTs are involved in a wide range of cellular functions, and defects of the microtubular system have emerged as a unifying hypothesis for the heterogeneous and variable clinical presentations of AD. MTs orchestrate their numerous functions through the spatiotemporal regulation of the binding of specialised microtubule-associated proteins (MAPs) and molecular motors. Covalent posttranslational modifications (PTMs) on the tubulin C-termini that protrude at the surface of MTs regulate the binding of these effectors. In neurons, MAP tau is highly abundant and its abnormal dissociation from MTs in the axon, cellular mislocalization and hyperphosphorylation, are primary events leading to neuronal death. Consequently, compounds targeting tau phosphorylation or aggregation are currently evaluated but their clinical significance has not been demonstrated yet. In this review, we discuss the emerging link between tubulin PTMs and tau dysfunction. In neurons, high levels of glutamylation and detyrosination profoundly impact the physicochemical properties at the surface of MTs. Moreover, in patients with early-onset progressive neurodegeneration, deleterious mutations in enzymes involved in modifying MTs at the surface have recently been identified, underscoring the importance of this enzymatic machinery in neurology. We postulate that pharmacologically targeting the tubulin-modifying enzymes holds promise as therapeutic approach for the treatment of neurodegenerative diseases.

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ABBREVIATIONS:

AD: Alzheimer's disease

Cryo-EM: cryo-electron microscopy

D: Aspartate

E: Glutamate

IFT: intraflagellar transport

MAP: MT associated protein

MAP2: MT-associated protein 2

MAPT: MT associated protein tau

MBD: MT-binding domain

MoA: mechanism of action

MTs: microtubules

NFT: neurofibrillary tangles

PET: positron emission tomography

PTMs: posttranslational modifications

RGCs: retinal ganglion cells

+TIP: MT plus-end tracking proteins

TTL: tubulin tyrosine ligase

TTLL: tubulin tyrosine ligase like

VASH: vasohibin

Y: Tyrosine

1. INTRODUCTION

Given the unprecedented speed at which the global population continues to age, finding therapeutics for the management of age-related indications and complications becomes urgent. Particularly, the number of people suffering from neurodegenerative diseases is increasing at an epidemic rate (Querfurth & LaFerla, 2010). According to the World Health Organisation the number of people living with dementia is expected to triple from 50 million today to 152 million by 2050 (World Health Organisation, 2017). Biochemically, AD is characterised by the accumulation in the brain of pathological amyloid plaques and neurofibrillary tangles (NFT). Due to the initial association between gene variants and plaque development, amyloid plaques have become the main target for pharmacological development (for review (Q. Wang et al., 2011)).

However, the prescient review by Steven Matsuyama and Lissy Jarvik, which was published thirty years ago (Matsuyama & Jarvik, 1989), proposed that defects associated with the microtubular system could explain most, if not all clinical presentations associated with AD. The accumulation of evidences on the role of microtubules (MTs) in fundamental neuronal processes further supports the hypothesis that impairment of MT-associated functions is a disease driver at the origin of the pathological changes observed in AD. Like the skeleton for the human body, each cell has an internal structure called cytoskeleton for maintaining its shape. MTs are the largest cytoskeletal filaments and are formed by polymerisation of α- and β-tubulin heterodimers that function as versatile modular building blocks to give rise to complex cellular structures with various morphologies. MT filaments have a distinct polarity, with ends that exhibit rapid polymerization at the plus (+) and slow polymerization at the minus (−) ends, respectively. MTs have a diameter of about 25nm, and are critical components of axonal projections that in humans can be as long as 1-meter long (e.g. sciatic nerve that extends from the spinal cord to the tip of the toes). In these structures, MT bundles are widely spaced and ordered in hexagonal arrays (Chen et al., 1992). Besides their structural function, MTs serve as tracks to molecular motors that transport essential cargoes. They rapidly can integrate

environmental cues by growing and shrinking, an essential molecular feature referred to as dynamic instability (Kristofferson et al., 1986).

In the context of neurological disorders including AD, finding a therapeutic that can preserve the function and integrity of neuronal MTs without inducing severe side effects holds the promise to be clinically useful for the management of these patients (Brunden et al., 2017; Kovalevich et al., 2016). MT-stabilising agents are among the most widely used drugs in cancer therapy. For instance, paclitaxel, sold under the brand name Taxol®, is a chemotherapeutic drug used for the treatment of various cancer types. The molecular mechanism by which Taxol® kills the cancer cells remains highly debated (Weaver, 2014). Initially, it was hypothesized that paclitaxel blocks cell division by affecting the highly dynamic spindle fibres and by activation of the spindle assembly checkpoint during mitosis. However, recent evidence suggests that paclitaxel intra-tumour concentrations are too low for blocking mitosis and that its cytotoxic effect occurs outside of mitosis, further increasing the ambiguity concerning its mechanism of action (MoA). In neurons, which are non-dividing terminally post-mitotic cells, MT-stabilising agents, such as paclitaxel, arguably might prevent MT depolymerisation and promote their stability. However, repositioning of these compounds as treatment for neurodegenerative disorders has not been successful. Despite very encouraging preclinical results with MT-stabilising drugs in animal models of AD (Barten et al., 2012; Makani et al., 2016; Ruschel et al., 2015; Zhang et al., 2012), the first-in-human phase 1 clinical trials were discontinued, presumably due to adverse effects (NCT01966666, 2019). A possible explanation is that many microtubule stabilisers are substrate of Pgp efflux pump and cannot sufficiently cross the blood brain barrier in humans, frequently inducing peripheral neuropathy in patients (Cavaletti & Marmiroli, 2010; Fellner et al., 2002; Gornstein & Schwarz, 2014; Hur & Lee, 2014). Hence, the lack of specificity of this approach and the drug-induced neuropathy seem to be major hurdles in drug development programmes. Moreover, recent studies using fluorescent paclitaxel and epothilone derivatives showed that human cells incubated with these compounds displayed cellular regions with unusual MT lattice conformation. These regions appeared at the growing MT ends that are in a pre-

catastrophe state and inhibit microtubule dynamics (Rai et al., 2020). The current literature converges toward the hypothesis that targeting the enzymes involved in MT posttranslational modifications (PTMs) could be clinically valuable as a neuroprotective treatment on its own or in combination with other therapeutics.

2. AN OUTLINE OF THE TUBULIN CODE

HUMAN RELEVANCE OF THE TUBULIN CODE

MTs provide the cell's structural framework but can also adopt a wide range of structures and behaviours to guide and organise various fundamental processes, including cell division, differentiation, motility and connectivity (Bulinski & Gundersen, 1991; Chang et al., 2002; Finkelstein et al., 2004; Gundersen & Bulinski, 1986, 1988). A central question in cellular biology is how all these different functions are executed or supported by the same filaments. It is becoming more and more evident that each MT function is dependent on the recruitment of a specific set of MT-associated proteins (MAPs) and molecular motors (McIlroy et al., 2020). To date, hundreds of proteins that bind to MTs have been identified, and new MAPs and related functions are still regularly discovered (Backer et al., 2012; Fassier et al., 2018; Goodson & Jonasson, 2018; Luke-Glaser et al., 2007; Mandelkow & Mandelkow, 1995; Y. Zhou et al., 2015). Many MAPs and molecular motors interact with the flexible tubulin C-terminal tails that protrude from the surface and thus are perfectly positioned to regulate protein binding (Ciferri et al., 2008; Lessard et al., 2019; Mizuno et al., 2004; Roll-Mecak & Vale, 2008; Skiniotis et al., 2004). Although small differences exist between α- and β-tubulin, both protruding C-terminal sequences are about 15 amino acid-long and contain a high number of negatively charged residues, such as glutamate (E) and to a lesser extent, aspartate (D). Conversely, the MT-binding domains found in many MAPs and molecular motors are mostly composed of positively charged amino acids, suggesting that the tails stabilise the association by electrostatic interaction. Thus, one way by which MTs can adapt to different functions is through

active modulation of the physicochemical properties of the protruding C-terminal tails. These modifications may fine-tune in a spatiotemporal manner the binding affinities of various MAPs that execute a specific biological function in the cell.

By analogy with the 'histone code' postulating that the transcription of genetic information is partly controlled by enzymatic modifications of histone proteins, the 'tubulin code' was proposed to explain the MT function specialisation (Verhey & Gaertig, 2007). After translation, tubulin dimers polymerise into MTs that are subjected to enzymatic modifications by covalent addition or removal of chemical groups/residues. These PTMs mostly affect the protruding C-terminal tails of both tubulins that extend at the MT surface. Reversible PTMs include ubiquitination, phosphorylation, glycylation, methylation, glutamylation, and detyrosination (for review (Carsten Janke & Magiera, 2020)). These enzymatic modifications are rapid and therefore, adjustable regulators of the interactions with cytoskeletal effectors. Since the proposal of the 'tubulin code' hypothesis, a growing number of studies provided evidence for the functional importance of these modifications (Kaul et al., 2014; Sirajuddin et al., 2014). An elegant example comes from an *in vitro* study showing that chemical changes on the tubulin C-terminus sequence affect both the velocity and processivity of the motor protein kinesin-1 (Sirajuddin et al., 2014). This observation was further confirmed by a more radical approach consisting of the complete removal of tubulin C-terminal tails using proteolytic digestion (Bhattacharyya et al., 1985; Serrano et al., 1984) (Z. Wang & Sheetz, 2000). Although the importance of biochemical diversity in the tubulin tails is underscored by the existence of numerous isoforms encoded by different tubulin genes (Chakraborti et al., 2016), this review will focus only on the functional relevance of the enzymatic modifications that target the C-terminal tails of tubulin in regulation of MAP binding in health and disease.

Alterations in tubulin PTMs have been associated with human pathologies affecting brain function and organisation. Infantile-onset neurodegeneration is the most compelling example of a human disease associated with aberrant levels of tubulin PTMs (Magiera, Bodakuntla, et al., 2018; Shashi et

al., 2018). This is a severe autosomal recessive neurodevelopmental disorder that affects the central and peripheral nervous system. It is characterised by global developmental delay, impaired intellectual development, poor or absent speech, and motor abnormalities that appear during the first year of life (Shashi et al., 2018). Recently, two studies revealed that a gene mutation in cytosolic carboxypeptidase 1 (CCP1), an enzyme that regulates the levels of tubulin polyglutamylation, is associated with the disease (Magiera, Bodakuntla, et al., 2018; Shashi et al., 2018). Detailed analysis of the pathological changes observed in patients with this disorder highlighted striking similarities with the phenotype of mice carrying the Purkinje cell degeneration (*pcd*) autosomal recessive mutation, including abnormalities in cerebellum and retina (Mullen et al., 1976). An additional observation illustrating the relevance of tubulin modifying enzymes in brain function and organisation came with the very recent report that defective tubulin detyrosination in humans causes structural brain abnormalities and cognitive deficiency (Z. Iqbal et al., 2019; Pagnamenta et al., 2019). Moreover, altered levels of tubulin PTMs were observed in brain specimens from patients with AD (Vu et al., 2017). Taken together, all these findings provided the "proof of concept" that deregulation of tubulin PTMs (e.g. glutamylation or detyrosination) can have deleterious effects on neural functioning in humans. Of note, many other pathologies, including cancer, heart failures and muscular dystrophy (Kerr et al., 2015), have also been linked to alterations of tubulin PTMs but fall outside the scope of this review.

TUBULIN DETYROSINATION IN NEURONS

Detyrosination was the first tubulin PTM to be described almost half a century ago (Barra et al., 1974). This PTM is specific to α-tubulin and removes the very C-terminal hydrophobic tyrosine residue (Y) encoded by most α-tubulins. Detyrosination leads to the unmasking of negatively charged glutamate residues (E), resulting in drastic changes in the physicochemical properties of the α-tubulin tail. Detyrosination is reversible and tyrosination is catalysed by an enzyme called Tubulin Tyrosine Ligase (TTL), which for identification was purified from brain tissue using classical biochemical and

immunology-based methods (Schroder et al., 1985). Knockout mice lacking TTL die perinatally due to abnormal neuronal wiring (Erck et al., 2005). The analysis of cultured *TTL*$^{-/-}$ neurons suggests that this phenotype could be explained by aberrant neurite outgrowth and premature axonal differentiation. Mechanistically, the observed defects correlate with mislocalization of the MT plus-end tracking protein (+TIP), called cytoplasmic linker protein 170 (CLIP170), caused by the almost complete absence of tyrosinated α-tubulin (Erck et al., 2005). Follow-up studies demonstrated that the reduced tubulin tyrosination and the very high levels of tubulin detyrosination have a broader impact and affect the binding of an entire subgroup of +TIPs that contain the CAP-Gly domain (Bieling et al., 2008; Peris et al., 2006). This domain is present in many MAPs, including CLIP-170 and CLIP-115 and also p150Glued that is a part of a MT-dependent motor complex called dynein (Peris et al., 2006; Weisbrich et al., 2007).

Unlike TTL that was discovered more than 30 years ago, the enzymes that catalyse detyrosination have been identified only very recently. Using different approaches, two independent studies showed that VASH1 and VASH2, two members of the vasohibin family, act as tubulin detyrosinases (Aillaud et al., 2017; Nieuwenhuis et al., 2017). In the approach originally developed by our laboratory, the α-tubulin C-terminal tail was chemically modified and used as a bait to biochemically enrich the detyrosinating activity contained in protein extracts obtained from brain tissue. This led to the identification of VASH1 and VASH2 and to the demonstration that they are key regulators of neuronal differentiation (Aillaud et al., 2017). The other group identified vasohibins as tubulin detyrosinases (Nieuwenhuis et al., 2017) by using a genetic screen (Carette et al., 2009). Apart from a bioinformatics study that suggested that vasohibins were related to an unusual class of cysteine proteases (Sanchez-Pulido & Ponting, 2016), the identification of vasohibin as tubulin detyrosinases was highly unexpected as they were originally considered to be secreted proteins involved in the regulation of vascularisation (Watanabe et al., 2004).

TUBULIN POLY-MODIFICATIONS IN NEURONS: GLYCYLATION AND GLUTAMYLATION

Two poly-modifications termed polyglycylation and polyglutamylation decorate the C-terminal tails of α- and β-tubulin. These modifications consist of the addition of either multiple glycine (G) or multiple glutamate (E) residues to the gamma-carboxyl groups of the primary sequence glutamates. Tubulin polyglutamylation, is broadly observed in neurons (Edde et al., 1990), while tubulin polyglycylation is restricted to cilia (Rogowski et al., 2009) and was recently reported to be involved in the control of the length of primary cilia (Gadadhar et al., 2017). On the basis of overlapping neurological defects observed in various ciliopathies, including Joubert syndrome, it has been suggested that primary cilia are also implicated in modulating neurogenesis, cell polarity, axonal guidance, and possibly adult neuronal function. Joubert syndrome is a autosomal recessive genetic ciliopathy characterised by congenital malformation of the brainstem, cerebellar vermis hypoplasia, oculomotor apraxia, intermittent hyperventilation, and delay in achieving motor milestones (Joubert et al., 1968). Initially considered as non-functional elements, primary cilia were later shown to modulate different morphogenic signalling pathways, including sonic hedgehog and Wnt (Huangfu et al., 2003) through the numerous receptors and ion channels they contain on their membrane. The MT-based core structure of cilia is called axoneme, and is evolutionarily conserved and shared by many species, from ciliates to mammals. Polyglutamylation and polyglycylation are particularly high on axonemal MTs (for review (Gaertig & Wloga, 2008)). Axonemes are the only known MT-based structures where these two poly-modifications coexist. Recently, it was reported that tubulin polyglutamylation is a major contributor to ciliary signalling, most likely by regulating intraflagellar transport (IFT) (He et al., 2018; Ikegami et al., 2006). IFT is a sophisticated transport system that is driven by kinesin-2 and cytoplasmic dynein, and is absolutely required for ciliary assembly and maintenance (Cole & Hammell, 1998). Kinesin-2 delivers all the components essential for the assembly of cilia, from the cell body where they are produced to the tip of cilium where the assembly takes place. Conversely, cytoplasmic dynein mediates the retrograde shuttling of the disassembled material that needs to be recycled (Kozminski et al., 1995; Pazour et al., 1999). Thus, it is likely that both poly-modifications regulate the efficiency of IFT-related molecular motors. Unlike

polyglutamylation, tubulin polyglycylation does not seem to be required for axoneme assembly, but might play a role in its maintenance (Rogowski et al., 2009), although both modifications compete for the same sites (Redeker et al., 2005; Wloga et al., 2009).

As the enzymes involved in polyglutamylation and polyglycylation share a homology domain with TTL, they are called tubulin tyrosine ligase like (TTLL). The human genome encodes 13 TTLLs, among which 9 are involved in polyglutamylation and 3 in polyglycylation (Rogowski et al., 2009; van Dijk et al., 2007). Tubulin polyglutamylation is reversible, and glutamate residues can be removed by members of the cytosolic carboxypeptidase (CCP) family (Rogowski et al., 2010). Therefore, the glutamylation level in a tissue is the result of the competition between the enzymes that catalyse the addition and removal of glutamate residues. TTLL1, the first glutamylase to be identified, was similarly to TTL also purified from brain tissue using classical biochemistry (C. Janke et al., 2005). Then, several studies showed that in neurons, TTLL1 is the main enzyme responsible for α-tubulin polyglutamylation. Surprisingly, TTLL1 knockout mice develop normally without any obvious neuronal defects (Vogel et al., 2010). This suggests that either TTLL1-catalysed tubulin glutamylation is dispensable for brain development, or that the remaining glutamylation on β-tubulin (catalysed by the TTLL7 glutamylase) is sufficient to fulfil the function of glutamylated tubulin in neurons (Ikegami et al., 2006). To unequivocally establish the functional importance of this PTM in brain development, double knockout mice lacking both TTLL1 and TTLL7 should be generated. *In vitro* studies have shown that polyglutamylation could be involved in regulating the binding of MT-associated proteins and motors (Bompard et al., 2018; Bonnet et al., 2001; Ikegami et al., 2006; Larcher et al., 1996). The original observation that polyglutamylation levels are critical for neuronal survival stems from studies in *pcd* mice that lack functional CCP1 (Rogowski et al., 2010). These mice display abnormally high levels of polyglutamylation that result in the degeneration of Purkinje cells in cerebellum, leading to ataxia. Two independent studies showed that Purkinje cell degeneration can be rescued by ablating the forward reaction encoded by TTLL1 glutamylating enzyme, clearly demonstrating that in *pcd*

mice, neurodegeneration is caused by tubulin hyperglutamylation (Berezniuk et al., 2012; Rogowski et al., 2010; van der Laan, Dubra, et al., 2019).

3. THE ROLE OF TUBULIN DETYROSINATION IN THE ESTABLISHMENT OF NEURONAL POLARITY

SPECIFICITIES OF AXONAL MTS

Neurons develop multiple dendrites, but only a single axonal projection. Dendrites contain microtubules of opposite polarity, while in the axon, MTs are uniformly oriented with their (+) ends distal to the body of the neuron (Yau et al., 2016). This specific MT organisation is critical for regulating the morphological characteristics of the projections, such as their relative lengths. Moreover, neuronal MTs are heterogeneous and contain different domains that differ in stability (Conde & Cáceres, 2009), composition, and PTMs as well as in how they interact with various MAPs (P. W. Baas & Black, 1990; Peter W. Baas et al., 2016). Axonal MTs are attached to the centrosome and released by the severing enzyme katanin for subsequent anterograde transport into developing processes. Inhibition of katanin by various experimental approaches blocks the release of MTs from the centrosome resulting in a marked increased in the length of MTs and severely compromised axonal growth (Ahmad et al., 1999; Karabay et al., 2004). The levels of katanin are high during rapid phases of axonal growth but diminish as axons reach their targets. Moreover the activity of the severing enzymes katanin and spastin are regulated by tubulin glutamylation (Lacroix et al., 2010; Sharma et al., 2007; Valenstein & Roll-Mecak, 2016). In growth cones, dynamic MTs act as sensors of cellular cues by extending the actin-rich peripheral domain in various directions (Goodhill et al., 2015; Michael Stiess & Bradke, 2011; Tanaka & Sabry, 1995). Some MTs interact with components of the cell cortex to activate signalling pathways required for regulating actin dynamics and axonal growth.

MTs have also been implicated in regulating the conversion of a motile growth cone into a synaptic terminal.

The principal microtubule-associated protein in axons is tau (MAPT), a natively disordered filamentous protein that is widespread in the central nervous system. Tau binding stabilises individual MTs and is responsible for the cross-linked structure of the axonal MT bundles (Conde & Cáceres, 2009). An early pathological hallmark of tau-related pathologies is the abnormal sorting of tau in the neuron somatodendritic compartment where hyperphosphorylated tau aggregates. Of importance, a previous *in vitro* study reported a role for differentially modified tubulin in tau binding (Saragoni et al., 2000). Overall, tau mislocalization and its invasion of dendrites coincides with the loss of cell polarity (Hoover et al., 2010), a mechanism underlying multiple brain diseases, such as neurodegenerative diseases.

MT STABILISATION IS ESSENTIAL FOR NEURONAL POLARITY

During neural development, the establishment of neuronal polarity is a key process that results in the formation of a single axon marked by the presence of tau, and of multiple dendrites characterised by the presence of MAP2 (Harada et al., 2002). A general increase in MT stability upon addition of low doses of the MT-stabilising agent paclitaxel (Taxol®) leads to the loss of neuronal polarity and the formation of multiple axons (Hammond et al., 2010). This was also observed in studies using a photoactivatable paclitaxel analogue showing that the local MT stabilisation in a neurite results in the differentiation of that projection into an axon, while the others become dendrites (Witte et al., 2008). Resembling paclitaxel-treated cells that develop multiple axons, cultured *Ttl*$^{-/-}$ neurons, which are characterised by abnormally high levels of detyrosination, also form multiple axons (Erck et al., 2005). Combined, these results suggest that similarly to paclitaxel, detyrosination might increase MT stability locally, and thus is likely to play a critical role in axonal determination. In support of this hypothesis, tubulin detyrosination inhibits the activity of kinesin-13 depolymerising motors (Helenius et al., 2006; Peris et al., 2009). Thus, high detyrosination levels might protect MTs from kinesin-13-

dependent depolymerisation, and increase their stability. This raises the important question of whether detyrosination could be the driving force of neuronal polarisation. A previous study demonstrated that the detyrosination level in the axon, especially in its initial segment, is much higher than in dendrites, and as such could increase the stability of axonal MTs. Furthermore, high tubulin detyrosination in the axon might function as positive signal to attract the kinesin-1 (KIF5) motor that localizes specifically to axons and plays an essential role in delivering all the components required to build this neuronal compartment (Kapitein et al., 2010; Konishi & Setou, 2009; van Beuningen & Hoogenraad, 2016). Tau is also localised specifically in the axon; however, at the moment, it is unclear whether axonal localisation of tau depends on tubulin PTMs, such as detyrosination. Future studies should investigate the relationship between the increased detyrosination levels in axonal MTs and tau localisation, and should precisely map the tau domain(s) responsible for this preferential axonal localisation.

The model suggesting that tubulin detyrosination might play a central role in neuronal polarisation is attractive, but does not explain how the specific increase of tubulin detyrosination in a neurite is achieved. This requires the local increase in the concentration or activity of detyrosinating enzymes in the neurite that will become the axon. Some studies have found a correlation between the proximity of a projection giving rise to the axon, and the cellular localisation of the centrosome (Higginbotham & Gleeson, 2007; M. Stiess et al., 2010). Although controversial, this observation suggests that one way to increase detyrosination is to localise the enzyme(s) involved in this PTM in the proximity of centrosomes. Strikingly, the two detyrosinating enzymes VASH1 and VASH2 are detected around centrioles and in their vicinity (Watanabe et al., 2004). During early neuronal development, vasohibins, which are concentrated around centrosomes, have preferential access to MTs in the neurite located close to the centrosome. The increased detyrosination level in this projection might promote the local MT stabilisation through kinesin-13 inhibition and possibly, the recruitment of structural MAPs, such as tau. In addition, detyrosination also serves as a signal for recruiting kinesin-1, which regulates the delivery of all the cargoes required for axon formation

(Konishi & Setou, 2009). For the model to work it requires that tubulin detyrosinating enzymes have a higher affinity for detyrosinated MTs leading to a generation of feed-forward loop. This model could contribute to the existence of fully detyrosinated MTs in the vicinity of tyrosinated MTs (Cambray-Deakin & Burgoyne, 1987; Robson & Burgoyne, 1989; Tas et al., 2017). Future *in vitro* experiments involving differentially tyrosinated MTs could provide initial experiments in support of this model. In conclusion, detyrosination might regulate all the major processes involved in neuronal polarisation. Considering the importance of detyrosination in neuronal polarity, tau mislocalization may be caused by deregulation of detyrosinating activities. More research is needed to precisely understand detyrosination role in this process.

4. THE MICROTUBULE-ASSOCIATED PROTEIN TAU AND NEUROPATHOLOGIES

ROLE OF TAU AS A MICROTUBULE-ASSOCIATED PROTEIN

More than twenty different human pathologies have been linked to tau dysfunction, including AD, Pick's disease, corticobasal degeneration, frontotemporal dementia, and glaucoma (Brunden et al., 2009; Chiasseu et al., 2016; Congdon & Sigurdsson, 2018; Kovacs, 2018; Tracy & Gan, 2018). These diseases are collectively called tauopathies and are characterised by abnormal accumulation of neurofibrillary tangles (NFT) in the brain and progressive neurodegeneration. From a biochemical perspective, NFTs are mostly composed of aggregated tau proteins. Tau is the most abundant structural MAP in neurons and constitutes more than 80% of the total protein mass bound to MTs (Kellogg et al., 2018). Alterations of tau physiological function are a common molecular mechanism in tauopathies.

The human *MAPT* gene contains 16 exons and transcript undergoes complex, regulated alternative splicing, giving rise to several mRNA species. MAPT transcripts are differentially expressed in the

nervous system, depending on stage of neuronal maturation and neuron type (Couchie et al., 1992, 1992; Goedert et al., 1992; Goedert, Spillantini, Jakes, et al., 1989; Nunez & Fischer, 1997; Y. Wang et al., 1993). In the adult central nervous system, MAPT transcripts undergo alternative splicing leading to the expression of six isoforms of the tau protein (Andreadis et al., 1992; Goedert, Spillantini, Jakes, et al., 1989; Goedert, Spillantini, Potier, et al., 1989). Importantly, exon 10 that encodes a microtubule-binding domain is also regulated by alternative splicing. In turn, splicing of exon 10 will impact the number of MT binding domains in the protein. When exon 10 is excluded, the resulting tau isoforms contain three MT-binding domains and are called Tau3R. In contrast, tau isoforms that have four MT-binding domains (when exon 10 is included) are called Tau4R. Overall, adult human CNS neurons express as much Tau3R as Tau4R (Goedert & Jakes, 1990), but this ratio displays regional disparities. Tauopathies are classified in three groups according to the predominant species of tau isoform that accumulates: 4R tauopathies (e.g., progressive supranuclear palsy, corticobasal degeneration), 3R tauopathies (e.g., Pick's disease), and 3R+4R tauopathies (e.g., AD) (Dickson et al., 2011; Irwin, 2016).

In AD, the most prevalent disease with a characterised tau malfunction, pathological tau accumulates in the brain decades before the detection of any clinical symptoms (Braak et al., 2006; Wharton et al., 2016). Moreover, a comprehensive literature analysis led by a consortium of clinicians concluded that among AD neuropathological modifications, NFT burden shows the best correlation with cognitive impairment severity in AD patients. This further substantiates the hypothesis that NFTs are drivers of neurodegeneration (Nelson et al., 2012). Moreover, in patients carrying a mutation in the *MAPT* gene, the presence of NFTs have been associated with the observed neurodegenerative abnormalities (Hong et al., 1998; Hutton et al., 1998; Strang et al., 2019). In AD neuropathology, NFT density and neuroanatomic localisation are crucial parameters. At the molecular level, NFTs are the result of altered tau function and behaviour. Dissociation from MTs is a critical event because tau pathological aggregation and hyperphosphorylation take place predominantly in the cytosol (Šimić et al., 2016). *In vitro*, tau overexpression leads to its missorting and charge alteration by

hyperphosphorylation (Zempel et al., 2017); however, the origin of pathological tau remains unclear. It is generally accepted that charge alteration underlies the switch-like response of signal transduction networks (H.-X. Zhou & Pang, 2018). Early studies already showed that tau phosphorylation strongly reduces its binding affinity for MTs due to structural changes and the attachment of multiple negatively charged phosphate groups (Jameson et al., 1980; Lindwall & Cole, 1984). As a result, the stability of the MT-based axonal projection is affected, leading to its disassembly and the release of excessive amount of tau protein that can then be further phosphorylated and aggregated. In turn, clearance of excess tau within the intracellular axonal compartment of the neuron is also essential. Previous work showed that in a mouse model of neurodegeneration, tau suppression resulted in recovery of memory function and increased number of neurons (Santacruz et al., 2005). Moreover, the post-mortem analysis of human brain specimens revealed that MT number and total length are significantly and selectively reduced in pyramidal neurons in patients with AD as compared to controls (Cash et al., 2003). In addition, tubulin extracted from brain tissues of patients with AD is less polymerisation-competent, suggesting that the intrinsic properties of tubulin subunits are changed in AD, possibly through changes in isotype expression and/or PTMs (K. Iqbal et al., 1976). On the basis of the above-mentioned, development of therapeutics that target tubulin modifying enzymes (i.e. the initial key process of tau dissociation from MTs) should be pursued alongside other strategies that focus on reducing the level of tau phosphorylation. In view of the unique sequence of the tubulin tails, modulating the levels of tubulin PTMs may provide additional advantage due to specificity of the approach. Overall, we hypothesize that disease-driving processes in AD might be caused by MT depolymerisation, leading to axonal retraction, loss of neuronal connectivity and ultimately cognitive decline. Moreover, it was previously reported *in vivo,* that local or systemic reduction of detyrosination accelerates axon regeneration and functional recovery (Gobrecht et al., 2016), further supporting the benefits of targeting detyrosination. In agreement, these authors suggested that pharmacological inhibition of MT detyrosinating enzymes may be a suitable clinical neuroprotective strategy.

TAU DOMAIN ARCHITECTURE AND PATHOLOGICAL PHOSPHORYLATION

At physiological pH, full-length tau is a dipole with clearly defined domains having opposite charge. Its acidic N-terminal domain (pI=4.0) is negatively charged, whereas the proline-rich domain (pI=10.3) and the microtubule-binding domain (MBD) (pI=9.8) are positively charged. At the C-terminus, there is a neutral domain that plays a regulatory role in stabilising the electrostatic interaction with MTs (Sillen et al., 2007). Most MAPs exhibit a similar domain organisation characterised by substantial disparities in acidity between the MBD and the other domains. This is well exemplified by the recently identified CSAP, a master regulator of tubulin glutamylation that possess a similar domain organisation (Bompard et al., 2018). Like tau and many other MAPs, CSAP contains a positively charged basic domain for strong electrostatic interaction between the MBD and the negatively charged residues at the MT surface. Furthermore, this model of MAP interaction is supported by the recently published near-atomic structure of physiological tau binding to MTs obtained by cryo-electron microscopy (cryo-EM) and computational modelling (Kellogg et al., 2018). Using a high resolution (atomic or near-atomic) structural biology technique, the study revealed that tau appears as a nearly continuous stretch on MTs, spanning over three tubulin dimers centred on α-tubulin. This mode of interaction explains tau stabilising function and the reduced off-rate of tubulin dimers, a spontaneous reaction blocked by tau-dependent stabilisation (Kellogg et al., 2018). Tau also is subjected to many PTMs (e.g. phosphorylation, acetylation, methylation, ubiquitination) (for review (Tapia-Rojas et al., 2019)). Phosphorylation, the most abundant PTM (so far 85 identified modifications sites) (Figure 2), adds many negatively charged residues to tau known to have significant effects on protein folding and interactions.

In AD, tau phosphorylation patterns change with disease progression (Augustinack et al., 2002). For instance, early phosphorylation has been linked to disruption of tau association with MTs and its relocation to the somatodendritic compartment (Hoover et al., 2010). By analysing the phosphorylation sites of full-length tau, we noticed two phosphorylation clusters flanking the MT

binding domain. One of these clusters is within the positively charged basic proline-rich domain of tau (Figure 2). During the early stages of AD, tau phosphorylation leads to overall charge inversion of this specific domain, from positive to negative. Moreover, it was previously shown that tau phosphorylation at Thr214, Thr231 and Ser235 in the basic proline region contributes to its dissociation from MTs (Ksiezak-Reding et al., 2003; Sengupta et al., 1998). This was confirmed in cultured cells and neurons that express hyperphosphorylated tau variants (Alonso et al., 2018; Eidenmüller et al., 2000). Accordingly, tau phosphorylation on Ser199, Ser202/205, Thr231 and Ser262 is associated with pre-tangles (i.e. newly formed tau aggregates that contain the early marks of the disease). In the phosphomimic tau triple mutant (Thr212/Ser235/Ser262 mutated to the negatively charged glutamate), the binding affinity to MTs is strongly reduced (Eidenmüller et al., 2000). Interestingly, the protruding anionic C-terminus of α-tubulin is functionally important for MT biology and tau association (Lefèvre et al., 2011). The recent cryo-EM high resolution model of the interaction between tau with the outer surface of MTs provided further structural insights into the residues involved in stapling together tubulin subunits and stabilizing the polymer (Kellogg et al., 2018).

A BIOPHYSICAL PERSPECTIVE: ELECTROSTATIC INTERACTIONS

Strikingly, tau phosphorylation sites are mainly clustered on both sides of the MBD (Figure 2). Therefore, we hypothesize that hyperphosphorylation of the proline-rich region flanking the MBD might greatly affect the overall electrostatic charge in the vicinity of the tubulin tails. At physiological pH, this proline-rich region contains various positively charged residues that electrostatically favour tau interaction with MTs via the negatively charged protruding C-terminal tails (Kellogg et al., 2018). However, tau hyperphosphorylation at Thr212, Thr214, Thr231 and Ser235 (within the proline-rich domain) leads to charge inversion and electrostatic repulsion (Alonso et al., 2010; Bancher et al., 1989; Köpke et al., 1993). This weakens tau interaction with MTs, alters the dynamics of association in favour of dissociation, thus representing an early stage of the neurodegenerative process.

Moreover, *in vitro* N-terminally-truncated tau that lacks these phosphorylation sites, binds more strongly to MTs than full-length tau (Derisbourg et al., 2015), further underscoring the importance of this domain in its interaction with MTs. PTMs that target the MTs can also modulate and alter the electrostatic repulsion between MTs surface and tau. The bulky hydrophobic tyrosine (Y) residue at the very end of the α-tubulin tail, which protrudes at the surface, may be of paramount importance because it obscures negatively charged glutamates (E). Therefore, enzymatic removal of this hydrophobic residue might directly affect the interaction between MTs and tau or phosphorylated tau. Likewise, tubulin polyglutamylation also results in increased exposure of negatively charged glutamate (E) residues at the MT surface. In this case, the length of the glutamate side chains might also be a relevant parameter since it is directly related to the overall negative charge of the tail. Moreover, the activity of the tubulin modifying enzymes on MTs appear to be regulated by the status of the modifications on the tubulin tails. Indeed, a crosstalk between detyrosination and glutamylation was recently proposed, opening the possibility that acting on a specific modification might impact the activity of another tubulin modifying enzyme (Mahalingan et al., 2020). Overall, we postulate that following its phosphorylation, the progressive loss of tau affinity for MTs is strengthened by tubulin detyrosination and/or polyglutamylation through exposure of negatively charged residues that increase the repulsive force between MTs surface and phosphorylated tau (Figure 3). It would be very surprising that charge alteration on tau impacts its association to MT's but that charge alteration on MTs does not.

TARGETING TAUOPATHIES FROM THE 'OTHER' SIDE

A detailed analysis of tau localisation in *pcd* mice in which tubulin is hyperglutamylated, as well as in Ttl-/- mice in which tubulin detyrosination level is increased should corroborate the above-mentioned therapeutic strategy (Figure 3). Likewise, a detailed analysis of tau localisation in patients carrying genetic mutations in the *CCP1* gene linked to altered tubulin glutamylation (Magiera, Bodakuntla, et al., 2018; Shashi et al., 2018) or genetic mutations in the *SVBP* gene that leads to

altered detyrosination (Z. Iqbal et al., 2019; Pagnamenta et al., 2019) would also provide valuable additional insights. Remarkably, genetic mutations in these two genes both result in severe neurological defects, suggesting overlapping molecular disturbances. Hence, we propose that pharmaceutical targeting tubulin glutamylases or detyrosinases should be clinically evaluated as target for treatment of neurological disorders. Targeting the enzymes that modify "the exterior" of MTs without directly affecting MT dynamics could prove to be a new mode of action that is therapeutically superior while displaying reduced toxicity.

Concerning tubulin glutamylases as therapeutic targets, some previously described phosphinic acid-based inhibitors have been assessed in an *in vitro* model (Liu et al., 2013; Mahalingan et al., 2020). Nevertheless, more work is needed to optimise potential compounds for preclinical development into drug candidates. The available structural data on glutamylases also should facilitate drug development (Garnham et al., 2015). Compared with detyrosinases, the identification of specific glutamylase inhibitors might be more challenging because they belong to a large family with nine different enzymes. On the other hand, the very recent identification of detyrosinases opens the way for developing therapies that specifically target these enzymes to modulate tau binding, while limiting the direct effects on MT dynamics. In this respect, targeting vasohibins as detyrosinases might improve two defective processes in AD: axonal retraction and possibly vascularisation and hypoperfusion. Vasohibins belong to the group of cysteine proteases that include an evolutionary conserved catalytic triad with a nucleophilic cysteine thiol (Aillaud et al., 2017; van der Laan, Lévêque, et al., 2019; Vicik et al., 2006). As many strategies have been developed to inhibit cysteine proteases (Silva et al., 2017; Vicik et al., 2006), the identification of an effective VASH1/2 inhibitor should be a feasible task. Moreover, the available structural data on VASH1 (Adamopoulos et al., 2019) and very recently on the microtubule-VASH1-SVBP complex obtained by Cryo-EM (Li et al., 2020), will accelerate and contribute to the process of rational drug design. Overall, in view of the importance of tubulin PTMs in human pathologies, molecules that modulate such PTMs should be

evaluated as a therapeutic strategy in neurological disorders, especially in the context of neurodegeneration.

5. CLINICAL PERSPECTIVES IN ALZHEIMER'S DISEASE

Currently, drug development for AD and their clinical evaluation remain challenging, costly and mostly unfruitful (Becker et al., 2008). In clinical trials, AD staging is critical, and the subjective nature of the available approaches, such as the Clinical Dementia Rating-Sum of Boxes (CDR-SB) score, further complexify the definition of robust inclusion criteria and the monitoring of disease progression. On the other hand, new tracers offer the possibility to study the brain accumulation of tau and beta-amyloid (two biochemical hallmarks of AD) *in vivo* in patients (Chun, 2018; Higuchi, 2019). Currently, the vast majority of drugs in development for AD treatment targets primarily tau and amyloid-beta aggregates, although the success of such strategies as monotherapy has not been demonstrated yet. In this respect, the very recent success of the anti-amyloid therapeutic antibody aducanumab (produced by Biogen) for AD treatment could be a major breakthrough, if independently validated. Although initially abandoned in Phase III (Biogen press release, 2019), a new analysis supports its potential benefit. After the previously published clinical data on aducanumab (Sevigny et al., 2016), this announcement is very encouraging because it would represent the first potential therapeutic with clinical significance for the management of AD. An independent clinical study to validate these findings must now be performed.

Concerning tau accumulation, the patterns of tracer retention correspond well with the Braak staging of neurofibrillary tau pathology (Scholl et al., 2016), although various technical challenges still need to be addressed (Leuzy et al., 2019). A very recent study using positron emission tomography (PET) with tau tracers (tau-PET) in patients with early symptomatic AD demonstrated that the specific distribution of the tau-PET signal is a strong indicator of the topography of future atrophy at the level of each single patient. Moreover, the relationship between baseline tau-PET signal and the

subsequent atrophy was particularly strong in younger patients. The authors concluded that tau pathology is a major driver of local neurodegeneration and highlighted the relevance of tau-PET as well as tau phosphorylation as a precision medicine tool to help predict disease progression in individual patients and design future clinical trials (Barthélemy et al., 2020; La Joie et al., 2020).

Although much pharmaceutical research and development is focusing on tau phosphorylation status, targeting tubulin modifying enzymes in the context of tauopathies has never been explored, despite accumulating evidence on the emerging link. Tau targeting strategies using kinase inhibitors have been mostly abandoned because of toxicity or lack of effect (Medina, 2018; van Dyck et al., 2019). Targeting tubulin PTMs holds the promise to be of interest in early tau-related events but also in later stages. For instance, inhibition of detyrosinases or glutamylases may limit the electrostatic repulsion and prevent tau dissociation from MTs. As tau mislocalization causes amyloid-beta toxicity (Ittner et al., 2010), addressing the disease driving mechanisms constitutes a superior therapeutic strategy. Future studies will determine whether the pharmacological inhibition of tubulin detyrosinases and glutamylases in the context of tau-related neurodegenerative disorders is beneficial for patients as stand-alone treatment or in combination with other strategies.

6. CONCLUSIONS

Neurodegeneration is a collective term to define the progressive loss of the structure, function, and ultimately death of neurons. To our knowledge, there is no drug on market or in clinical trials designed to target PTM of MTs. Recent studies showed that accumulation of PTMs regulates MT dynamics and proposed that it is the driving factor in the induction of tau-mediated neuronal damage (Qu et al., 2017). Dissociation of tau from MTs is a very early event in tauopathies, and tau hyperphosphorylation weakens the interaction and increases tau concentration in the cytosol. Overall, it is tempting to hypothesize that inhibition of tubulin modifying enzymes, including detyrosinases and glutamylases, may reduce the level of negatively charged amino acids exposed at

the MT surface, thus decreasing the electrostatic repulsion of phosphorylated tau in early and also late disease stages. The field has witnessed recent breakthroughs, including the identification of tubulin detyrosinases, the clear association with human pathologies, and the first near-atomic cryo-EM description of tau binding to MTs. All these findings indicate that acting on tubulin modifications is a suitable therapeutic approach. Bulky hydrophobic aromatic residues, such as tyrosine, can obscure the negatively charged glutamate residues. Therapeutic strategies that target tau-MT interaction need more investigations. Combinatorial approaches in which kinase inhibitors are associated with inhibitors of enzymes that target tubulin PTMs, could be clinical advantageous. Development of therapeutics targeting disease drivers hold the promise to be clinically efficient to preserve neurons from death and halt degeneration. Once memory is lost, one cannot recover it.

Conflict of Interest Statement:

A patent entitled “Methods for purifying proteins having a tubulin carboxypeptidase activity and peptidic based inhibitors thereof” has been filed by CNRS. KR, KH and SvdL are shareholders of MT-act, a preclinical stage spin-off company of the Institute of Human Genetics, Montpellier, France.

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Figure Legends

Figure 1: The 'tubulin code'. The C-terminal tails of both α- and β-tubulin are subject to various chemical modifications. Polyglutamylation and polyglycylation occur on both α- and β-tubulin tails. On the other hand, detyrosination (i.e. removal of the C-terminal tyrosine residue) concerns only α-tubulin. In brain tissue, detyrosination and polyglutamylation are abundant. Conversely, polyglycylation is almost absent in neurons and is specific to cilia and flagella. A comprehensive Snapshot on these modifications has been recently published (Magiera, Singh, et al., 2018).

Figure 2: Schematic drawing of tau domain architecture with the isoelectric points (pI) of each domain. In physiological conditions (pH±7.4), the domain in red is negatively charged, the domains in blue are positively charged, and the domain in green is electrostatically neutral. Tau has four domains, including a basic MT binding domain with four different repeats called R1-R4. The four major phosphorylation regions are shown in yellow below the schematic representation of tau. Of note, phosphorylation sites are mostly located on both sides of the MT binding domain.

Figure 3: Electrostatic repulsion model based on the recent near-atomic structure of MT-tau interaction (Kellogg et al., 2018). The colour code for the tau protein is the same as in Figure 2. Black/red arrows indicate electrostatic forces. The initial phosphorylation of tau in the proline-rich and the N-terminal domains leads to alterations of the protein overall electrostatic charge (early stage) and repulsion from the tubulin C-terminal tails protruding at the surface. This is followed by tau dissociation and hyperphosphorylation that in combination with increased tubulin detyrosination and glutamylation, leads to stronger electrostatic repulsion (late).

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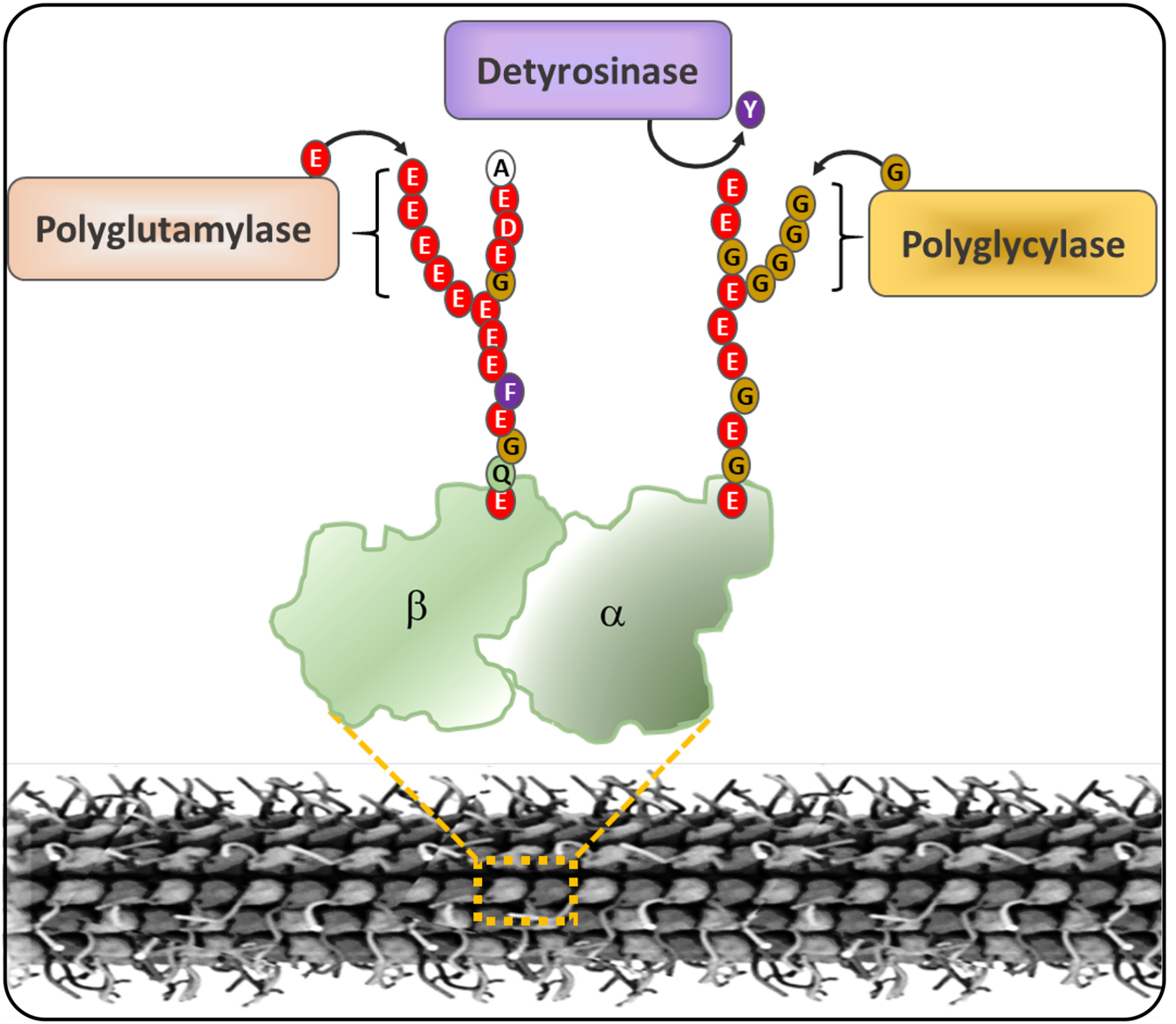
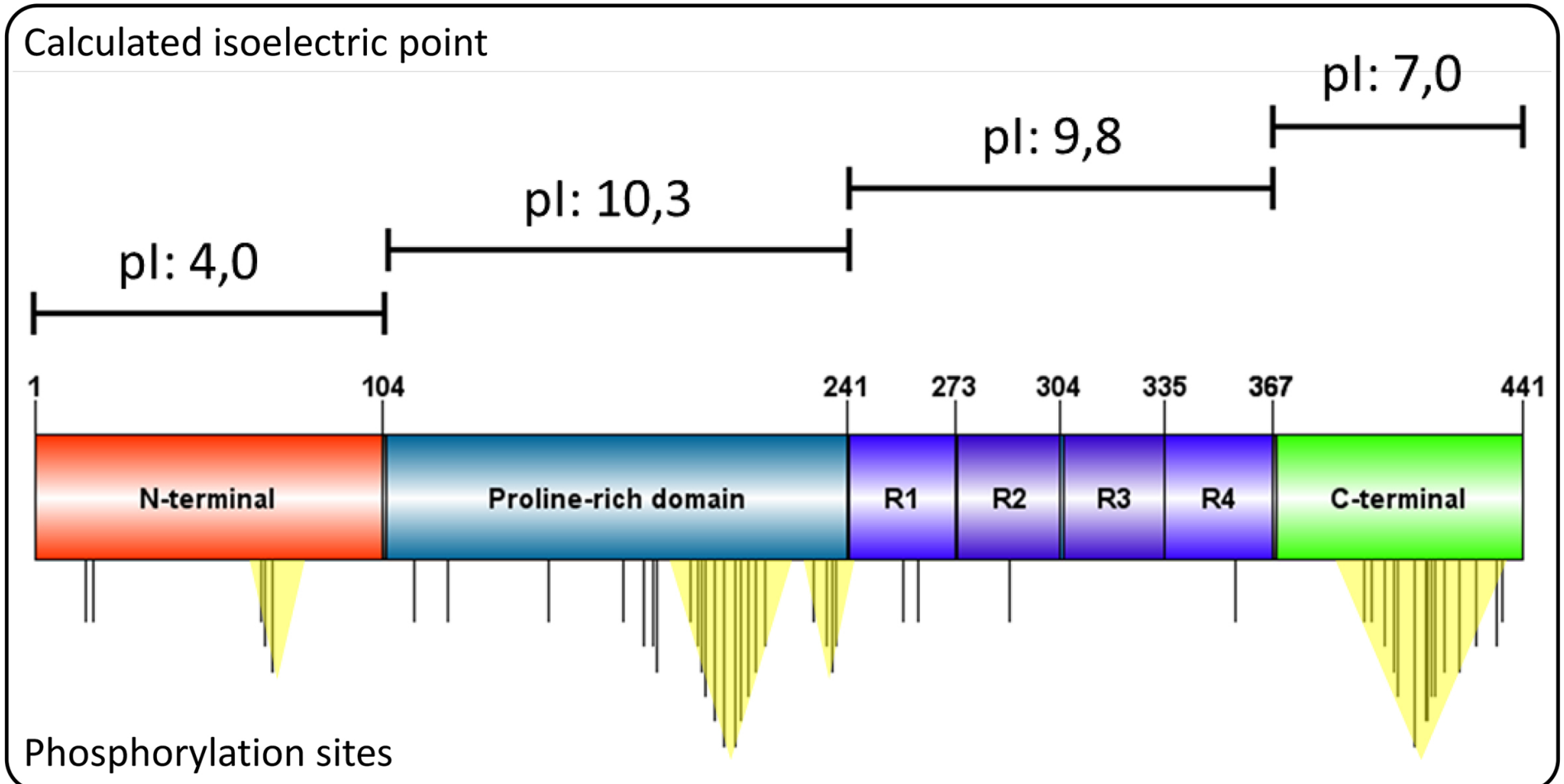


Figure 1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pI: 10,3
pI: 9,8
pI: 7,0
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104
241
273
304
335
367
441
N-terminal
Proline-rich domain
R1
R2
R3
R4
C-terminal
Phosphorylation sites

Figure 2

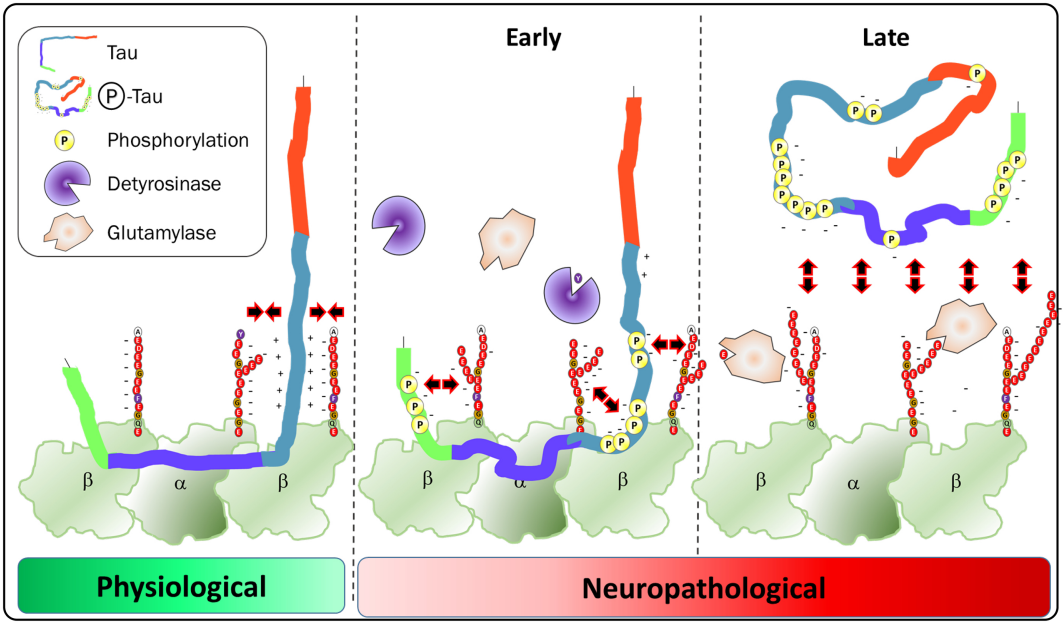
Tau
Ⓟ-Tau
Phosphorylation
Detyrosinase
Glutamylase
Early
Late
β
α
β
Physiological
Neuropathological

Figure 3