DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Microtubule-targeting agents: a therapeutic strategy in neurodegenerative diseases

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Nuno Miguel Ferreira Morais Apóstolo

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Abstract

Microtubule instability is a common feature among several neurodegenerative diseases. Abnormal genetic or environmental changes induced to tubulin or microtubule-related molecules such as MAPs, motor proteins, microtubule +TIPs or even microtubule severing enzymes are associated with reduced stability and increased dynamicity of microtubules in degenerating neurons. Microtubules form the main tracks that serve intracellular transport of cargos like synaptic proteins, mitochondria and polyribosomes covering long distances in neurons. In addition, it is now known that microtubules are important players regarding the development and maintenance of dendritic spines. Overall, microtubules have a central role keeping neurons in shape concerning their role in neuronal morphology, intracellular transport and synaptic plasticity, uncovering the reason why microtubule-related deficiencies are frequent in neurodegenerative diseases. Consequently, MTA are used as part of a therapeutic strategy to neurodegenerative diseases where they are intended to stabilize degenerating microtubules and prevent neuronal loss. Here we characterized the effect of Taxol, Epothilone D and Noscapine as regards to their ability to stabilize microtubules, using primary hippocampal cultures. We showed that these drugs are able to increase microtubule stability, although with different mechanisms of action, by increasing the relative amount of polymerized and acetylated tubulin. Moreover these drugs were capable of inducing neurite extension. Finally, we showed that there is a slight decrease in microtubule stability in an *in vitro* tau-aggregation AD model.

Keywords: Microtubules; Microtubule post-translational modifications; Synaptic plasticity; Neurodegenerative diseases; Microtubule-targeting agents.

Resumo

A presença de microtúbulos instáveis é um fenómeno recorrente em várias doenças neurodegenerativas. Alterações anormais, de origem genética ou ambiental, induzidas na tubulina ou em moléculas relacionadas com os microtúbulos tais como MAPs, proteínas motoras, +TIPs dos microtúbulos ou mesmo enzimas responsáveis por cortar os microtúbulos, estão associadas com a reduzida estabilidade e hiperdinâmica dos microtúbulos em neurónios que degeneram. Os microtúbulos constituem grande parte das estruturas responsáveis por apoiar o transporte celular de materiais tais como proteínas da sinapse, mitocôndrias e poliribossomas, por vezes durante longas distâncias em neurónios. Além disso, recentemente foi descoberto que os microtúbulos são responsáveis também por suportar o desenvolvimento e a manutenção de espículas dendríticas. Em conjunto, os microtúbulos têm um papel central no que toca à manutenção de neurónios saudáveis tendo em conta o seu papel no suporte da morfologia neuronal, no transporte intracelular e na plasticidade sináptica, percebendo-se assim, o porquê de alterações anormais nos microtúbulos e proteínas relacionas serem frequentemente observáveis em doenças neurodegenerativas. Desta forma, existem compostos que são usados como estratégia de terapia em doenças neurodegenerativas com o objectivo de estabilizar microtúbulos susceptíveis de degenerar e assim prevenir morte neuronal. Neste projecto caracterizou-se o efeito do Taxol, da Epotilona D e da Noscapina tendo em conta a capacidade que estes compostos apresentam em estabilizar microtúbulos, usando culturas primárias do hipocampo. Mostrou-se que estes compostos são capazes de aumentar a estabilidade dos microtúbulos, apesar de usarem diferentes mecanismos, tendo em conta o aumento na quantidade de tubulina polimerizada e acetilada. Além disso, estes compostos conseguiram promover o crescimento de neurites. Finalmente, mostrou-se que há um ligeiro decréscimo na estabilidade dos microtúbulos num modelo *in vitro* da doença de Alzheimer baseado na agregação da proteína tau.

Palavras-chave: Microtúbulos, Modificações pós-traducionais em microtúbulos; Plasticidade sináptica; Doenças neurodegenerativas; Compostos que interagem com microtúbulos.

Abbreviations

+TIPs – Plus-end tracking proteins

AD – Alzheimer's disease

AK – Adenylate kinase

ALS – Amyotrophic lateral sclerosis

AMPARs – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

ASD – Autism spectrum disorders

AVV – Adeno-Associated Virus

BBB – Blood-brain barrier

BDNF – Brain-derived neurotrophic factor

BSA – Bovine Serum Albumin

CaMKII – Ca^{2+}/calmodulin-dependent protein kinase II

DIV – Days in vitro

DMSO – Dimethyl sulfoxide

GFP – Green Fluorescent Protein

GPCRs – G protein-coupled receptors

GSK-3β – Glycogen synthase kinase-3β

HBSS – Hank's balanced salt sodium solution

HD – Huntington's disease

HRP – Horseradish peroxidase

hWT – human Wild Type

LTD – Long-term depression

LTP – Long-term potentiation

MAPs – Microtubule-associated proteins

MDA – Microtubule-destabilizing agent

MEM – Minimum Essential Medium

MMA – Microtubule-modulating agent

MSA – Microtubule-stabilizing agents

MTA – Microtubule-targeting agents

NEO – Neurite Outgrowth

NFTs – Neurofibrillary tangles

NGS – Normal Goat Serum

NMDARs – *N*-Methyl-D-aspartate receptors

PBS – Phosphate Buffered Saline

PD – Parkinson's disease

PFA – Paraformaldehyde

PSD – Postsynaptic density

PTMs – Post-translational modifications

RT – Room temperature

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1. Introduction

1.1 Dendritic spines

1.1.1 Structure and function

Glutamatergic synapses are the major excitatory synapses occurring in the mammalian central nervous system. These types of synapses take place at special post-synaptic membrane protrusions named dendritic spines. Dendritic spines vary in size (0.5 – 2 μm long), are motile and acquire different sizes and shapes, ranging from long, thin filipodia-like protrusions to mushroom-shaped spines: filopodia, thin, stubby and mushroom (Goellner and Aberle, 2012) (Figure 1). A typical mushroom spine contains three compartments: a bulbous head contacting the axon, a constricted neck in the middle and a delta-shaped base at the junction with the dendrite. Conversely, thin spines have longer necks and narrower heads, whereas stubby spines lack a neck and are formed by a dense patch of branched actin (Korobova and Svitkina, 2010) (Figure 1). The heads of spines contain an Arp2/3 complex-dependent actin branched network shaping the volume of the spine head, whereas the spine neck is probably maintained by actin filament bundle associated with myosin so it can contract (Korobova and Svitkina, 2010). These are not permanent structures but rather reflect a continuum of shapes that dynamically change over time (Rochefort and Konnerth, 2012).

During early synaptogenesis, dendritic shafts (the axis of dendrites) are covered with transient filopodia that grow and shrink trying to meet a developing axon. When they find activity-dependent signals, synaptogenesis is triggered and filopodia change their shape and undergo maturation (Portera-Cailliau et al., 2003). As synapse formation progresses the numerous dendritic filopodia are gradually replaced by spines (Matus, 2005). Spine density reaches its maximum level during late development when synaptic plasticity is at its height and then decreases to a relatively stable level throughout adulthood in normal individuals (Zhang and Benson, 2000). Nonetheless, studies using multiphoton microscopy over days to months in living mice have confirmed that spines and their synapses can form and retract throughout adulthood (Grutzendler et al., 2002, Trachtenberg et al., 2002) establishing the idea that adult brains can retain the capacity to form new synapses and thereby remodel its circuitry throughout life.

The morphology of spines can directly affect functional communication between neurons (Bourne and Harris, 2007, Harms and Dunaevsky, 2007). Enlarged spine heads correlate with an increased size of the postsynaptic density (PSD) (Tada and Sheng, 2006), while spine surface area, spine volume, bouton volume, and number of presynaptic vesicles are all highly correlated with synaptic area and therefore synaptic strength (Harris and Sultan, 1995, Schikorski and Stevens, 1999, Fiala et al., 2002). PSD is a compartment where most of the molecular diversity of excitatory synapses is settled and where the initial signal transduction events take place in response to presynaptic inputs inducing synaptic work. It is directly apposed to the active zone (pre-synaptic terminal) and perfectly matched with it in size and shape. Nearly all dendritic spines contain a PSD which is composed of a complex matrix of postsynaptic receptors (ionotropic glutamate receptors, G protein-coupled receptors – GPCRs, and tyrosine kinase receptors), cell adhesion molecules, scaffolding proteins, signaling molecules and cytoskeletal elements involved in synaptic signaling and plasticity, altogether forming a condensed “proteinaceous disk-like structure” (Nimchinsky et al., 2002, Sheng and Hoogenraad, 2007).

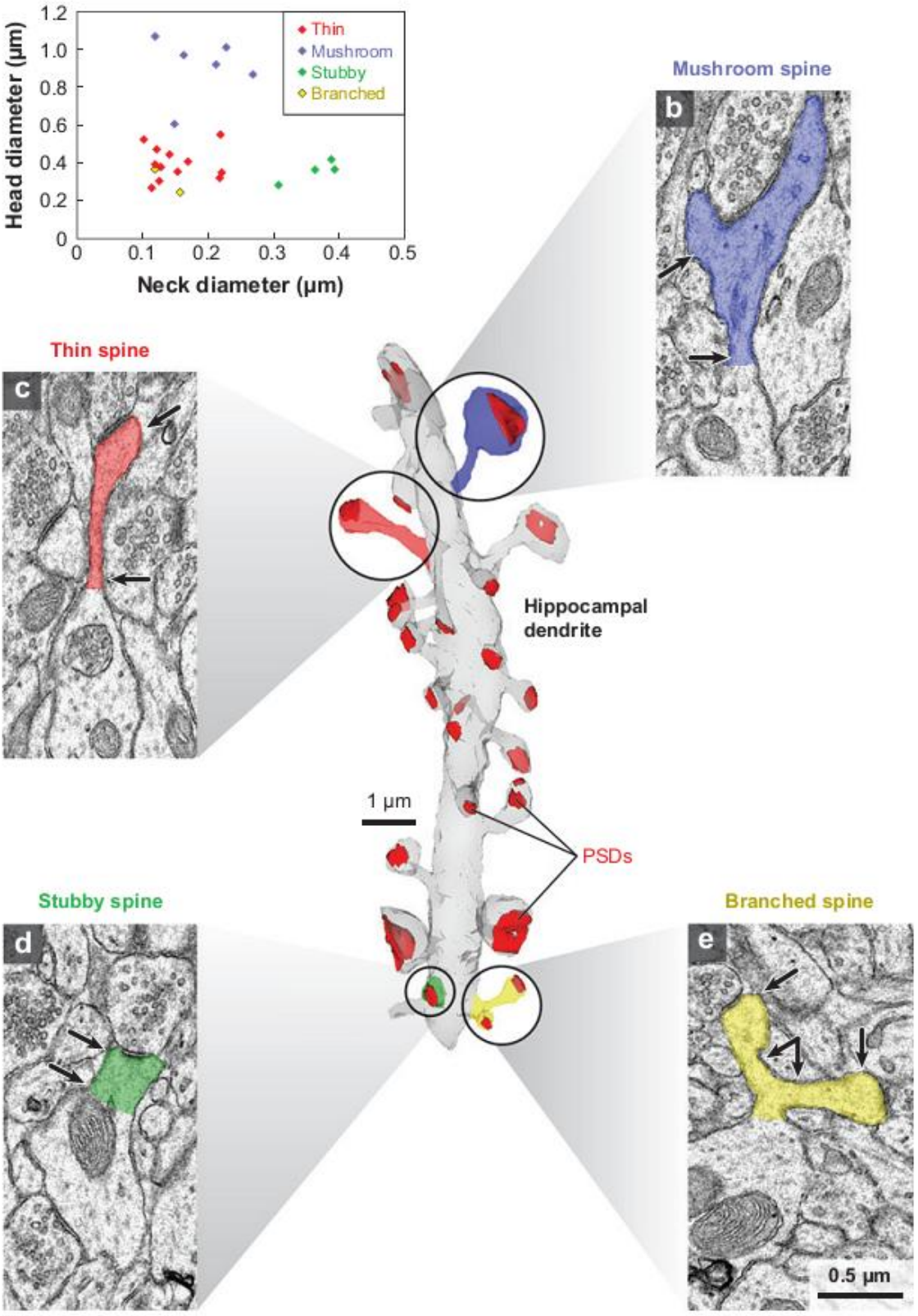


Figure 1 – (From Bourne, J. N., & Harris, K. M., Annual review of neuroscience, 2008) Variability in spine shape and size. A three-dimensional reconstruction of a hippocampal dendrite (gray) illustrating different spine shapes including mushroom (blue), thin (red), stubby (green), and branched (yellow). PSDs (red) also vary in size and shape.

Actin filaments are often directly connected with membrane-embedded receptors, thereby linking synaptic inputs to structural modifications. In fact, cooperation between actin filaments and microtubules is thought to be important in dendritic spine morphology and synaptic plasticity (Hoogenraad and Akhmanova, 2010). Consequently, it is believed that synaptic inputs occurring in spines activate receptors that in turn may interact with scaffolding proteins, effector proteins (kinases) and/or actin filaments that sequentially interact with microtubules that will keep the flow of information necessary for spine rearrangement (Priel et al., 2010). The spine neck, on the other hand, seems to restrict this flow of information by hampering diffusional exchange of signaling molecules to dendritic spines in the neighborhood (Koch and Zador, 1993), localizing biochemical changes to a particular synapse (Bliss and Collingridge, 1993). Moreover, the spine neck serves as a barrier to suppress Ca^{2+} leakage from the spine head to the dendritic shaft since Ca^{2+} is an important activator of synapse-specific regulatory mechanisms including cytoskeleton remodeling (Nimchinsky et al., 2002).

In summary, dentritic spines are small sites spread over dendritic branches that concentrate specific proteins needed to receive and transmit information to the soma coming from pre-synaptic terminals of connected neurons.

1.1.2 A site of synaptic plasticity

Synaptic plasticity in the mammalian central nervous system is required to support highly dynamic processes such as learning and memory. Although these processes are often considered together, learning is considered the process by which the nervous system improves its adaptation to the environment, whereas memory represents a process by which this information is stored in neurons or in the connections between them (Priel et al., 2010). As previously said, there is a strong correlation between the size of the spine and the strength of the synapse, making spine remodeling an attractive structural mechanism underlying learning and memory once synaptic strength differences/favored synaptic connections between groups of synapses are thought to be the molecular foundation behind these processes. It is interesting to think that filopodia may extend and retract when looking for a target axon as a correlate to learning – readiness to learn something new while trying to adapt to the environment – while spines mature and enlarge preserving and favoring specific signals – information stored. The idea that adaptations within the intraneuronal matrix, rather than or in addition to changes to interneuronal connectivity, are involved with learning and memory is consistent with species-specific patterns of connective plasticity. That is, human pyramidal neurons were proven to have more extensive dendrite arbors and spine densities compared to mouse pyramidal neurons (Benavides-Piccione et al., 2002) and accordingly possess more extensive intraneuronal cytoskeletal matrices (Priel et al., 2010). Moreover, spatial learning (Moser et al., 1997) and exposure to enriched environments (Kozorovitskiy et al., 2005) alter hippocampal spine numbers and lead to improvements in the performance during several learning tasks (Bruel-Jungerman et al., 2005). Experience plays an important role eliminating excessive and imprecise synaptic connections formed early during development but is also responsible for the formation of new ones (Lichtman and Colman, 2000, Zuo et al., 2005). So, it is clear that synaptic plasticity is correlated with alterations to dendritic spines (number, size, shape or composition) where cytoskeleton elements play an important role. However, because synapses undergo rapid changes in response to environmental perturbations, it is unknown how dynamic synaptic circuits maintain indelible memories for a lifetime. With that in mind, one group recently showed that two populations of stable

spines are important for maintaining lifelong memories: (1) a small fraction of new spines induced by novel experience together with (2) spines formed early during development that remain after experience-dependent pruning represent a unique and stable physical entity for lifelong memory storage (Yang et al., 2009) (Figure 2).

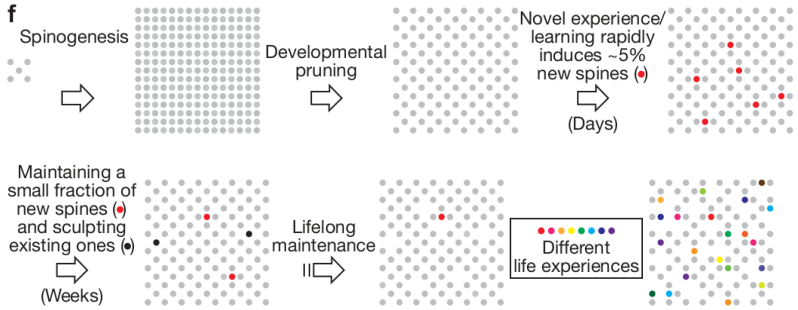


Figure 2 – (From Yang, G., et al, Nature, 2009) Schematic summary of spine remodeling and maintenance throughout life. After birth there is a great production of spines while some are eliminated right after during development due to neuronal connections refinement. Spines that survive are stable throughout life. New experiences are responsible for the formation of new spines as well as for pruning already existing spines that survived developmental pruning. Different experiences are responsible for the accumulation of a specific set of spines during life, and thus, despite dynamic plasticity, dendritic spines can provide a structural basis for learning and memory storage.

Memory is seen as a physical substrate, as something substantive and concrete. As such, a convincing molecular correlate for memory is the reorganization of the cytoskeleton within spines, as already mentioned specifically microtubules and actin filaments, responsible not only to connect structures in the cell body to synapses allowing the exchange of important building blocks, trophic signals and cellular waste but also to adapt and maintain the spine conformation according to the input received. Structural changes in the cytoskeleton that supports both pre and post-synaptic terminals are accompanied by an increase or decrease in synaptic strength, consequently, they potentiate/decrease the release of neurotransmitters or favor/hamper activation of certain synaptic contacts over others, respectively (Priel et al., 2010, Dent et al., 2011). Besides, it is known that large spine heads are generally stable, express large numbers of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and contribute to strong synaptic connections. By contrast, spines with small heads are more motile, less stable, and contribute to weak synaptic connections (Matsuzaki et al., 2004, Holtmaat et al., 2006).

Synaptic plasticity occurring in dendritic spines is bidirectional, that is, accompanied by enlargement or shrinkage of the spine head, as a result of an input frequency-dependent shift in the F-actin/G-actin equilibrium (Okamoto et al., 2004). As already stated, this capacity may be the molecular basis of memory and learning processes since it has been suggested that large spines represent “memory spines” and small spines represent “learning spines” (Kasai et al., 2003). Moreover, there is an overwhelming amount of evidence showing that synapses are plastic and undergo short- and long-term modifications during developmental refinement of neural circuits in learning and memory (Kandel, 1997, Malenka and Bear, 2004, Flavell and Greenberg, 2008). Long-term potentiation (LTP) is the best characterized form of such plasticity, which is observed at excitatory synapses in the CA1 region of the hippocampus (Nicoll and

Malenka, 1995). Indeed, there is a large amount of literature documenting the enlargement of spine heads as well as the emergence of new spines after the induction of LTP, whereas spine shrinkage and elimination are considered to be a key step in long-term depression (LTD) (Matsuzaki et al., 2004, Nagerl et al., 2004, Okamoto et al., 2004, Zhou et al., 2004). Interestingly, changes in synaptic strength and spine morphology share common early steps, including activation of *N*-Methyl-D-aspartate receptors (NMDARs) and calcineurin in the case of LTD, but diverge in later steps, with phosphoprotein phosphatase 1 activity required for LTD but not for spine shrinkage. The latter is mediated by cofilin activity (protein that triggers F-actin depolymerization) (Zhou et al., 2004), further supporting the idea of an association between synaptic plasticity and dendritic spine morphology remodeling, at least in the first steps. Besides, modifications in the number and activity of membrane surface neurotransmitter receptors are considered to be a key event underlying synaptic modification in dentritic spines. For example, the intracellular domain of AMPARs is phosphorylated to increase ion conductance during early LTP (Benke et al., 1998). To generate a long-lasting LTP, however, it is necessary to increase the number of postsynaptic glutamate receptors on the postsynaptic surface. These postsynaptic changes appear to be reversed during LTD, including dephosphorylation of AMPARs and their removal from the postsynaptic membrane (Gu and Zheng, 2009). Also, blocking AMPARs exocytosis prevents the induction of LTP, whereas blocking endocytosis prevents the induction of LTD (Bredt and Nicoll, 2003).

Individual synapses exhibit site-specific plasticity, which, in its long-term form, requires somatic transcripton and translation as well as local protein synthesis (Martin et al., 1997, Kandel, 2001). Early LTP is short-lasting and requires post-translational modifications of synaptic proteins but is independent of protein synthesis, while late LTP represents the long-lasting phase of LTP that is both transcriptional and translational dependent (Voronin et al., 1995, Reymann and Frey, 2007). Thus, in order to establish late LTP and LTD it is required that specific material is transported to specific synapses – the “synaptic tag and capture” hypothesis. This hypothesis postulates that modifications activated synapses include the formation of a molecular “tag” that can facilitate the capture of specific material being delivered throughout the dendritic arbors to dendritic spines (Frey and Morris, 1998, Redondo and Morris, 2011). This process is possible due to the active transport of gene products that require three critical components such as cytoskeletal tracks (formed by microtubules and actin), molecular motors (kinesin, dynein, and myosin), and cargos (Liu et al., 2012). In fact, in addition to transcriptional activation in the nucleus and local protein synthesis at the synapse, the coordinated upregulation of kinesin-mediated transport is also a critical component for long-term learning-related plasticity (Puthanveettil et al., 2008). According to this view, proteins carried by kinesins in an anterograde fashion are used to induce immediate synaptic changes while mRNAs are subsequently used to maintain these changes.

In summary, synaptic plasticity is thought to be crucial supporting unique brain skills as learning and memory, and remodeling of the cytoskeleton is of extreme importance to this plastic phenomena. Therefore, a better understanding of the molecular and cellular mechanisms underlying synaptic plasticity is of great value to understand brain development and function under both physiological and pathological conditions.

1.2 The role of cytoskeleton in synaptic plasticity

1.2.1 Microtubules

1.2.1.1 Structure and function in neurons

Microtubules are formed from the association of dimers of α- and β-tubulin into protofilaments; 13 protofilaments further interact side by side to make up a hollow tube. The head to tail association of α- β heterodimers is responsible for the microtubule intrinsic polarity, where the plus end (or faster growing end) shows a β monomer while the minus end (or slow growing end) shows an α monomer. *In vivo*, proteins such as γ-tubulin bind to the minus ends of microtubules promoting nucleation of tubulin dimers but also capping this terminals leaving the plus ends responsible for microtubule elongation (Zheng et al., 1995). Each tubulin dimer has two GTP molecules non-covalently bound, one in each monomer, but however, only one is exchangeable with free GTP, the one in β-tubulin. The presence of GTP enhances the polymerization process, however, hydrolysis of GTP to GDP by an intrinsic β-tubulin GTPase domain occurs subsequently to microtubule polymerization (Carlier et al., 1989). The fast addition of GTP-bound tubulin dimers to microtubules is responsible for the formation of a GTP cap in the plus end where GTP molecules from β-tubulin remain with three phosphate groups promoting stabilization of the straight conformation in protofilaments, and consequently, microtubule growth is induced. Loss of the cap results in the transition from growth to shrinkage (catastrophe), whereas reacquisition of the GTP cap results in a transition from shortening to growth (rescue). This characteristic dynamic behavior, termed “dynamic instability”, allows a rapid remodeling of microtubules (Mitchison and Kirschner, 1984). GPCRs are thought to regulate this dynamics *in vivo* by mobilizing G protein (Gα and/or Gβγ) subunits to bind to microtubules. In addition, receptor-independent activators of G proteins signaling also mediate a diverse range of signals within the cell responsible for rearranging the microtubule network. This dynamic ability of microtubules to quickly polymerize and depolymerize are critically involved in cell division and differentiation, cell motility, intracellular transport, cell morphology, and recently it is known that in neurons it may also support synaptic plasticity (Desai and Mitchison, 1997, Roychowdhury and Rasenick, 2008). Microtubules are key determinants of neuronal polarity (Kapitein and Hoogenraad, 2011) and form the transport highways for cargo trafficking in axons and dendrites in neurons (Hirokawa and Takemura, 2005). They show intracellular variations in their density, orientation and post-translational modifications (PTMs) but also in their interacting partners such as motor proteins, microtubule associated proteins (MAPs), severing enzymes and microtubule plus-end tracking proteins (+TIPs). As further explored below, the occurrence of different sets of microtubules and binding partners is what confer neuronal polarity and assure the transport of cargoes to specific subcellular compartments present in such extensive and polarized cells as neurons including growth cones and dendritic spines (Kapitein and Hoogenraad, 2011).

1.2.1.2 Microtubule post-translational modifications

MAPs bind to microtubules and regulate their stability and function by modifying their interaction with motors proteins as well as other important proteins involved in transport and cytoskeleton

rearrangement (Liu et al., 2012). Besides MAPs, direct enzymatic modifications on α- and β-tubulin are also thought to alter microtubule stability and function: microtubule PTMs. These modifications are capable of generating different sets of microtubules and consequently different microtubule-associated functions by altering the way microtubule polymers can interact with proteins complexes that regulate specific cellular processes. Microtubule PTMs occur in already polymerized tubulin. Mature, long-lived microtubules accumulate more modifications as compared to dynamic microtubules (Westermann and Weber, 2003, Hammond et al., 2008), so, microtubule PTMs are normally associated with stable microtubules, but they do not promote microtubule stabilization *per se*, at least directly (Baas and Ahmad, 2013). However, some PTMs are thought to further increase microtubule stability by reducing the activity of microtubule depolymerases (Peris et al., 2009). There are several microtubule PTMs known (Hammond et al., 2008, Janke and Kneussel, 2010, Janke and Bulinski, 2011) divided into two groups: mono-modifications and poli-modifications. Mono-modifications include detyrosination, acetylation and phosphorylation, while glutamylation and glycylation are part of poli-modifications (Figure 3). Detyrosination is the process by which a tyrosine residue in the C-terminal of α-tubulin is removed (Ikegami and Setou, 2010). This tyrosine can be replaced (retyrosination) after the tubulin dimer is removed from the microtubule lattice by the action of tubulin tyrosine ligase. Freshly polymerized tubulin dimers are tyrosinated in the α-tubulin subunits by default, allowing for dynamic microtubules to be detected by making use of antibodies against tyrosinated α-tubulin. The detyrosinated/tyrosinated tubulin state can alter the interaction of microtubules with molecular motors and +TIPs. Accordingly, tyrosinated microtubules are more prone to recruit +TIPs (Infante et al., 2000, Peris et al., 2006), and thus regarded as dynamic microtubules, since microtubule +TIPs are responsible to promote microtubule growing/shrinking. In their turn, detyrosinated microtubules (more stable microtubules) are enriched in axons (Hammond et al., 2008) and interestingly have more affinity for kinesin-1 (Dunn et al., 2008, Konishi and Setou, 2009). This suggests that microtubule PTMs can influence intracellular cargo transport and sorting (Kapitein and Hoogenraad, 2011). Although it is known that detyrosinated tubulin is present mainly in axons, recent work showed that the turnover of microtubules in axons and dendrites is similar (similar stability). Probably, microtubule lifetime is not the predominant cause for the axonal enrichment of detyrosinated microtubules, and instead the activity or concentration of modifying enzymes differ between these two compartments (Hammond et al., 2010). Glutamylation and glycylation involve the addition of short or long chains of glutamate and glycine aminoacids, respectively, into glutamate residues in the C-terminal of both α- and β-tubulin. These poli-modifications in the C-terminal tail of both tubulin monomers may be responsible of tagging microtubules and also alter the way they interact with proteins, like severing proteins, MAPs and motor proteins (Larcher et al., 1996, Bonnet et al., 2001), thus influencing microtubule function. Acetylation, the addition of an acetyl group on lysine 40 of α-tubulin, is common in microtubules and can be found on long-lived, stable microtubules (Hammond et al., 2008). This modification as well can be responsible for structural alterations in microtubules and consequently change their role in cellular processes like cargo transport, as kinesin-1 binds with higher affinity to acetylated microtubules *in vitro* (Reed et al., 2006). In summary, tubulin PTMs can influence microtubules by regulating their stability and/or structure, and the recruitment of microtubule interacting proteins such as MAPs, molecular motors, +TIPs, severing proteins and other proteins that may in the future show a relevant role in microtubule dynamics regulation. The occurrence of a diverse set of microtubule PTMs and their complex combinations form different patterns of PTMs, leading to the hypothesis that cells possess a “tubulin code” (Westermann and Weber, 2003, Verhey and Gaertig, 2007) or a “microtubule code” (Janke and Kneussel, 2010) that can guide cell effectors to operate on specific locations, regarding that different cell compartments have different subsets of microtubules (Janke and Kneussel, 2010).

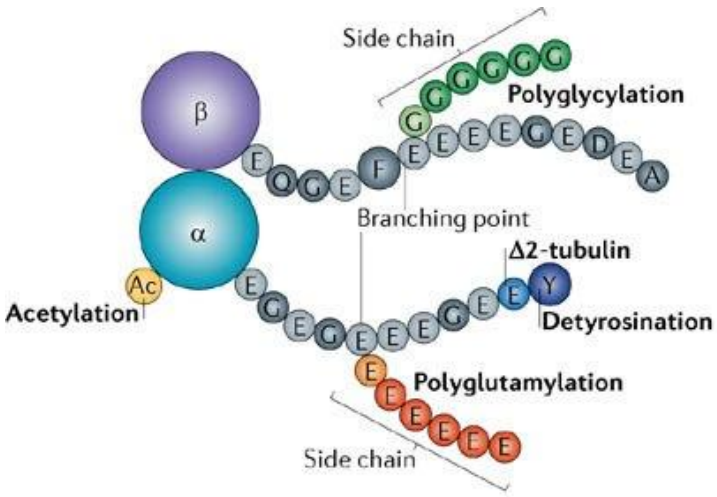


Figure 3 – (From Janke, C. et Bulinski, J. C., Nat Rev Mol Cell Biol, 2011) Schematic representation of α- and β-tubulin post-translational modifications. Carboxy-terminal tails of both subunits are represented as amino acid sequences. Both α-tubulin and β-tubulin can be modified by polyglutamylation and polyglycylation on different Glutamate residues within those tails. Together with detyrosination at the C terminus of α-tubulin, these modifications are specific to the C-terminal tails of tubulin. Acetylation of Lysine 40 is localized at the amino-terminal domain of α-tubulin.

Both spatial and temporal differential composition of microtubules could promote specific cargos to be transported to specific sites at specific time points, for example. Indeed, it was shown that synaptic activity can regulate tubulin PTMs changing the set of proteins targeted to neurites (Maas et al., 2009). This could be the case also for spines, where activity-dependent modifications in microtubules could recruit a restrict group of proteins to be delivered into dendritic spines and be of great importance in synaptic plasticity.

1.2.1.3 Microtubules support synaptic plasticity

In developing neurons, actin filaments and microtubules act together to guide and support the growth and differentiation of axons and dendrites. In contrast to these well-studied examples of microtubule-actin cooperativity, it is widely accepted that in dendrites of mature neurons the two cytoskeletal domains are spatially separated; while actin filaments are predominately concentrated in spines, stable microtubules are confined to the dendritic shaft and do not branch off into spines. Accordingly, studies examining mature dissociated hippocampal neurons have suggested that microtubules cannot enter in dendritic spines (Kaech et al., 1997, Kaech et al., 2001), but recent reports (Gu et al., 2008, Hu et al., 2008, Mitsuyama et al., 2008, Jaworski et al., 2009) showed the capture of the plus ends of dynamic microtubules inside spines. Growing microtubules specifically accumulate a set of factors, the already mentioned +TIPs, at their ends. Moreover, they can be used as tools to visualize growing microtubule ends even within dense microtubule networks (Jaworski et al., 2009). Among +TIPs, proteins of the EB

family directly interact with the majority of other known +TIPs and have been implicated as key regulators of microtubule-associated signaling pathways (Akhmanova and Steinmetz, 2010). Importantly, in contrast to EGFP-α-tubulin, that incorporates throughout microtubules allowing one to image all microtubules within a living neuron, EB3-EGFP labels the fast growing plus ends of polymerizing microtubules, but not paused or depolymerizing microtubules (Stepanova et al., 2003) and thus can be used to specifically target growing microtubules that may enter in spines. An EB3 binding partner, p140Cap, was identified to bind to a Src kinase substrate and F-actin binding protein, cortactin (Jaworski et al., 2009) (Figure 4), in spines, demonstrating a possible mechanism of actin-microtubule interaction there. The binding ability of EB3 to drebrin may also contribute to the interaction between microtubule and actin filaments (Geraldo et al., 2008). The interaction of microtubule plus-ends containing EB3, with drebrin and cortactin may therefore represent a link for signaling between microtubules and the actin cytoskeleton within dendritic spines, which can be of key importance to understand local changes of spine and synapse structure during plasticity. The reason it was thought microtubules in spines were less abundant might be due to the fact they are very sensitive to disruption, and very dynamic, so, it was supposed that intraspinal microtubules depolymerized during conventional fixation methods once studies using microtubule-conserving fixation methods or live experiments were a success showing intraspinal microtubules. Westrum and Gary were the first to observe microtubules in spines, associated with the PSD, with the aid of enhanced microtubule preservation techniques (Westrum and Gray, 1976). Different approaches to track spines by labeling neurons with microtubule-associated protein 2 (MAP2) failed (Kaech et al., 2001). This could be due to the fact that MAP2 does not label the dynamic ends of microtubules, but rather the more stable sections of microtubules that are present in the dendritic shaft (Hu et al., 2008). Thus, it now seems that stable microtubules are predominantly present as bundles in dendritic shafts whereas dynamic microtubules can enter dendritic spines. The association of microtubules with the PSD before mentioned suggested that microtubules may have a direct role in synaptic plasticity and consequent spine remodeling upon activity or the absence of it. Indeed, recent studies showed that after LTP-induction on hippocampal slices, microtubules of the dendritic shaft ramified into spines that were specific to the stimulated postsynaptic membranes (Mitsuyama et al., 2008). Moreover, the frequency of microtubules polymerizing into spines was observed to increase after activation of synaptic NMDARs, and NMDAR-dependent spine enlargement was dramatically enhanced in spines targeted by microtubules (Merriam et al., 2011). Conversely, a study was published showing that chemical LTD decreases microtubule dynamics in the dendritic shaft as well as the frequency of microtubule spine invasions (Kapitein et al., 2011). Since increases in spine size are known to depend on actin polymerization (Okamoto et al., 2004), and now, that microtubule and actin dynamics work hand with hand, it is perhaps not surprising that microtubule invasions into spines contribute to spine enlargement during LTP. Importantly, inhibition of microtubule dynamics with Nocodazole (drug that inhibits microtubule polymerization) markedly inhibited microtubule invasion of spines and abolished the increase in spine size that followed synaptic NMDARs activation (Merriam et al., 2011). Another study showed that Taxol (drug that stabilizes microtubules) can potentiate the effects of brain-derived neurotrophic factor (BDNF) on spine formation, and, on the other hand, Nocodazole completely blocked the effect of BDNF (Gu and Zheng, 2009). These findings further suggest that microtubules play an important role in spine development and plasticity.

Furthermore, in an elegant study conducted in living neurons Hu et al. discovered that almost 10% of spines were targeted by microtubules per hour in a long term time lapse imaging experiment, indicating many spines on a neuron may be targeted by microtubules over a day. In addition, in all types of dendritic

protrusions examined (filopodia, stubby spines, thin spines and mushroom-shaped spines) microtubules were capable of rapidly extending into and out of the full extent of the protrusion, but did it more frequently and for longer periods on mature spines, suggesting that microtubule invasion of spines may function to maintain spine structure (Hu et al., 2008). Surprisingly, the same authors discovered that even in mature hippocampal and cortical neurons with 63 days *in vitro* (DIV) microtubules remained dynamic, meaning that the ability to extend into spines is probably maintained later in life. It is known that dynamic instability enables microtubules to explore different cellular locations for potential interacting structures and signaling components. A productive interaction may stabilize this "highway" between two distant locations within the cell allowing them to communicate and change important components. Indeed, the function microtubules serve by transiently target dendritic spines is likely to involve transport of essential proteins into and out of spines, since microtubules are the major long-distance transport machinery inside all cells (Liu et al., 2012). Microtubules that reach spines might be biochemically "tagged" after a connection is established and perhaps tubulin PTMs may play a role, as previously said. This "tagging" could result in the specific kinesin-mediated delivery and/or dynein-mediated removal of receptors, structural proteins, mRNA, GTPase effectors or organelles that may be required for synaptic development and plasticity (Kneussel and Loebrich, 2007, Jacob et al., 2008, Dent et al., 2011). It is interesting to think of spines like isolated cities that rely on a road network (microtubules and actin) so that the working class (activity-dependent effector molecules and building blocks) can get into the city and do their job (spine remodeling). In fact, polyribosomes were found to be recruited into spines after LTP induction (Ostroff et al., 2002). Additionally, microtubules are also involved in vesicle trafficking of neurotransmitter receptors and mitochondria to dendritic spines (Gu and Zheng, 2009) (Figure 4). The coordinated regulation of axonal transport in pre and post-synaptic neurons has been identified as a critical mediator of long-term learning-related plasticity (Puthanveettil et al., 2008) and this idea is concordant with Mitsuyama's lab hypothesis, the "endless memory amplifying circuit", where they propose that retrograde transport of Ca^{2+}/calmodulin-dependent protein kinase IV from spines to the nucleus could activate specific transcription factors leading to anterograde products such as AMPARs and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) to be translocated to stimulated postsynaptic membranes (Goellner and Aberle, 2012, Mitsuyama et al., 2012) according to the synapse "tagging" theory already mentioned. This group also states that the translocation of proteins to transmit signals from stimulated synapses to the nucleus appears as a more appropriate and selective mechanism to form memories, in contrast to signal transmission by action potentials and calcium waves that could affect adjacent non stimulated synapses as well.

Thus, microtubules are no longer seen only as important components regarding cell structure maintenance and integrity, shaping and allowing transport of cargo along cells, but in addition, as a dynamic, plastic structure involved in neuronal polarity (Hoogenraad and Bradke, 2009) and synaptic plasticity capable of accumulating modifications that code for specific signals.

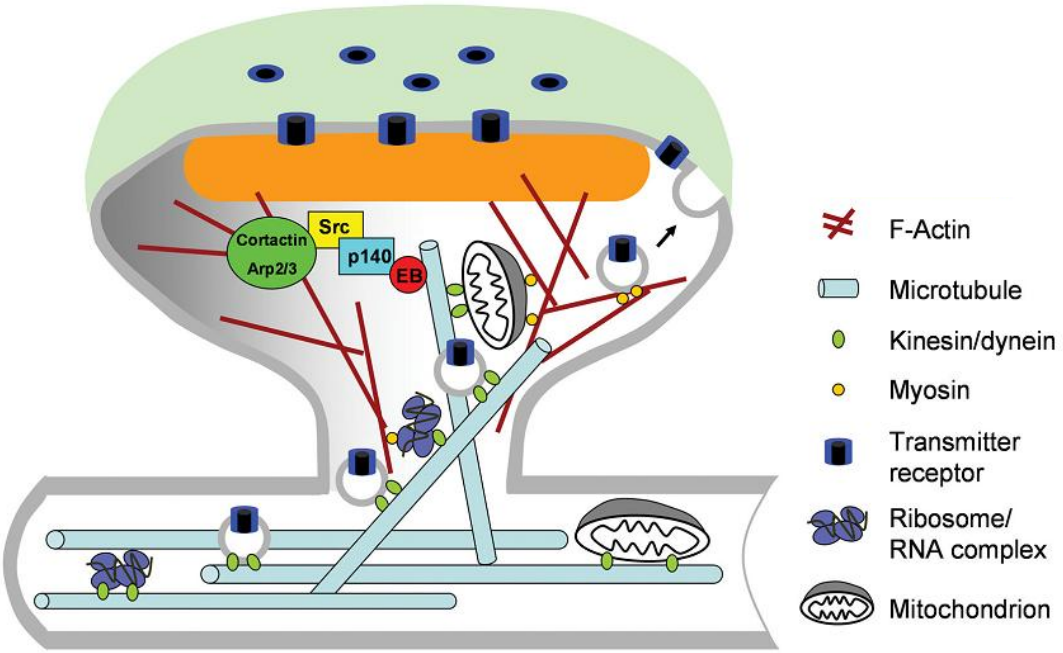


Figure 4 – (From Gu, J., Zheng J., Q., *Open Neurosci J.* 2009) A schematic diagram illustrating potential functions of microtubules in dendritic spines. In addition to the proposed microtubule regulation of actin filaments through p140Cap, Src kinase and cortactin, microtubules may also be involved in delivering membraneous organelles (e.g. mitiochondria and receptor-containing vesicles), as well as ribosome/RNA complexes, to the dendritic spine. It is likely that microtubules and actin filaments cooperate in the delivery of these cargos into spines and in the regulation of spine structure and function.

1.2.2 Actin

Actin is particularly abundant in axonal growth cones and dendritic spines (Hotulainen and Hoogenraad, 2010). Within spines, actin is present as a soluble pool of monomeric G-actin and as polymerized F-actin filaments that confer the characteristic spine shape. In the presence of Mg^{2+}, K^{+} or Na^{+} ions G-actin assembles into long, helical F-actin polymers (Frieden, 1983). Long filaments are predominantly present in the spine neck while short, branched actin filaments are found in the spine head (Kapitein and Hoogenraad, 2011). Like microtubules, actin filaments also have intrinsic polarity: the barbed end (the fast-growing end) and the pointed end (slow-growing end) with its ATP binding site exposed; the barbed end pointing to the plasma membrane in the presynaptic and postsynaptic regions (Kapitein and Hoogenraad, 2011, Liu et al., 2012). Whether ADP or ATP is bound to the actin monomer affects polymerization into filaments and their association to actin-binding proteins (Priel et al., 2010). Concerning dendritic spines, actin filaments are generally considered as mediators of synapse dynamics being the predominant cytoskeletal element there (Fifkova and Delay, 1982). Decoration of actin filaments with myosin II confirmed that actin filaments were the major cytoskeletal component of spines (Korobova and Svitkina, 2010). Older results had shown that actin filaments in spines are highly dynamic and that rapid changes in spine shape and size can be driven by actin (Fischer et al., 1998). Recent results concordantly state that spine structure changes through the reorganization of the actin network (Matsuzaki et al., 2004, Okamoto et al., 2004, Honkura et al., 2008). In its turn, actin network is regulated by GTPases belonging to the Rho family (Martino et al., 2013), a class of hydrolases expressed in

eukaryotic cells that includes Rho, Rac, and Cdc42 subfamilies (Etienne-Manneville and Hall, 2002). Activation of Rho GTPases produces a substantial increase in spine density on both basal and apical dendrites of hippocampal CA1 pyramidal neurons (Martino et al., 2013). Modifications on dendritic spine morphology concerning actin rely on specific motor proteins named myosins. Myosins are enriched in the PSD, where they translocate along actin filaments regulating their contractility and by this means spine shape (Osterweil et al., 2005, Ryu et al., 2006). Live-cell imaging studies *in vitro* and *in vivo* have established that spines are plastic and undergo activity-dependent changes in morphology, which are believed to be controlled by the actin network. Indeed, actin polymerization is coupled with spine formation/enlargement during LTP, whereas LTD involves spine shrinkage through actin depolymerization (Fukazawa et al., 2003, Okamoto et al., 2004, Zhou et al., 2004). Another study showed that CaMKII, RhoA and Cdc42 are activated during LTP, and in particular long-lasting, spine-specific Cdc42 activation plays an important role maintaining spine structure for long periods (Korobova and Svitkina, 2010).

Thus, intraspinal actin and microtubule dynamics are thought to be of extreme importance during spine development, changing and maintaining the structure of synapses undergoing LTP or LTD, both known as molecular correlates of learning and memory.

1.3 Microtubule instability: an important player in brain diseases

Several neurodegenerative diseases including Alzheimer's disease (AD), other tauopathies, Parkinson's disease (PD) and Huntington's disease (HD) are known to display microtubule instability, and consequently, defective intracellular transport (Brunden et al., 2009, Sudo and Baas, 2011, Franker and Hoogenraad, 2013, Hinckelmann et al., 2013, Millecamps and Julien, 2013, Esteves et al., 2014, Smith et al., 2014). In healthy neurons, pre and post-synaptic structures require a functional microtubule network capable of a competent intracellular transport work in order to exchange specific material with the neuronal soma, sometimes throughout very long distances (Kapitein and Hoogenraad, 2011). This particular neuronal demand is important to establish efficient synaptic connectivity and assure overall brain functioning, and that is probably why dysfunctional microtubules are a common feature among neurodegenerative diseases. Moreover, it is now clear that microtubules have an important role in dendritic spine formation and maintenance as already discussed, further highlighting microtubule's importance in neurons.

Particularly in AD, dendritic spine loss is observed in the hippocampus and throughout the cortex of patients (DeKosky and Scheff, 1990, Walsh and Selkoe, 2004, Knobloch and Mansuy, 2008). Such alterations are thought to be responsible for cognitive deficits before the absence of neuronal loss. Several pieces of evidence, mentioned above in the microtubule section, suggested that spine elongation may be caused by microtubule polymerization; conversely, synapse loss or spine loss observed in AD may be caused by the depolymerization of intraspinal microtubules (microtubule instability). Indeed, it is known that amyloid-β, an hallmark abnormal protein in AD, activates glycogen synthase kinase-3β (GSK-3β) (Terwel et al., 2008) and the activated form of GSK-3β causes the abnormal hyperphosphorylation of tau, consequently leading to depolymerization of axonal microtubules, resulting in the impairment of axonal transport (Iqbal et al., 2009). Tau, a major MAP in neurons, plays an important role in the outgrowth of neuronal processes and development of neuronal polarity by promoting microtubule

assembly and stabilization affecting microtubule dynamics and consequently intracellular transport (Lee et al., 2001, Kapitein and Hoogenraad, 2011, Morris et al., 2011). Normal tau is mainly present in the axon bound to microtubules, but hyperphophorylated tau has low affinity for microtubules, is prone to aggregation into neurofibrillary tangles (NFTs) (Brunden et al., 2009) (Figure 5), and distributes to the somatodendritic compartment decreasing the efficiency of axonal transport in neuropathies (Konzack et al., 2007). In dendrites, tau aggregates are able to sequester other MAPs (Alonso et al., 1997). In the process, disruption of intraspinal microtubules might happen due to the loss of the microtubule-preserving effect inherent to MAPs (Mitsuyama et al., 2012). Actually, the brains of patients with AD and many other central nervous system disorders, such as fronto-temporal lobar degeneration, Pick's disease, corticobasal degeneration and progressive supranuclear palsy, contain inclusions comprised of tau (Brunden et al., 2009), suggesting that microtubule instability might be universal among these disorders. In addition, it is suggested that amyloid-β is a putative intraspinal microtubule depolymerizer capable of inducing spine loss and synaptic dysfunction, ultimately leading to the cognitive deficits associated with AD (Mitsuyama et al., 2009, Zempel et al., 2010). Moreover, it is thought that overactivation of a NMDA-calcineurin-GSK-3β pathway may indicate a mechanism by which synapses degenerate in AD, since amyloid-β oligomer-induced spine loss and dendritic dystrophies can be prevented by calcineurin inhibition (Wu et al., 2010).

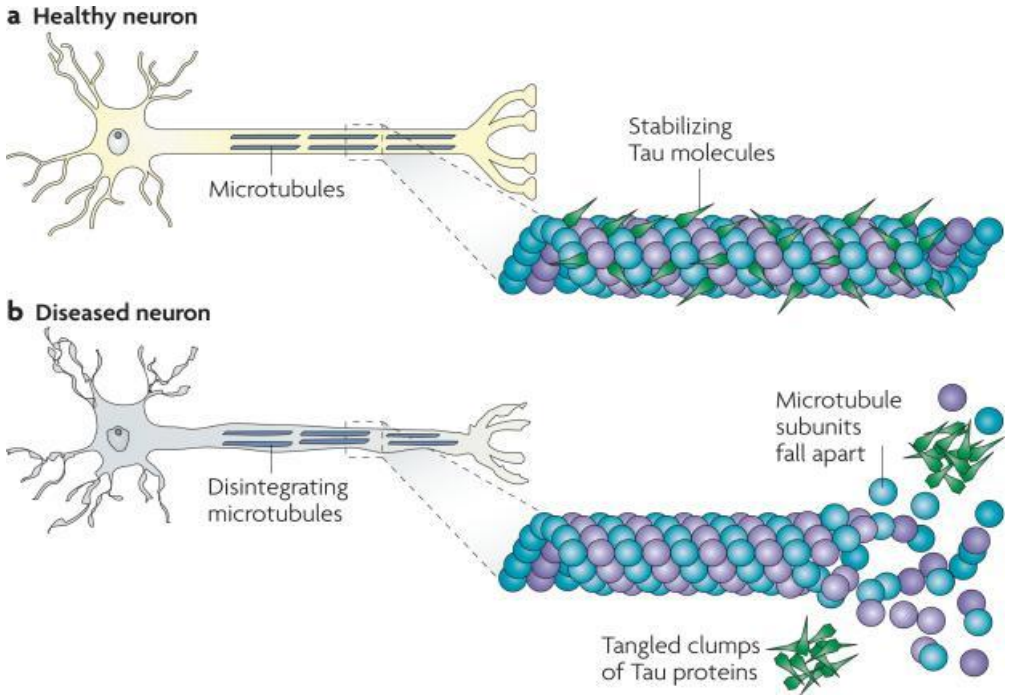


Figure 5 – (From Brunden, K. et al., Nat Rev Drug Discov., 2009) Tau in healthy neurons (**a**) and in tauopathies (**b**). **a** - Tau is particularly abundant in axons that stabilizes microtubules and regulates the spacing between them. Stable microtubules are required to support traffic of cellular cargos along neuronal processes. **b** - It is thought that tau function is compromised in AD and other tauopathies. This probably results from both tau hyperphosphorylation, which reduces the binding of tau to microtubules, and the sequestration of hyperphosphorylated tau into NFTs, which reduces the amount of tau that is available to bind microtubules. The loss of tau function leads to microtubule instability and reduced axonal transport, which could contribute to neuropathology.

It is interesting to think that this NMDA-calcineurin-GSK-3β pathway as well as being responsible to induce alterations in spine morphology during LTD in normal physiological conditions, may also be responsible for spine shrinkage and/or loss when deregulated, like in the case of AD where this pathway is overactivated. Finally, in patients with AD, a reduced microtubule density is observed in pyramidal neurons compared with age-matched controls, bringing up the concept that drug-induced stabilization of microtubules could be beneficial in AD and other tauopathies (Brunden et al., 2009), although the traditional microtubule-stabilizing agents (MSA) including taxanes have poor blood-brain barrier (BBB) penetration (Ballatore et al., 2007).

Other neurological disorders as Autism spectrum disorders (ASD) and Schizophrenia are characterized by marked disruptions in information processing and cognition, and recent studies support altered synaptic connectivity and plasticity in the brains of affected individuals (Glantz and Lewis, 2000, Tackenberg et al., 2009, Hutsler and Zhang, 2010). In this regard, some schizophrenic and bipolar patients were reported to have decreased spine density (Figure 6) in pyramidal cells of temporal and frontal cortex (Garey et al., 1998). Besides, smaller spines have been reported in the striatum of schizophrenics (Roberts et al., 1996). Furthermore, MAP-2 and -3 are found to be abnormally expressed and there is altered phosphorylation of MAP1B in schizophrenia (Blanpied and Ehlers, 2004) potentially showing that the microtubule network could also be affected and responsible at some point in the disease-causing mechanism. Fragile X brain is characterized by an elevated spine density (Figure 6), showing elongated, tortuous spine morphologies which are thought to result from pruning deficits (Irwin et al., 2001). Moreover, lack of fragile X mental retardation protein has been shown to result in filopodia-like immature spines and altered synaptic plasticity in fragile X-syndrome, possibly through the deficient regulation of MAP1B translation (Lu et al., 2004). In another mental retardation disease, Down syndrome, there is a decrease in the number of muschroom-shaped spines (Blanpied and Ehlers, 2004).

As previously said, microtubule PTMs are emerging as important regulators of microtubule dynamics and interaction with MAPs, motor proteins and +TIPs. Thus, deficient microtubule PTMs may also be associated with neurological disorders. In fact, depression is associated with increased detyrosination and deacetylation, alterations that are thought to lead to spine decrease in size and density (Wong et al., 2013). Moreover, poli-glutamylation is thought to enhance tau interaction with microtubules in normal conditions (Boucher et al., 1994). So, tau binding to microtubules could also be disturbed in AD due to deregulated tubulin PTMs, something not studied yet.

In summary, there are several brain diseases where microtubules are unstable, many due to deficiencies in MAPs, and thereby efficient intracellular transport and synaptic plasticity is compromised. As dendritic spines are fundamental structures in the brain, it is reasonable to think that a breakdown in any neuronal process responsible to fuel or support them can alter normal brain connectivity. Consequently, it is wise to take them into account in therapeutic strategies. Specifically, drugs that target microtubules, thereby reducing microtubule instability, might be able to restore the normal function of intracellular transport and respective support in synaptic plasticity. Accordingly, MSA would aim to promote spine maturation and restore spine stability in ASD, fortify existing synapses and restore spine plasticity in schizophrenia, or prevent dramatic spine loss in AD (Penzes et al., 2011). So, a role for microtubules in spine development and plasticity could open up new windows in the study of the molecular and cellular mechanisms underlying several brain disorders. Given that many brain disorders are associated with abnormal spine morphology or density, it would be interesting to confirm if microtubules are involved in disease-causing mechanisms, and in that case, if microtubule-based therapeutic strategies would be of help.

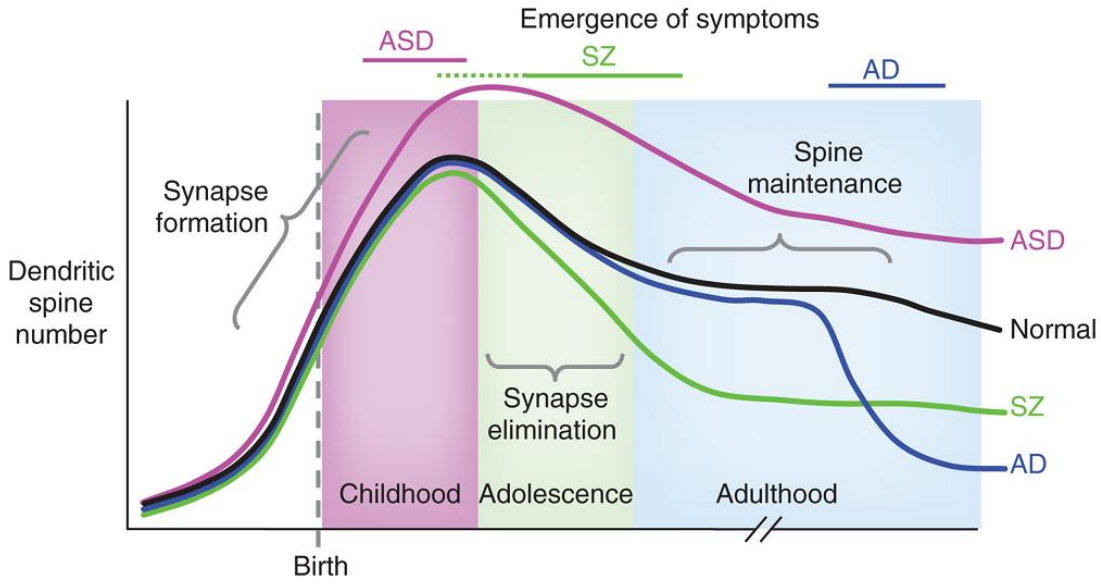


Figure 6 – (From Penzes, P. et al., *Nat Neurosci.*, 2011) This graph relates dendritic spine number *versus* age, in a normal subject (black), in ASD (pink), in schizophrenia (green) and in AD (blue). Bars across the top indicate the period of emergence of symptoms and diagnosis. In normal subjects, spine numbers increase before and after birth; spines are selectively eliminated during childhood and adolescence to adult levels. In ASD, exaggerated spine formation or incomplete pruning may occur in childhood leading to increased spine numbers. In schizophrenia, exaggerated spine pruning during late childhood or early adolescence may lead to the emergence of symptoms during these periods. In AD, spines are rapidly lost in late adulthood, suggesting perturbed spine maintenance mechanisms that may underlie cognitive decline.

1.4 Microtubule-targeting agents

1.4.1 What are they?

Several drugs target α- or β-tubulin, forcing conformational alterations in the tubulin dimer consequently altering microtubule structure, the so called microtubule-targeting agents (MTA). Depending on the drug, such conformational changes on tubulin can facilitate microtubule assembling, disassembling or even stabilize microtubule length within a range (without promoting assembling or disassembling) (Amos, 2011). These molecules have varied structure, can be natural or synthesized, and are nowadays used in several occasions in cancer chemotherapy (Amos, 2011) due to their ability to compromise normal microtubule "dynamic instability", crucial phenomenon in dividing cells.

Among drugs that promote microtubule assembling, Taxol (also known as Paclitaxel) is probably the most famous, belonging to the Taxanes class of MSA. Taxol binds to β-tubulin subunits in a pocket on the luminal surface of the microtubule lattice and counteracts the effect of GTP hydrolysis (that would facilitate depolymerization) (Amos and Lowe, 1999, Prota et al., 2013). At high concentrations Taxol overstabilizes microtubules compromising microtubule dynamics completely, whereas at low concentrations it selectively compromises catastrophe events, this way favoring the overall

polymerization of microtubules at plus ends (Derry et al., 1995, Derry et al., 1997). Epothilone D is another well studied MSA from a different class, the Epothilones. Epothilones bind near the Taxanes site on β-tubulin and that is probably why Taxol and Epothilone D have a similar mechanism of action, as both promote microtubule assembly and suppress microtubule "dynamic instability" (Kamath and Jordan, 2003, Perez, 2009). Both drugs bind along the microtubule length, strengthening contacts between adjacent tubulin dimers within protofilaments and also by stabilizing lateral contacts between protofilaments (Khrapunovich-Baine et al., 2011). At high concentrations, MSA are thought to generate new nucleation sites that promote assembling of new microtubules in various directions (De Brabander et al., 1981, Masurovsky et al., 1981).

Differently, Noscapine is a drug that does not promote microtubule assembly or disassembly. This drug is known to bind specifically and stoichiometrically to tubulin. Unlike Taxanes and Epothilones, Noscapine does not significantly promote microtubule polymerization and does not alter the tubulin polymer/monomer ratio. Instead, Noscapine modulates microtubule dynamics by reducing growing/shortening rates and increasing the percentage of time that microtubules spend in a steady-state, thus stabilizing the microtubule length within a range (Landen et al., 2002, Landen et al., 2004). Although Noscapine stabilizes microtubules, does it in a distinct way comparing to Taxol and Epothilone D (MSA), therefore this drug is considered to be a microtubule-modulating agent (MMA) instead of a MSA.

Finally, Nocodazole is one well-known example of a microtubule-destabilizing agent (MDA). At high concentrations, this drug binds free tubulin monomers and lower their capacity to assemble onto the microtubule polymer, thereby shifting the balance between polymer and free tubulin toward depolymerization (Baas and Ahmad, 2013).

1.4.2 Microtubule-targeting agents as a therapeutic strategy in neurodegenerative diseases

Considering the major role played by microtubules in intracellular transport and synaptic plasticity, it is wise to consider them as a therapeutic target in neurodegenerative diseases where microtubule degeneration and subsequent dendritic spine deficiencies lead to a decrease in the number of functional synapses. Accordingly, neuropsychiatric disorders presenting cognitive deficits associated with abnormal spine density and shape could also benefit from this therapeutic approach.

One among several of therapeutic strategies on neurodegenerative diseases focuses on MTA (Figure 7). MTA have been studied and used for a while in chemotherapy, so, much information is known already for some of these drugs. Concerning MSA, high doses are used in chemotherapy and side-effects as peripheral neuropathy and neutropenia have been reported (Mielke et al., 2006, Scripture et al., 2006, Reyes-Gibby et al., 2009, Bedard et al., 2010). However, low doses of MSA are used in studies regarding neurodegenerative diseases. MSA were already tested in *in vitro* and *in vivo* models of Amyotrophic lateral sclerosis (ALS), PD, HD, AD and other tauopathies (Fanara et al., 2007, Brunden et al., 2010, Shemesh and Spira, 2011, Das and Miller, 2012, Zhang et al., 2012, Brunden et al., 2013, Cartelli et al., 2013). They are intended to stabilize and promote polymerization of existing microtubules in an attempt to counteract microtubule degeneration and associated negative effects: disrupted intracellular transport, synaptic plasticity and overall neuron morphology.

Taxol is a strong MSA used already in neurodegenerative disease models (Zhang et al., 2005, Michaelis et al., 2006, Sengottuvel and Fischer, 2011), however it is not suitable for the treatment of diseases of the central nervous system since it does not readily cross the BBB (Fellner et al., 2002, Brunden et al., 2012). Conversely, Epothilone D is brain penetrant, and was preferred among other MSA of the Epothilones class of MSA due to its pharmacokinetic and pharmacodynamic properties (Brunden et al., 2011). Accordingly, Epothilone D accumulates in the brain, and this may be an advantage as it might allow for prolonged drug activity in the brain, decreased drug doses and treatment frequency and at the same time minimizing peripheral exposure (Brunden et al., 2010). Epothilone D, at much lower doses than used in human cancer treatment, was able to improve axonal microtubule density and decreased axonal dystrophy in tau transgenic mice, leading to an alleviation of cognitive deficits without adverse side effects (Brunden et al., 2012). Furthermore, Epothilone D showed beneficial effects on synaptic function and behaviour in a mouse model of schizophrenia (Andrieux et al., 2006) showing the possibility of using MSA in neurological disorders also.

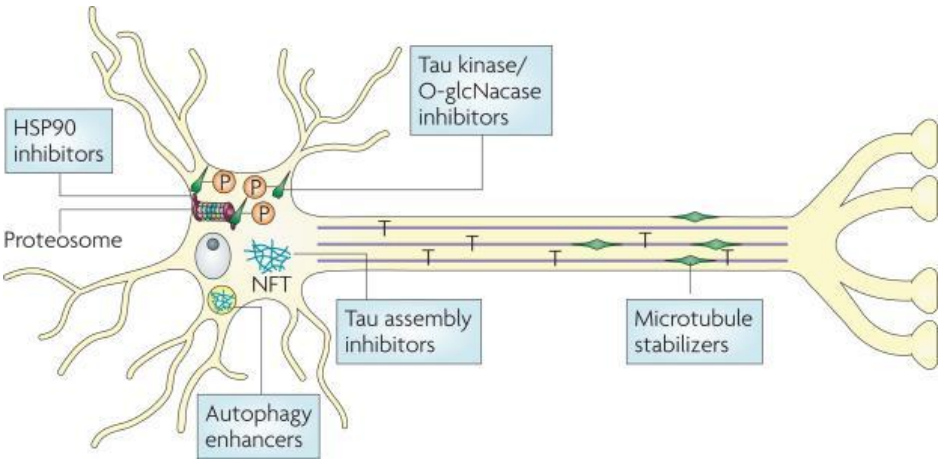


Figure 7 – (From Brunden, K. et al., Nat Rev Drug Discov., 2009) Schematic illustration of recent strategies to reduce neurodegeneration in the case of tauopathies. The use of MSA focuses on the negative effects of tau loss-of-function. Abnormal hyperphosphorylated tau has low affinity for microtubules and aggregates into NFTs. MSA are intended to recover microtubule stability lost in tauopathies due to loss of tau-associated microtubule stabilization. Moreover, this strategy could be useful in other neurodegenerative diseases where microtubule instability is present.

Although beneficial effects were observed in neurodegenerative disease models with MSA, doubts were raised about the negative effects induced by MSA regarding microtubule overstabilization. MSA promote abnormal microtubule nucleation and assembly for high concentrations as already mentioned before. Moreover, rather than simply stabilizing and condensing microtubules, long-term MSA administration induce microtubule polar reconfiguration (Shemesh and Spira, 2010). This is of extreme importance because polarization of microtubules and neuronal polarization are parallel events, and interfering with the regulation of microtubule stability disrupts proper establishment of neuronal polarity (Witte et al., 2008). Accordingly, alterations in the microtubule polarity patterns of axons and dendrites could have profound negative consequences in the normal operation of intracellular transport and synaptic plasticity

(Kapitein and Hoogenraad, 2011, Baas and Mozgova, 2012). This made scientists rethink the therapeutic strategy and establish that it would be important to normalize microtubule dynamicity without the overstabilization effect (Brunden et al., 2013). Therefore, drugs capable of mildly stabilizing microtubules without promoting microtubule polymerization, nucleation of new microtubules or overstabilization are now being pursued. MMA seem to suit this profile, as they do not promote microtubule polymerization and are able to mildly stabilize microtubule length while reducing overall dynamicity. Noscapine, a MMA, is a common antitussive agent, already used in cancer treatment without toxicity (Landen et al., 2002, Landen et al., 2004), can be orally administered, has no reported side-effects, crosses the BBB and minimally affects normal dividing tissues and peripheral nerves (Landen et al., 2004). This makes Noscapine a nice candidate to reduce microtubule instability in neurodegenerative diseases. Actually, Noscapine was shown to stabilize hyperdynamic microtubules in an ALS mouse model (Fanara et al., 2007).

It is important to know that MTA will not bring back the normal microtubule dynamics, and most likely the neuron is not able to fully recuperate from microtubule-related injuries. However, these drugs should be able to maintain microtubules stable and prevent severely microtubule degeneration. Furthermore, MTA affect the entire microtubule system. So, in the future, one should be able to target specifically instable microtubules and promote microtubule stabilization in a more physiological way, without compromising microtubules dynamics. Accordingly, strategies focused in microtubule-related proteins would be of interest as they should confer specificity to the treatment while targeting natural molecular mechanisms responsible for regulation of microtubule dynamics. Turn the focus into molecular targets such as the enzymes that affect the microtubule PTMs or the microtubule +TIPs would probably create strategies more specific to a set/section of microtubules. In the case of the enzymes responsible for microtubule PTMs one should be able to manipulate microtubule stability at a subcompartmental level, once these PTMs show a specific pattern regarding cellular localization; while using microtubule +TIPs would create the possibility of controlling dynamic microtubules that enter dendritic spines, without promoting overstabilization, possibly regulating dendritic spines morphology.

1.5 Experimental goals

The first goal of this project was to characterize the effect of Taxol, Epothilone D (both MSA) and Noscapine (MMA) on the microtubule stability of rat primary hippocampal neurons by quantification of alterations in microtubule PTMs induced by these drugs. According to the literature, MSA should induce accumulation of microtubule PTMs, as would be expected with greater stability. This means that microtubules would have a higher concentration of acetylated tubulin and detyrosinated tubulin, and by opposition less tyrosinated tubulin (Baas and Ahmad, 2013). However, Taxol and Epothilone D have a different mechanism of action comparing to Noscapine, so, different profiles were expected regarding their effect on microtubule PTMs. Determining the effect these drugs have on microtubule PTMs would allow for future simple screenings of new MTA based on their effect on microtubule PTMs, having in mind that good candidates would increase microtubule stability without massively increasing microtubule polymerization, induce overstabilization and completely block dynamicity. After, we sought to find the

ability of these drugs to promote neurite extension in primary neurons. Finally, we wanted to determine the stability of microtubules in an *in vitro* tau-aggregation AD model by evaluating the PTMs of the microtubule network, in an attempt to understand if this would be a good model to test in the future the ability of Taxol, Epothilone D and Noscapine to recover microtubule stability.

2. Materials and Methods

2.1 Materials

2.1.1 Antibodies

Name	Company	Catalog number	Dilution used
Anti-acetylated tubulin	Sigma Aldrich	T-6793	1:10000
Anti-chicken Alexa 647	Life Technologies	A21449	1:500
Anti-detyrosinated tubulin	Millipore	AB3201	1:2500
Anti-human Tau 10	Homemade		0.5 µg/mL
Anti-MAP2	Abcam	Ab5392	1:10000
Anti-mouse Cy3	Jackson Immuno Research	115-165-146	1:200
Anti-rabbit Alexa 488	Life Technologies	A11008	1:200
Anti-tyrosinated tubulin	Sigma Aldrich	T-9028	1:2500
Anti-α tubulin	Sigma Aldrich	T-6199	1:2500
Anti-β III Neuronal specific tubulin	Covance	PRB-435P	1:2500
Anti-β tubulin	Sigma Aldrich	T-4026	1:2500
ECL anti-mouse IgG	GE Healthcare LifeSciences	NA9310	1:3000
ECL anti-rabbit IgG	GE Healthcare LifeSciences	NA934	1:3000

2.1.2 Biological and chemical material

Name	Company	Catalog number
0.5 % Trypsin-EDTA	LifeTechnologies	15400
B-27 serum-free supplement	LifeTechnologies	17504-044
Bovine Serum Albumin (BSA)	Sigma Aldrich	A4503
DAPI	Life Technologies	D1306
Dimethyl sulfoxide (DMSO)	Merck Millipore	1029311000
DTT	Invitrogen	D-1532
EGTA	Sigma Aldrich	E4378
Embryos from pregnant Rat (WistarCrl:WI) E18-19	Charles River Laboratories	2308816
Epothilone D	Johnson&Johnson	JNJS 54299076
Glucose	Merck Millipore	1083421000
Glutaraldehyde	Sigma Aldrich	G5882
Hank's balanced salt sodium solution (HBSS) [without Ca2+,Mg2+]	LifeTechnologies	14175
Heparin	Sigma Aldrich	H-5284

HEPES (liquid)	LifeTechnologies	15630-122
HEPES (powder)	Sigma Aldrich	H7523
Horse Serum	LifeTechnologies	26050088
Hydrochloric acid	Merck Millipore	1090571000
L-glutamine	LifeTechnologies	25030-024
Magnesium Cloride	Sigma Aldrich	M8266
Minimum Essential Medium (MEM)	LifeTechnologies	31095-052
Neurobasal Medium	LifeTechnologies	21103-049
Nocodazole	Johnson&Johnson	JNJS 1580540
Normal Goat Serum (NGS)	Sigma Aldrich	G9023
Noscapine	Johnson&Johnson	JNJS 30396964
Paraformaldehyde (PFA)	Sigma Aldrich	76240
PBS 10x	Roche Applied Science	11666789001
Penicilin/Streptomycin	LifeTechnologies	15140
Phosphate Buffered Saline (PBS)	Sigma Aldrich	D8537
PIPES	Sigma Aldrich	P8203
Potassium chloride	Merck Millipore	1049380500
Potassium dihydrogen phosphate	Merck Millipore	1048731000
Sodium acetate	Sigma Aldrich	S2889
Sodium bicarbonate	Sigma Aldrich	S-5761
Sodium borohydride	Sigma Aldrich	213462
Sodium chloride	Sigma Aldrich	S-9625
Sodium hydroxide	Merck Millipore	1091371000
SuperSignal West Dura Extended Duration Substrate	ThermoScientific	34076
Taxol	Johnson&Johnson	JNJS 17129515 JNJS 4795960
ToxiLight™bioassay kit	Lonza	LT07-117
Triton X-100	Sigma Aldrich	T8787

2.1.3 Laboratorial material and equipment

Name	**Company**	**Catalog number**
12-Channel Pipettor 5-50 µL and 50-300 µL	VWR	
96-well Microplates	Greiner BioOne	655906
Analytical Balance	Sartorius	
Biological Safety Cabinet EF/S	Telstar Clean Air	
Cell Voyager CV 7000	Yokogawa	
Centrifuge Allegra 6	Beckman Coulter	366802

Benchtop		
CO_2 Incubator Hera cell 150	Thermo Scientific	51026281
Disposable Polystyrene Serological Pipets	Fischer-Scientific	
Easy-grip cell culture dish, 100x20 mm	Corning	353003
Easy-grip cell culture dish, 35x10 mm	Corning	353001
Falcon 15 mL	Corning	352196
Falcon 50 mL	Corning	352070
FIREBOY plus	Integra-Biosciences	144000
FluoroskanAscent™ FL MicroplateFluorometer and Luminometer	Thermo Scientific	
IKA MS1 Shaker	Sigma Aldrich	Z404047
Leica DMI4000 B	Leica Microsystems	
Magnetic Stirrer/Heater	IKA	0003622000
MIAS-2 Multimode Microscopy Reader	Digilab	
Pasteur Pipette	Volac	D812
PIPETBOY acu 2	Integra-Biosciences	155000
Pipette tips	Eppendorf	
Pipettes P2, P10, P20, P100, P200 and P1000	Gilson PIPETMAN Classic	
Quantum EX Cartridge	Millipore	QTUM000EX
Stericup-GP Filter 0.22 µM	Millipore	SCGPU05RE
Vi-Cell Counter XR	Beckman Coulter	383556
Zeiss LSM 510 META	Zeiss	

2.2 Methods

2.2.1 Primary hippocampal cultures

Pregnant rats (Wistar Crl:WI) at gestational day E18-19 were sacrificed by decapitation and embryos collected to petri dishes with pre-warmed HBSS/Hepes buffer (7mM HEPES and 1% Penicillin-Streptomycin, at 37°C). After all hippocampi were isolated under a dissecting microscope they were transferred to a 15mL falcon already with 4.5mL of pre-warmed HBSS/Hepes plus 500µL of 10x concentrated trypsin and incubated for 10-15 minutes at 37°C to promote chemical neuronal dissociation. Next, 3 washing steps were done using 3-5mL of pre-equilibrated (37°C and 5% CO_2) MEM-Horse medium (10% Horse serum, 0.6% glucose in MEM-medium 1x) before resuspending the hippocampi in 3mL of MEM-Horse medium and mechanically dissociate the tissue first with a sterile glass Pasteur pipette with a normal tip diameter followed by a similar pipette with a smaller tip diameter. Then, cells were centrifuged at 1000 rpm for 5 minutes and resuspended with 2-3mL of MEM-Horse medium. Finally, cells were counted using an automatic counter and plated in poly-D-lysine pre-coated 96-MW microplates with µclear bottom using a cell density of 10 000 or 20 000 cells per well. After 4

hours minimum, MEM-Horse medium was replaced by pre-equilibrated Neurobasal medium (2% B27 supplement and 2mM L-glutamine).

2.2.2 Transduction of primary hippocampal cultures and addition of pre-formed fibrils

Primary hippocampal cultures plated in poly-D-lysine pre-coated 96-MW microplates with µclear bottom using a cell density of 10 000 per well were transduced with Adeno-Associated Virus (AVV) serotype 6 to overexpress either human Wild Type (hWT) tau, hP301L tau or Green Fluorescent Protein (GFP), driven by the hSYN1 promoter. Appropriate transduction units of virus concerning a multiplicity of infection of 100 were diluted in Neurobasal medium and directly added to the medium after 3 DIV. A truncated form of human tau prone to aggregation containing only the 4 microtubule-binding domains, K18, with a P301L point mutation, K18P301L (expressed in bacteria and purified) was provided by Wouter Bruinzeel, Tibotec. Furthermore, tau seeds were formed by *in vitro* fibrilization (Calafate, S., "Tauopathy seeding models as a platform for tau aggregation and clearance study", Master Thesis, University of Coimbra, 2012) and added at 7 DIV (in addition to transduction). Briefly, 40 µM of K18P301L was mixed with 40 µM of heparin and 2 µM of DTT in 100 mM sodium acetate buffer at pH of 7.0. The mix was incubated at 37°C for 48 to 72 hours and further centrifuged at 100 000g during 30 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in sodium acetate until the desired concentration and then stored at -80°C or immediately used. Immediately before use pre-formed fibrils at 40 µM were diluted to 5 µM with sodium acetate solution and sonicated with a probe tip by 25 pulses, 1 second each pulse with 10 seconds pause between each. Finally, they were added into the medium at a final concentration of 25 nM.

2.2.3 Drug treatment

Drug powders were resuspended with sterile DMSO under the flow in order to produce stock solutions after stored at -20°C with the following concentrations: Taxol 1 mM, Epothilone D, Noscapine and Nocodazole 10 mM. The day of treatment (at least 1 day after plating) the final drug concentrations were prepared in pre-equilibrated supplemented Neurobasal medium starting from correspondent 100-fold concentrated fresh drug solutions previously prepared diluting stock solutions with DMSO (final DMSO concentration was always <1%). Afterwards, old medium was replaced by 100 or 200 µL of medium with drug treatment depending on how long the neurons would be in culture, less or more than 8 DIV respectively.

2.2.4 Adenylate Kinase toxicity assay

The cell toxicity assay was performed using ToxiLight^{TM} bioassay kit instructions. Briefly, medium samples of 50 µL/well from plated cells treated with Taxol, Epothilone D, Noscapine or Nocodazole (after 1 or 7 DIV for 24 hours) were collected before fixation for further analysis by In-Cell ELISA. After all reagents (including medium samples) were at room temperature (RT) the adenylate kinase (AK) detection reagent was reconstituted in assay buffer and incubated for 15 minutes at RT. Then, 25 µL of each sample were transferred to 96-MW microplates with µclear bottom and 65 µL of AK detection reagent was added to each sample for 5 minutes. Finally, plates were measured using Fluoroskan Ascent^{TM} FL Microplate Fluorometer and Luminometer.

2.2.5 In-Cell ELISA

Primary hippocampal neurons were fixed with 0.5% glutaraldehyde/0.5% Triton X-100 in PHEM buffer (Schliwa and van Blerkom, 1981) for 10 minutes at RT with an additional permeabilization step with 0.5% Triton X-100 in PHEM buffer for 30 minutes. Reduction of background autofluorescence followed using 1 mg/mL of $NaBH_4$ in PHEM buffer for 10 minutes. Next, neurons were blocked with 5% NGS/0.1% BSA in PBS buffer for 30 minutes. Primary antibodies were incubated overnight at 4°C in 1% NGS/0.1% BSA in PBS while Horseradish peroxidase (HRP)-attached secondary antibodies were incubated for 1 hour RT in 0.1% BSA in PBS. There were 3 washing steps using 0.1% BSA in PBS after incubation with primary and secondary antibodies, 10 minutes each step. Finally, SuperSignal West Dura Chemiluminescent Substrate was used for 5 minutes to develop the signal. Bioluminescence was measured in a microplate luminometer.

2.2.6 Immunocytochemistry

Primary hippocampal neurons were fixed with 4% PFA in PBS for 10 minutes and washed 2 times with PBS for 10 minutes under the hood at RT. Afterwards, permeabilization of neurons was accomplished using 0.1% Triton X-100 in PBS during 10 minutes followed by a step of washing with PBS for 10 minutes. Next, neurons were blocked with 5% NGS/0.1% BSA in PBS for 30 minutes. From this point 0.1% BSA in PBS was used as buffer for labeling and washing steps. Primary antibodies were incubated overnight at 4°C. After 3 washing steps for 10 minutes, secondary antibodies were incubated for 1 hour at RT away from light and excess antibody was rinsed. Nuclei were stained using DAPI in 0.1% BSA for 5 minutes. Finally, another washing step was done and neurons remained in 0.1% BSA in PBS buffer. Images were obtained using Zeiss LSM 510 META confocal microscope or a normal fluorescence microscope Leica DMI4000 B. For high content screening purposes automated images were taken using the MIAS-2 Multimode Microscopy Reader or the Cell Voyager CV 7000.

2.2.7 Image Analysis

To study morphological alterations such as neurite outgrowth, number of neurites and ramification points, images from the MIAS-2 Multimode Microscopy Reader and the Cell Voyager CV 7000 were analyzed with the Neurite Outgrowth (NEO) assay software from DCI Labs. The 2-channel 2FLUO assay was used combining DAPI + β-III Tubulin or DAPI + MAP2 to quantify neurite outgrowth, number of neurites and ramification points.

2.2.8 Statistical Analysis

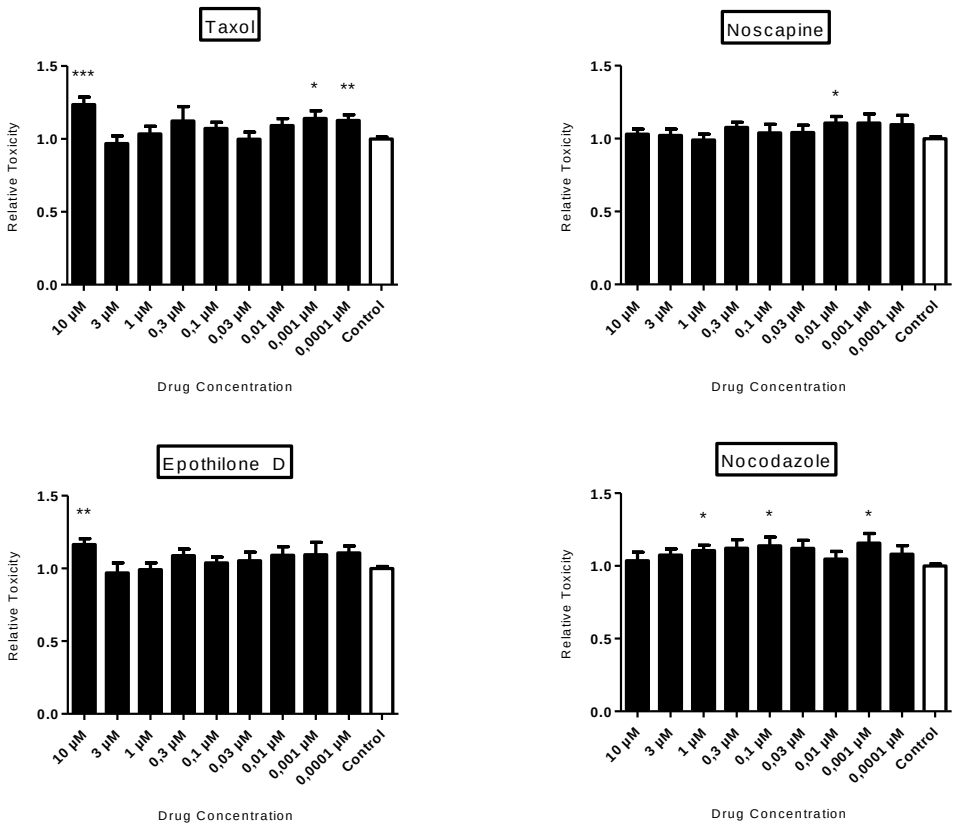
Comparison between different sets of data was performed using the Unpaired T-test with Welch's correction or the Two-way ANOVA + Bonferroni post-tests.

3. Results

3.1 Measurement of cytotoxicity induced by Taxol, Epothilone D, Noscapine and Nocodazole

To check cell toxicity induced by Taxol, Epothilone D, Noscapine and Nocodazole (MTA), a cytotoxicity assay was performed in primary hippocampal neurons (cells used in all the experiments). Basically the assay measures the amount of AK that leaks into the medium as a result of damage to cell membrane integrity consequently reporting possible drug-induced cytotoxicity. After 1 or 7 DIV neurons were treated for 24 hours and medium samples collected for the assay before neurons were fixed. There was no pronounced toxic effect induced by any drug as toxicity levels did not change much when different drug concentrations were compared to the control, from 0.0001 µM up to 10 µM (Figure 8). However, Taxol and Epothilone D showed a trend to be toxic after 10 µM as toxicity levels increased approximately 20% at this concentration. It is important to mention that a positive control (cell membrane destabilizing agent like Triton X-100 or stress-inducing agent like hydrogen peroxide) is lacking in order to confirm that the assay is working correctly.

A- Neurons treated at 1 DIV for 24 hours



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B- Neurons treated at 7 DIV for 24 hours

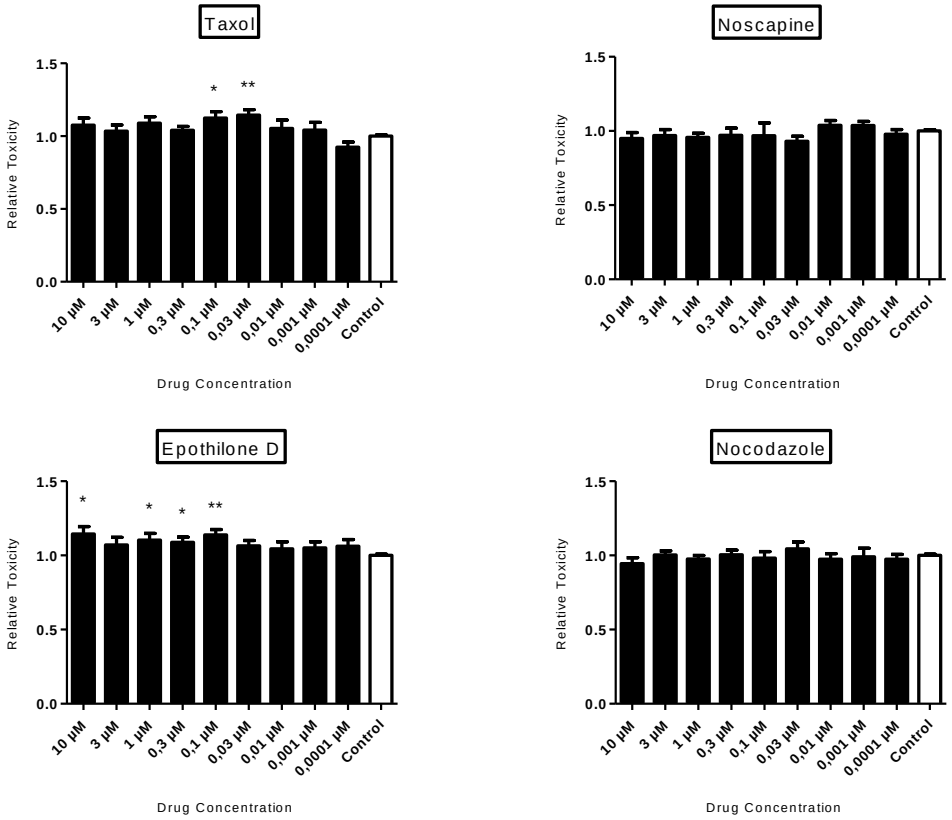


Figure 8 – Cytotoxicity assay by measurement of AK release of neurons – The toxicity induced by Taxol, Epothilone D, Noscapine and Nocodazole was tested in neurons by a bioluminescence method. **A-** Quantification of the toxicity levels normalized to control (mean ± SEM) in neurons treated at 1 DIV for 24 hours; **B-** Quantification of the toxicity levels normalized to control (mean ± SEM) in neurons treated at 7 DIV for 24 hours. Statistical analysis by T-test + Welch's correction, n=2.

3.2 Intracellular localization of microtubule PTMs

The goal of this experiment was mainly to determine the intracellular localization of the acetylated and tyrosinated tubulin. We confirmed the presence of acetylated tubulin (associated with old, stable microtubules) in dendritic shafts as wells as in the axon (Figure 9 A) and interestingly its absence in more distal parts of the dendrites (Figure 9 A, arrows), where microtubules with a more dynamic character should be present. Tyrosinated tubulin was present in freshly polymerized microtubules, associated with dynamic microtubules, mainly in growth cones but also in more distal parts of dendrites (Figure 9 B, arrowheads), overlapping with sites where acetylated tubulin was absent.

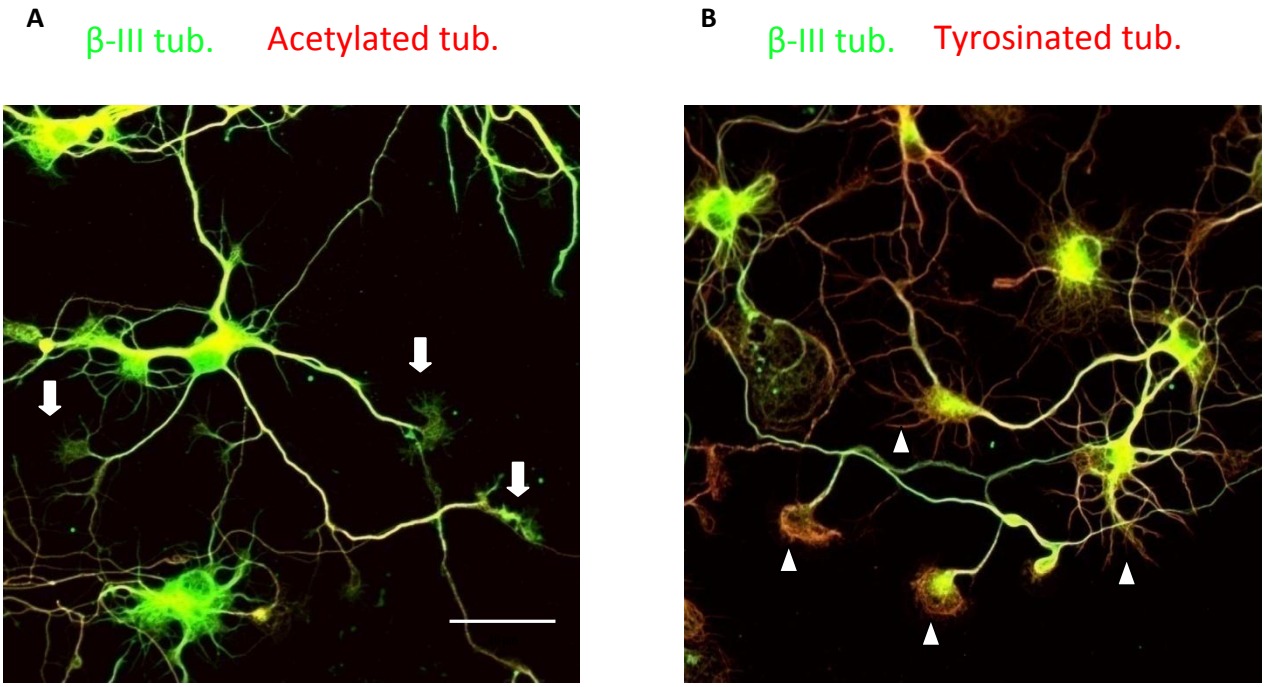


Figure 9 – Intracellular localization of microtubule PTMs – A, B, Representative images of rat hippocampal neurons 7 days in culture labeled for: (**A**) rabbit anti-β-III tubulin (green) and mouse anti-acetylated tubulin (red), white arrows show the absence of acetylated tubulin in dentritic distal sites while the soma and main processes including the axon show a yellow labeling due to the overlapping localization of β-III tubulin and acetylated tubulin where stable microtubules are; (**B**) rabbit anti-β-III tubulin (green) and mouse anti-tyrosinated tubulin (red), white arrowheads show sites of intense labeling of tyrosinated tubulin including growth cones and tips of dendrites where microtubules are predominantly dynamic. Scale bar, 40 µm.

3.3 Quantification of microtubule PTMs after treatment with MTA

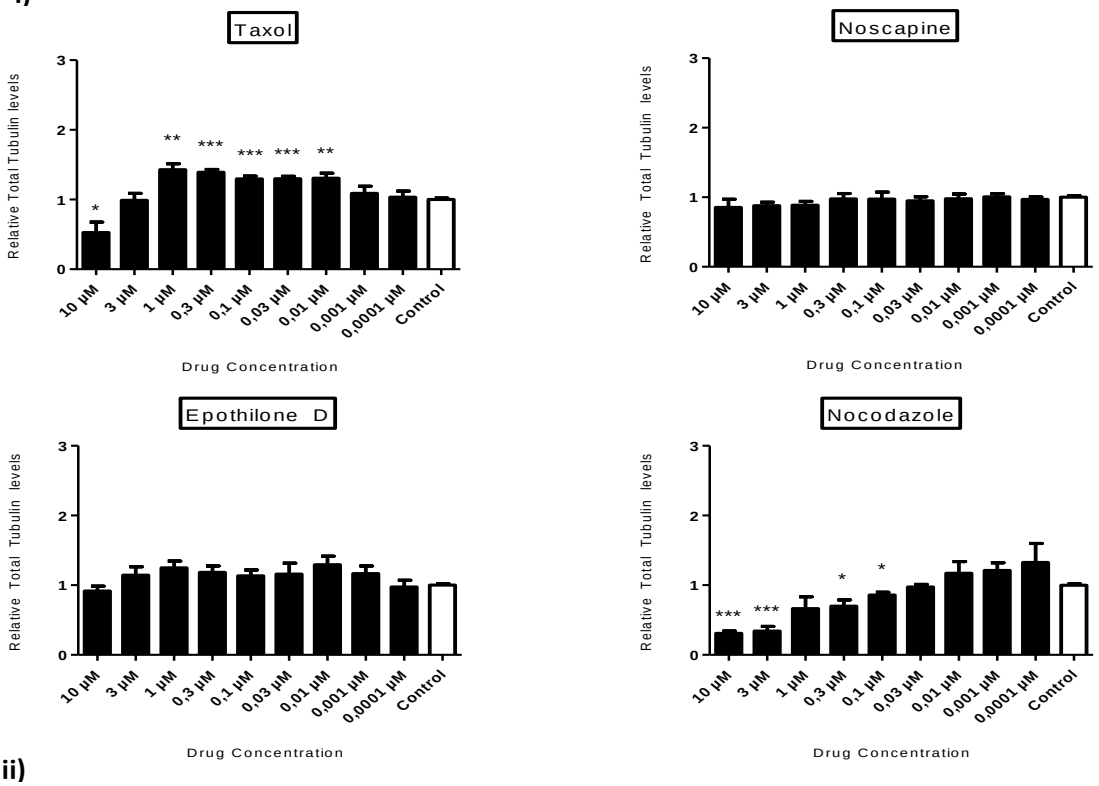
We quantified changes in the amount of polymerized tubulin as well as in microtubule PTMs induced by Taxol, Epothilone D, Noscapine and Nocodazole in neurons by In-Cell ELISA to infer about these drugs' ability to enhance microtubule polymerization and/or stability. Nocodazole is used as a negative control, it is known as a microtubule destabilizing agent, the opposite effect of Taxol, Epothilone D and Noscapine. Primary hippocampal neurons were treated at different time points, either after 1 or 7 DIV, for 24 hours, and then fixed and permeabilized at the same time in order to wash away the non-polymerized tubulin. This way, we were able to quantify only the polymerized α- or β-tubulin (total tubulin) and respective PTMs.

Neurons treated at 1 DIV for 24 hours by Taxol or Epothilone D increased their relative levels of total tubulin (Figure 10 Ai) and acetylated tubulin (in some cases up to more than two times the control) in a dose-dependent manner (Figure 10 Aii), although the effect of Epothilone D on the relative levels of total tubulin was not statistically significant. Moreover, these drugs induced a significant decrease (almost to half the control) in the relative levels of tyrosinated tubulin also in a dose-dependent manner (Figure 10 Aiii), even though for the two lowest concentrations used there was a trend to increase these microtubule PTM. Noscapine was not able to induce an increase in the relative levels of total tubulin (Figure 10 Ai). Nevertheless, it induced an increase in the relative levels of acetylated tubulin (Figure 10 Aii) (not as pronounced as the effect induced by Taxol and Epothilone D) but also in the relative levels of tyrosinated tubulin (Figure 10 Aiii). In both cases Noscapine did not show a dose-dependent response like Taxol and Epothilone D but instead its effect reached a "plateau" where increasing concentrations of the drug did not change the relative levels of acetylated or tyrosinated tubulin, confirming a different microtubule-stabilization mechanism of action in comparison to Taxol and Epothilone D. Interestingly, Nocodazole, in spite of being a microtubule-depolymerizing agent, slightly increased the relative levels of the total, acetylated and tyrosinated tubulin levels for the lowest concentrations used (Figure 10 Ai,ii and iii). However, this effect is reversed at higher concentrations where this drug massively decreases the relative levels of the total, acetylated and tyrosinated tubulin in a dose-dependent manner as expected. Last but not least, the relative levels of detyrosinated tubulin were not altered by any drug for the concentrations used (Figure 10 Aiv). Not even Nocodazole, known to destabilize microtubules and decrease microtubule length, was able to alter the relative levels of detyrosinated tubulin.

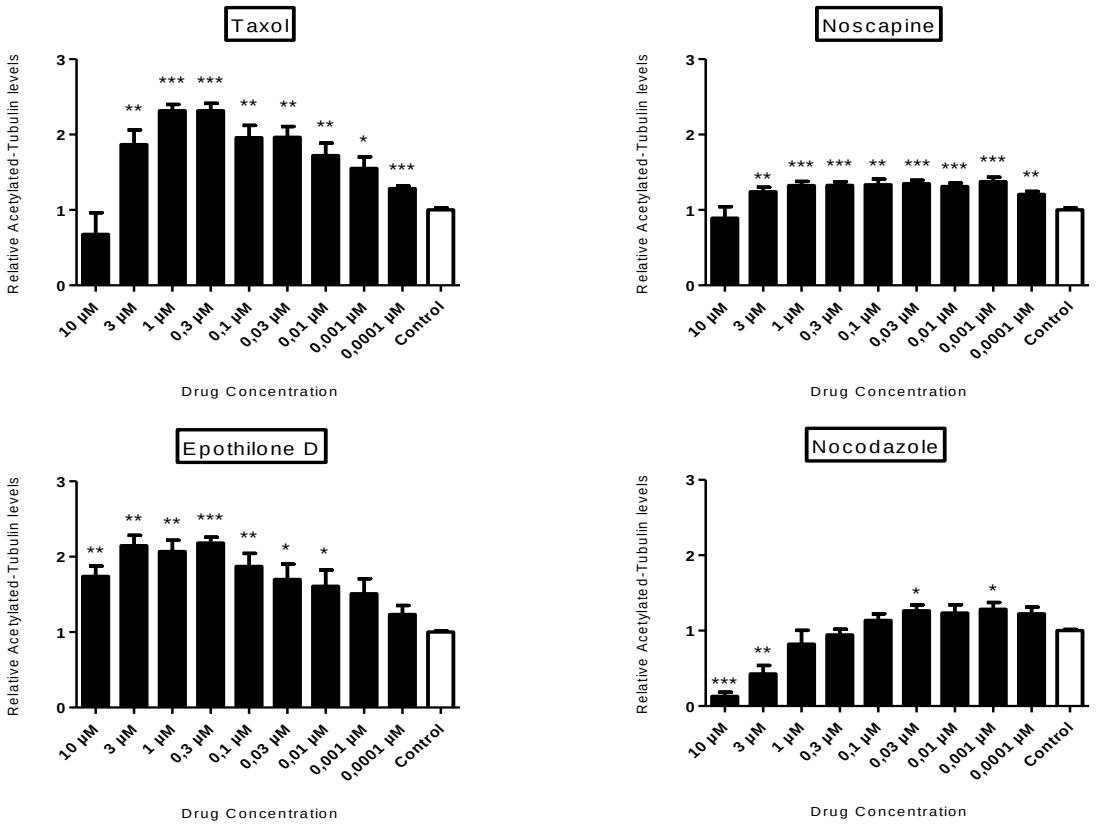
Neurons treated for 24 hours after 7 DIV were not susceptible to changes in the relative levels of total and acetylated tubulin when Taxol, Epothilone D and Noscapine were used (Figure 10 Bi and ii). However, Taxol and Epothilone D induced a small decrease in the relative levels of tyrosinated tubulin for concentrations higher than 0,03 μM in opposition to Noscapine that did not promote such decrease, further confirming a different mechanism of action in comparison to Taxol and Epothilone D (Figure 10 Biii). Nocodazole, in contrast to the previous drugs, could keep its effect after 7 DIV significantly decreasing the relative levels of total, acetylated and tyrosinated tubulin as expected (Figure 10 Bi, ii and iii). Here too no drug was able to change the relative levels of detyrosinated tubulin for the concentrations used (Figure 10 Biv).

A- Neurons treated at 1 DIV for 24 hours

i)



ii)



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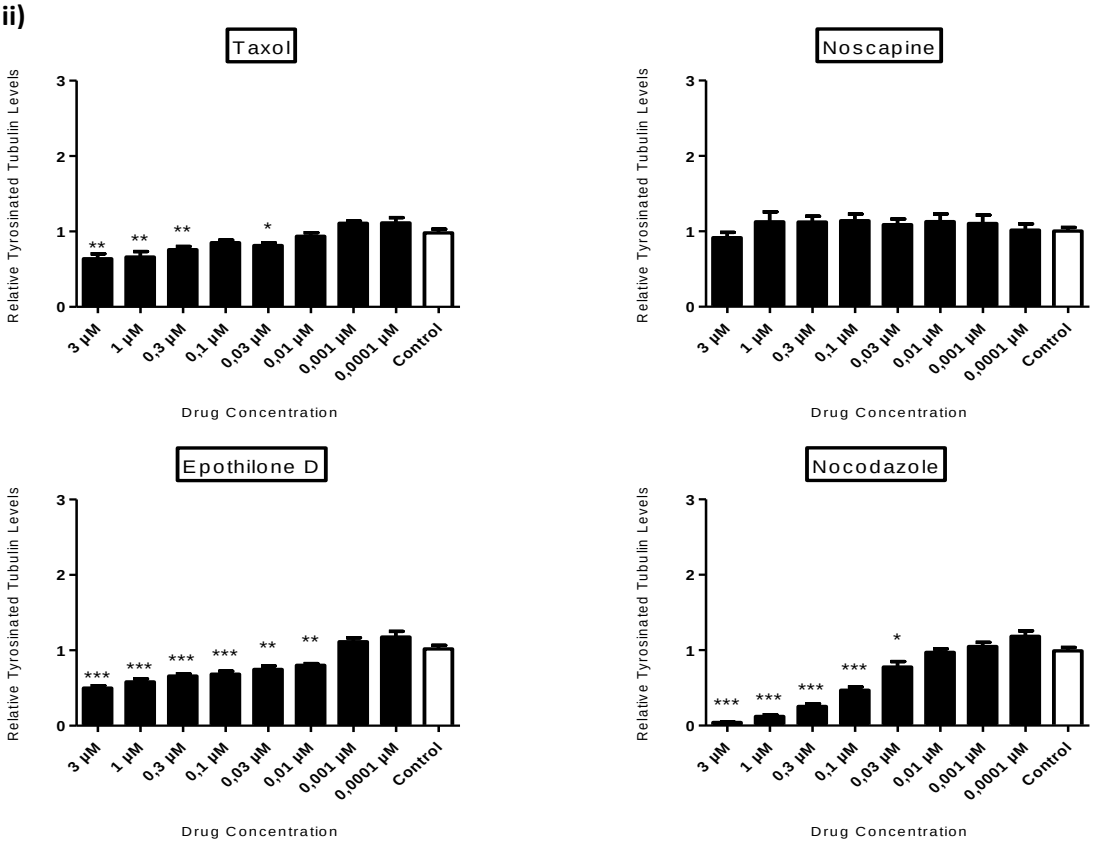
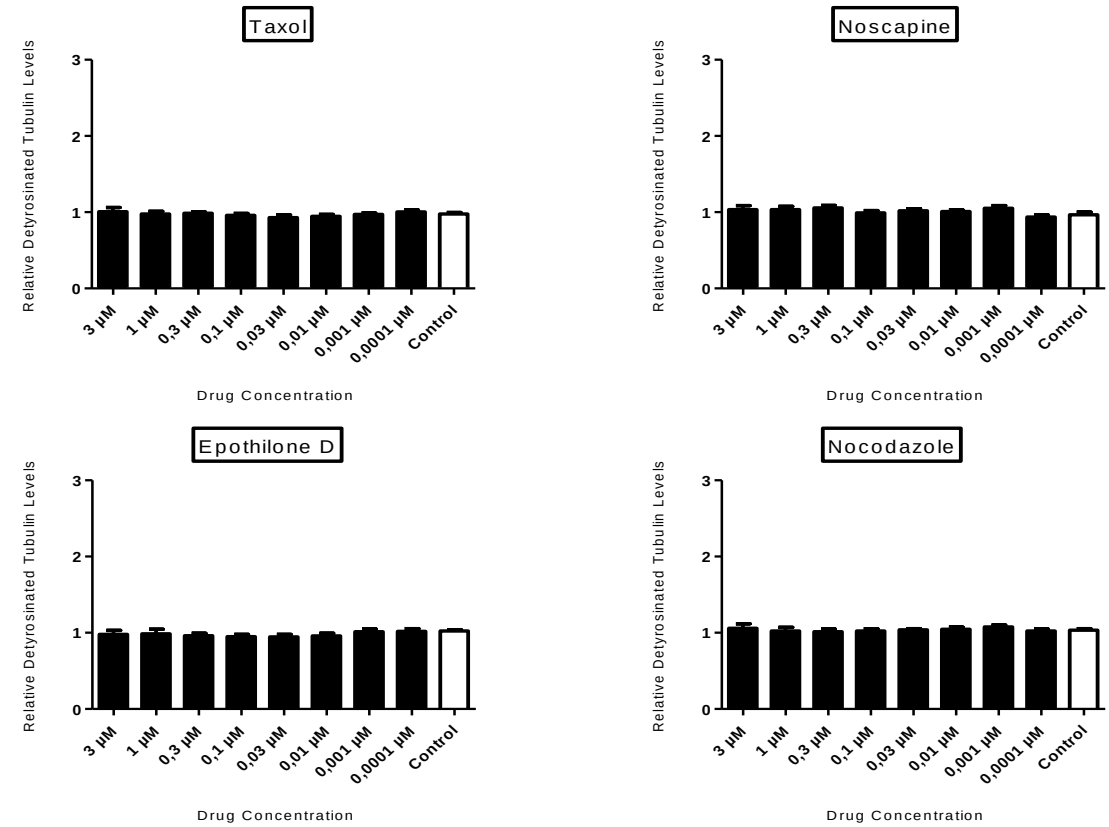
iii)



iv)



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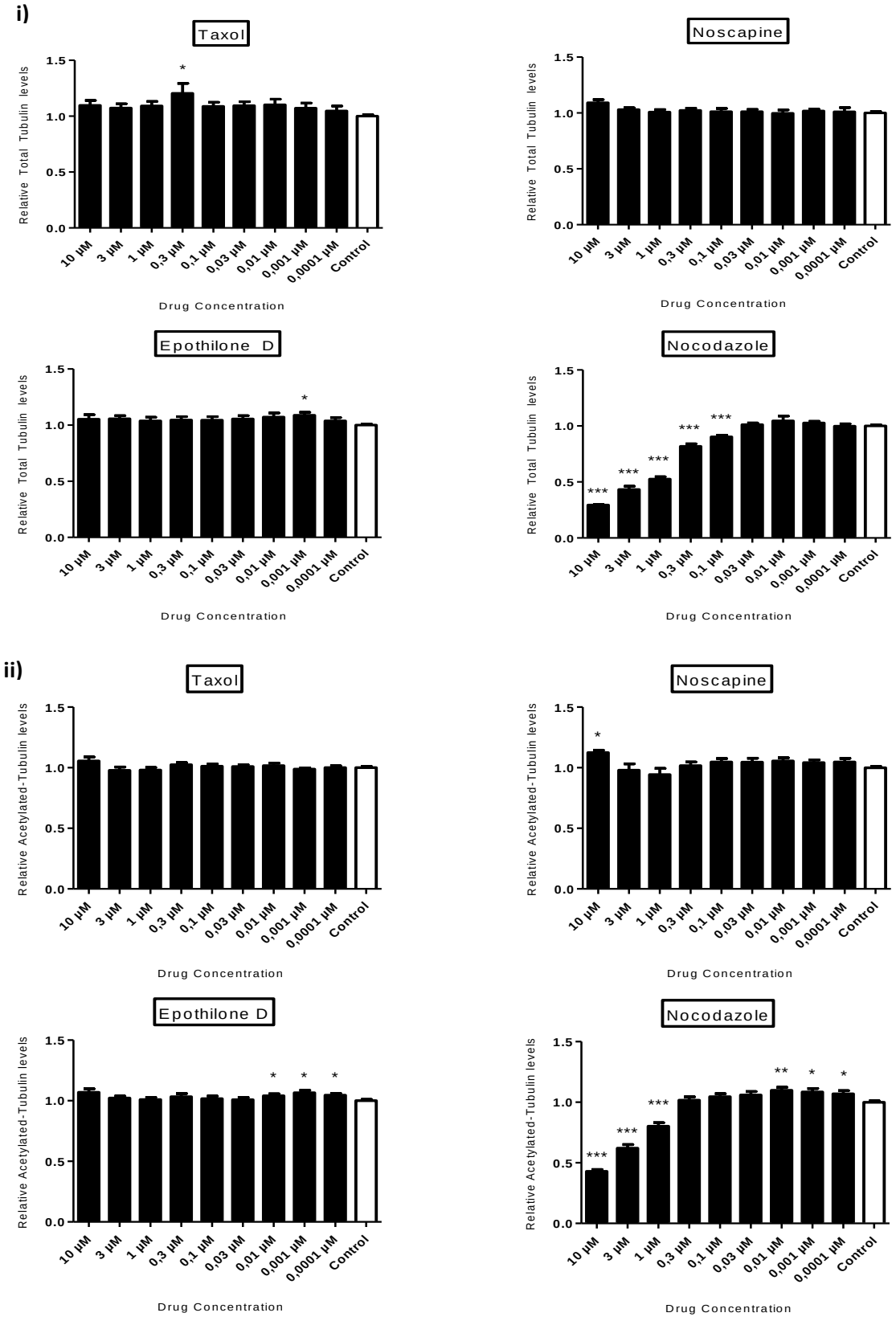
B- Neurons treated at 7 DIV for 24 hours



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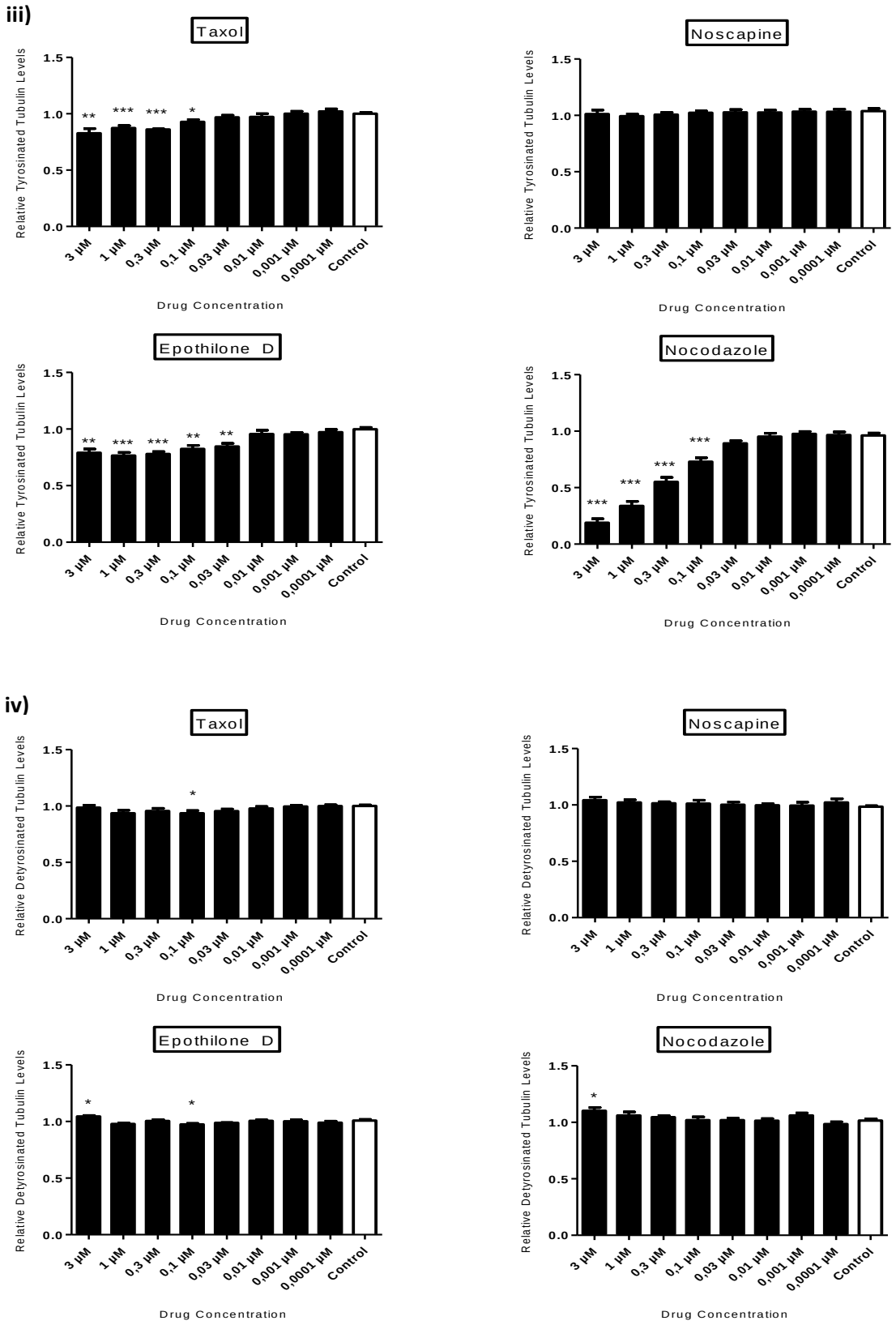

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3.4 Characterization of the effect of MTA on neuronal morphology

3.4.1 MTA effect on neurite length, number and ramification points

In order to study the effect of Taxol, Epothilone D and Noscapine on neuronal morphology, primary hippocampal neurons were either treated at 1 DIV and fixed at 7 DIV or treated at 7 DIV and fixed at 14 DIV. The reason for the long treatment duration was to be sure drugs could induce significant changes in neuronal morphology since similar experiments with treatments lasting just 1 or 3 days did not show any effect (data not shown). Moreover, the drug concentration range used was broader for Taxol and Epothilone D (0.01nM to 1μM) compared to Noscapine and Nocodazole (0.001 μM to 1 μM) because the first drugs showed toxicity for higher concentrations, observed by loss of positive nuclei (which are nuclei - DAPI positive - included in a soma attached to neurites - β-III Tubulin positive - a specific neuronal marker). After fixation neurons were labeled for β-III Tubulin and DAPI to track neurites and nuclei, respectively (for examples see Figure 11 Ai and Bi). Software that can automatically analyze neurite length, number and branching points was subsequently used (Figure 11 Aii,iii and Bii,iii) (NEO, DCI Labs).

Neurons treated at 1 DIV and fixed at 7 DIV with Taxol and Epothilone D showed a moderate increase in neurite length/pos. nucleus and in the number of branches/pos. nucleus when 1 nM of compound was used (Figure 12 A). Higher concentrations induced a decrease in the number of pos. nuclei meaning that neurons started to suffer drug-related toxicity and died. Noscapine was also able to increase the neurite length/pos. nucleus and the number of branches/pos. nucleus (Figure 12 A), although to a lesser extent, and at higher concentrations (10 to 1000 times higher) comparing to Taxol and Epothilone D. This means that Taxol and Epothilone D are more powerful regarding the analyzed parameters, but Noscapine is better tolerated by neurons since there was no drug-related toxicity seen for the concentrations used (no decrease in positive nuclei, neurite length or neurite number).

Figure 10 – Quantification of the effect induced by Taxol, Epothilone D, Noscapine and Nocodazole on microtubule PTMs – Primary hippocampal neurons were treated for 24 hours after 1 (**A**) or 7 DIV (**B**) and the effect induced by these drugs on polymerized tubulin and some tubulin PTMs was quantified by In-Cell ELISA. **A, B i, ii, iii** and **iv** are groups of graphs showing the quantification of the levels of total tubulin (α- or β-tubulin) (**i**), acetylated tubulin (**ii**), tyrosinated tubulin (**iii**) and detyrosinated tubulin (**iv**) normalized to the respective controls (mean ± SEM) for the respective treatment time points. Taxol, Epothilone D and Noscapine can induce alterations in microtubule PTMs in neurons at 1 DIV, increasing the relative levels of acetylated tubulin and suggesting that these drugs indeed promote microtubule stabilization. However, that effect is lost when the treatment is done after 7 DIV. Interestingly, Taxol and Epothilone D showed a similar profile regarding their effect on microtubule PTMs, confirming that these drugs share a similar mechanism of action, but different from the mechanism of Noscapine, that exhibited a different profile (did not induce an increase on the relative levels of total tubulin not even a decrease in the tyrosinated tubulin). Conversely, Nocodazole exerted its effect after 1 and 7 DIV, decreasing the relative levels of the total, acetylated and tyrosinated tubulin but interestingly, did not change the relative levels of detyrosinated tubulin probably due to the resistance of the population of microtubules or microtubule portion bearing this PTM to this drug. Statistical analysis by T-test + Welch's correction, n=2 or 3.

Neurons treated at 7 DIV for 7 days respond differently. Taxol and Epothilone D could not induce an increase in neurite length/pos. nucleus, number of neurites/pos. nucleus or number of branches/pos. nucleus and instead provoked the opposite effect for high concentrations (Figure 12 B). It might be that the number of positive nuclei decreases due to drug-related toxicity for concentrations >1 nM, and consequently, the total neurite length decreases also as less neurons are present.

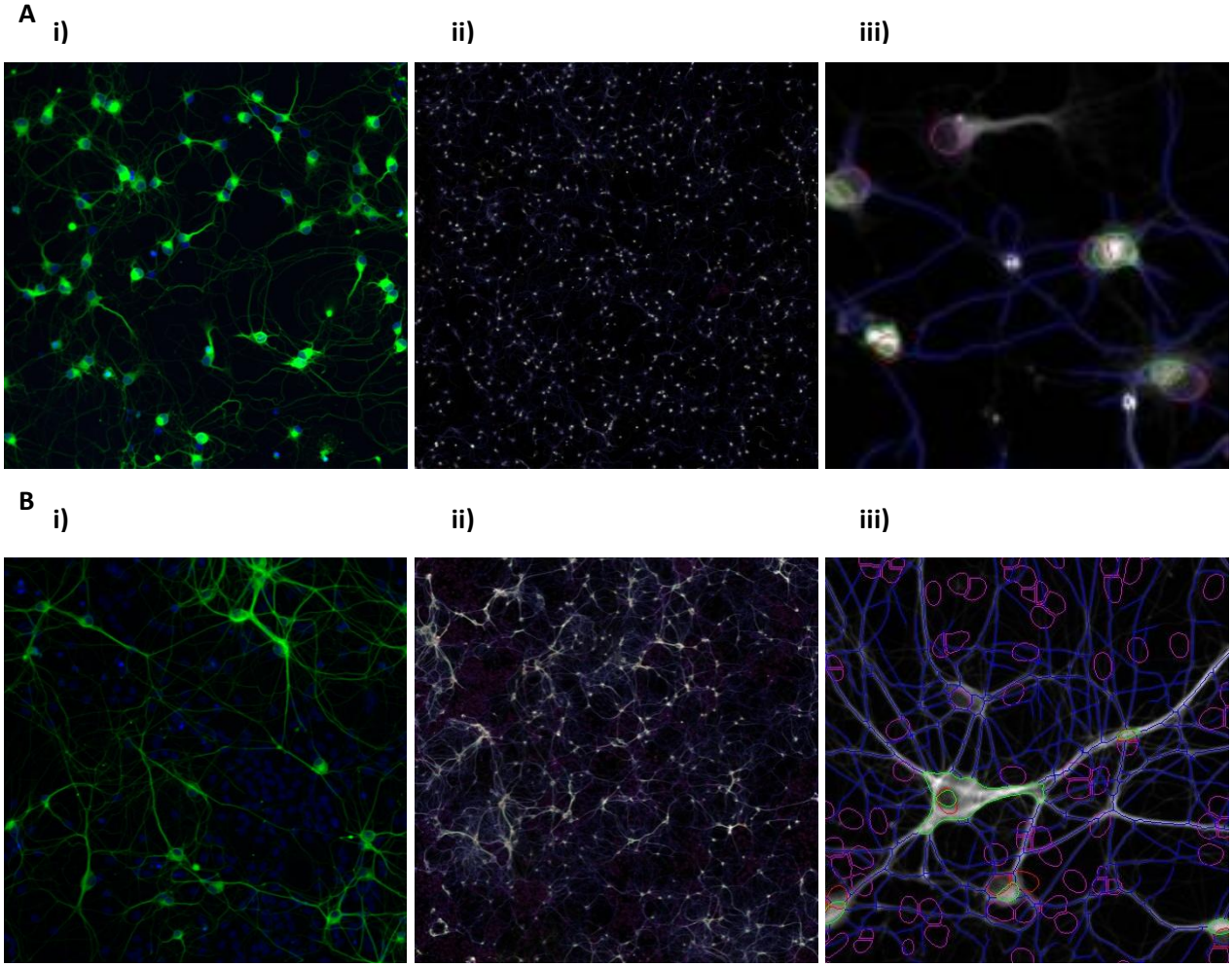
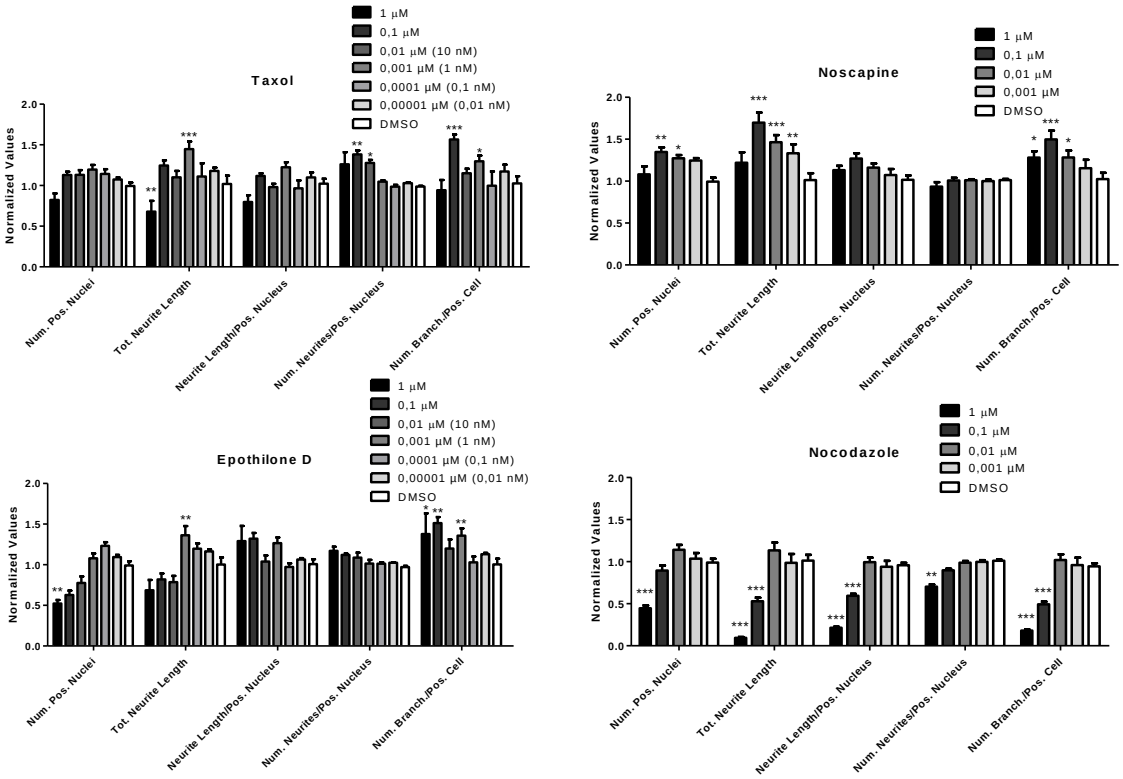


Figure 11 – Example of measurement of neurite length, number of neurites and branching by a high-content screening approach – A, B, Representative images of non-treated rat hippocampal neurons (control condition) fixed after 7 DIV (**A**) or after 14 DIV (**B**). **Ai** and **Bi**: neurons labeled for β-III tubulin (green) and DAPI (blue). Images taken with a 20x objective, each image represents a tile. **Aii** and **Bii**: a set of sixteen tiles for the same condition are acquired by the microscope and then juxtaposed and analyzed by the software NEO, DCI Labs. **Aiii** and **Biii**: expanded images of **Aii** or **Bii**, respectively. The software tracks the nuclei and separates "negative" nuclei (pink circles) from "positive" nuclei (red circles). Nuclei (stained with DAPI) overlapping with cell bodies connected with neurites (stained with anti-β-III tubulin) are classified as being "positive" nuclei and most likely belong to neurons. Other nuclei are classified as being "negative". Neuronal somas are surrounded by green circles and neurites tracked in blue.

A-Neurons treated at 1 DIV and fixed at 7 DIV



B-Neurons treated at 7 DIV and fixed at 14 DIV

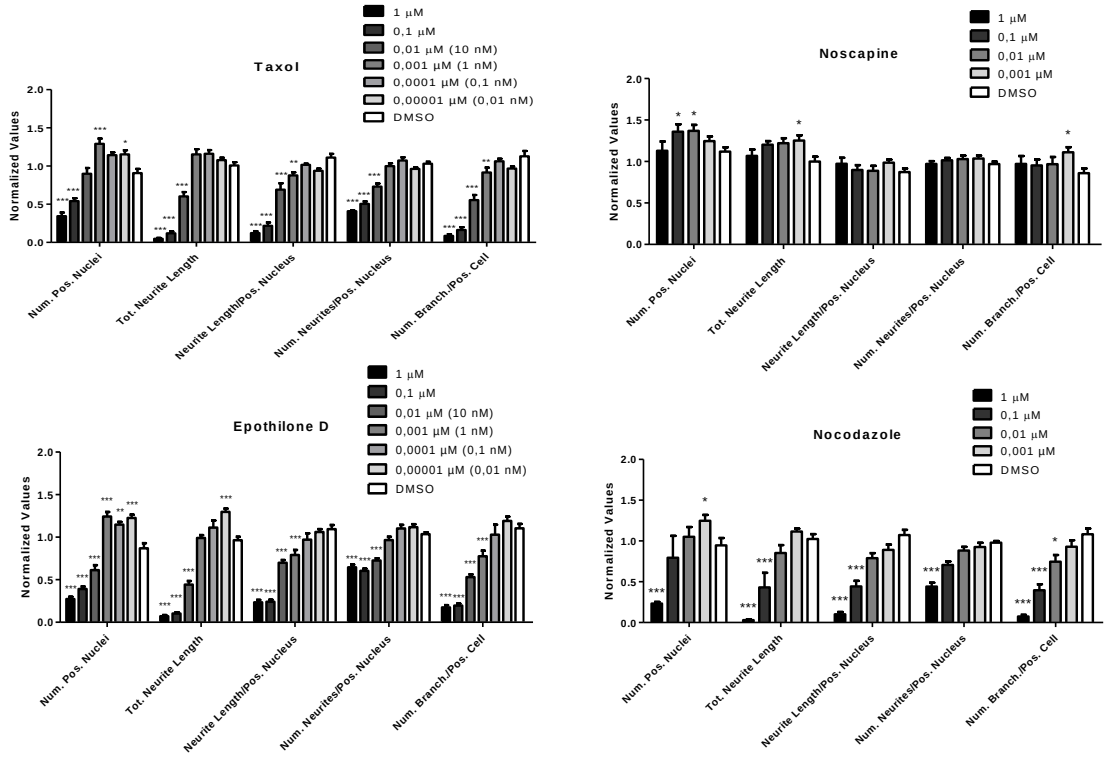


Figure 12 – For caption please refer to page 44.

Noscapine was also incapable of promoting neurite length extension or branching when administered after 7 DIV (Figure 12 B), however, it did not induce toxicity for the drug concentration range used, contrary to Taxol and Epothilone D, as already observed when treatment started at 1 DIV (Figure 12 A). Nocodazole decreased neurite length/pos. nucleus, the number of neurites/pos. nucleus and the number of branches/pos. nucleus for the higher concentrations used in both treatments starting at 1 or 7 DIV during 6 or 7 days respectively (Figure 12 A and B).

Figure 12 – Quantification of neurite length, number of neurites and branching alterations induced by Taxol, Epothilone D, Noscapine and Nocodazole by a high-content screening approach – Primary hippocampal neurons were either treated after 1 DIV for 6 days (**A**) or after 7 DIV for 7 days (**B**). Several images were acquired with a 20x objective in an automated microscope and the effect induced by these drugs on neuronal morphology alterations was analyzed by the software NEO as previously mentioned. **A, B:** graphs showing the quantification of neuronal morphology alterations induced by the above mentioned drugs, normalized to the respective controls (mean ± SEM) for the respective treatment time points. Taxol, Epothilone D and Noscapine induced a moderate increase in the neurite length/pos. nucleus and in the number of branches/pos. nucleus when neurons were treated after 1 DIV for 6 days, although Noscapine was used in higher concentrations. This effect is lost for neurons treated at 7 DIV for 7 days. Nocodazole was able to decrease the neurite length/pos. nucleus, the number of neurites/pos. nucleus and the number of branches/pos. nucleus in both treatment time points. Statistical analysis by Two-way ANOVA + Bonferroni post-tests, n=3.

3.4.2 MTA effect on dendrites length, number and ramification points

Here we used the same approach as in the previous experiment except with one alteration: MAP2, a specific dendritic marker, was used instead of β-III Tubulin so one could track dendrites instead of all neurites (for examples see Figure 13 Ai and Bi). This way, morphological parameters analyzed concern dendrites only (Figure 13 Aii, iii and Bii, iii). Maximum concentration used for Taxol and Epothilone D was 10 nM due to observed toxicity in the previous experiment for higher concentrations. Noscapine and Nocodazole were used with the same concentrations as previously.

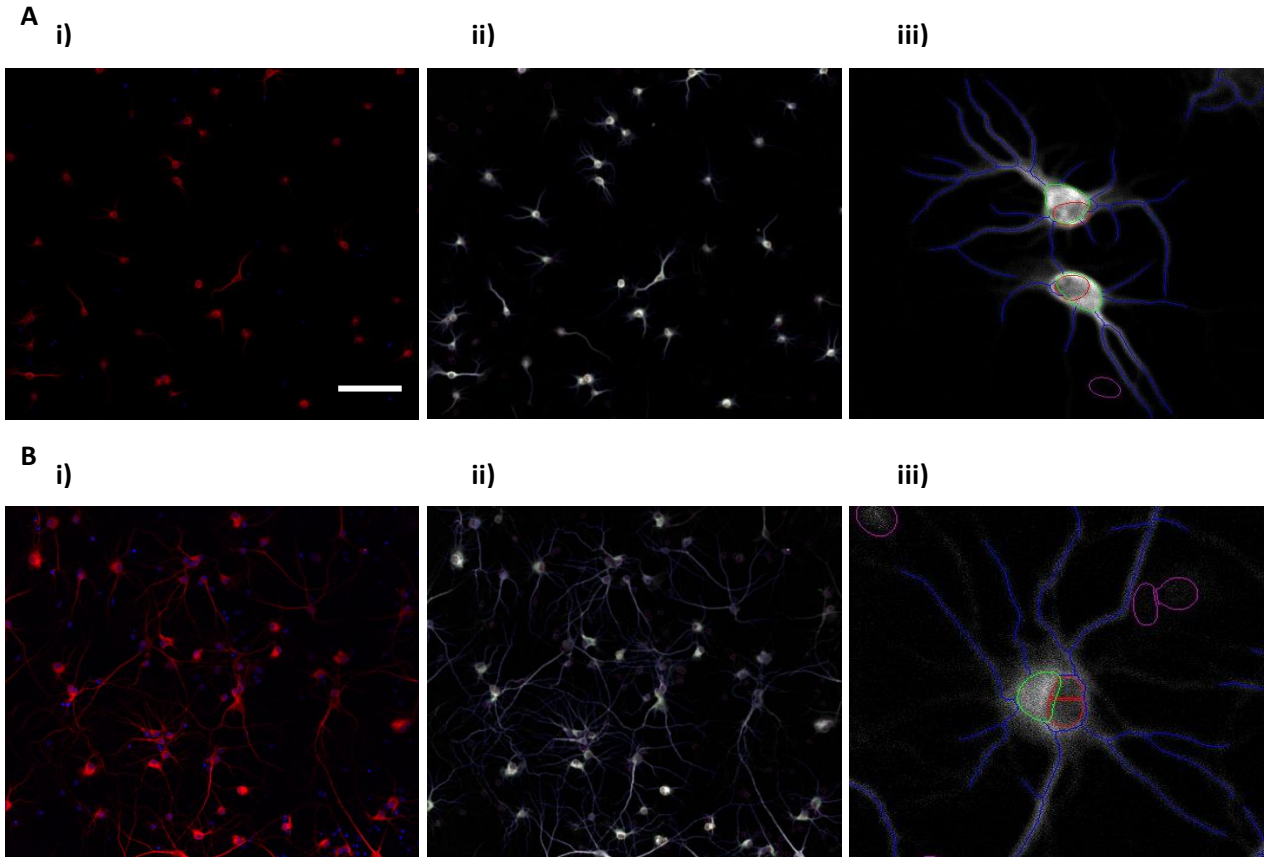


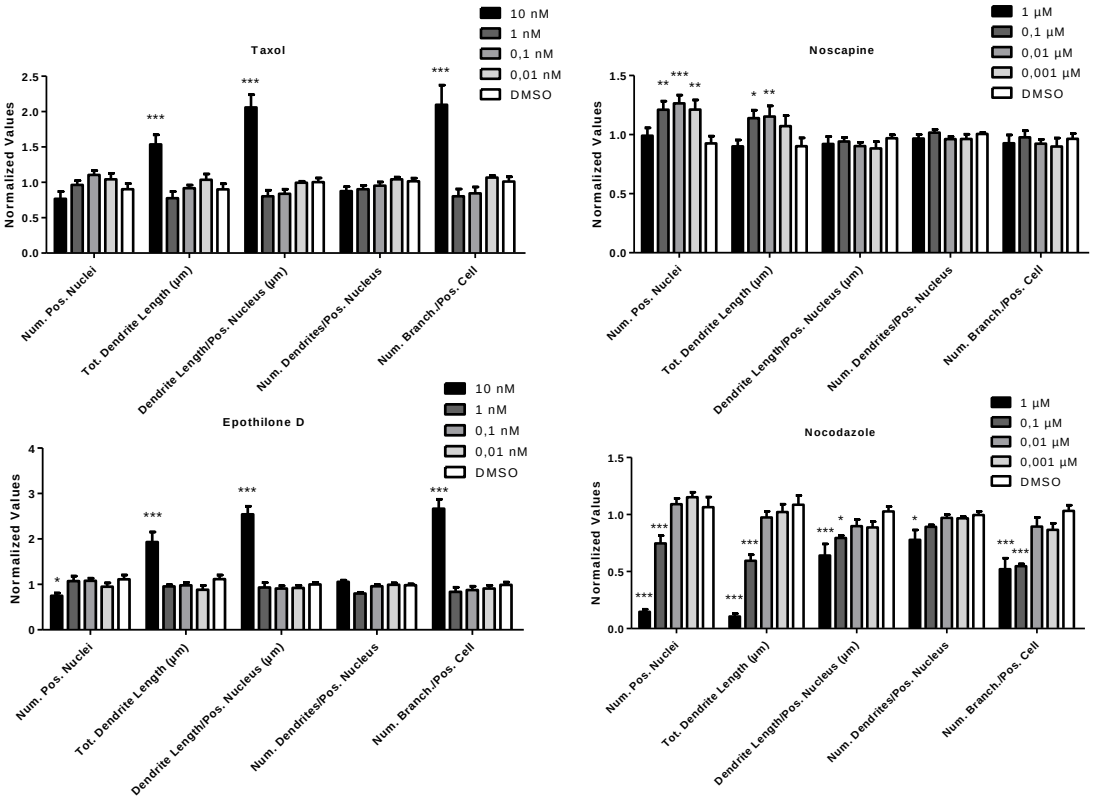
Figure 13 – Example of measurement of dendrite length, number of dendrites and branching by a high-content screening approach – A, B, Representative images of non-treated rat hippocampal neurons (control condition) fixed after 7 DIV (**A**) or after 14 DIV (**B**). **Ai** and **Bi**: neurons labeled for MAP2 (red) and DAPI (blue). Images taken with a 20x objective, each image represents a tile. **Aii** and **Bii**: a set of four tiles for the same condition are acquired by the microscope and then analyzed by the software NEO, DCI Labs. **Aiii** and **Biii**: expanded images of **Aii** or **Bii**, respectively. The software tracks the nuclei and separates "negative" nuclei (pink circles) from "positive" nuclei (red circles). Nuclei (stained with DAPI) overlapping with cell bodies connected with dendrites (stained with anti-MAP2) are classified as being "positive" nuclei and most likely belong to neurons. Other nuclei are classified as being "negative". Neuronal somas are surrounded by green circles and dendrites tracked in blue. Scale bar, 400 µm.

Neurons treated at 1 DIV with Taxol, Epothilone D and Noscapine and fixed at 7 DIV did not show any alterations induced by these drugs regarding dendrite length/pos. nucleus, the number of dendrites/pos. nucleus or the number of branches/pos. nucleus (Figure 14 A). Strangely, Taxol and Epothilone D at 10 nM (maximum concentration used for these drugs) abruptly increased both dendrite length/pos. nucleus and the number of branches/pos. nucleus to over two times the control.

Neurons treated at 7 DIV and fixed at 14 DIV showed a moderate increase in the dendrite length/pos. nucleus and in the number of branches/pos. nucleus when treated with low concentrations of either Taxol or Epothilone D (Figure 14 B). Noscapine was able to promote an increase in dendrite length/pos. nucleus, in the number of branches/pos. nucleus but also in the number of dendrites/pos. nuclei, although higher concentrations of this drug were used compared to Taxol and Epothilone D as already mentioned (Figure 14 B).

Finally, Nocodazole as expected induced a decrease in dendrite length/pos. nucleus, in the number of dendrites/pos. nucleus and in the number of branches/pos. nucleus, both when treatment started after 1 DIV or 7 DIV, for 6 or 7 days respectively (Figure 14 A and B).

A-Neurons treated at 1 DIV and fixed at 7 DIV



B-Neurons treated at 7 DIV and fixed at 14 DIV

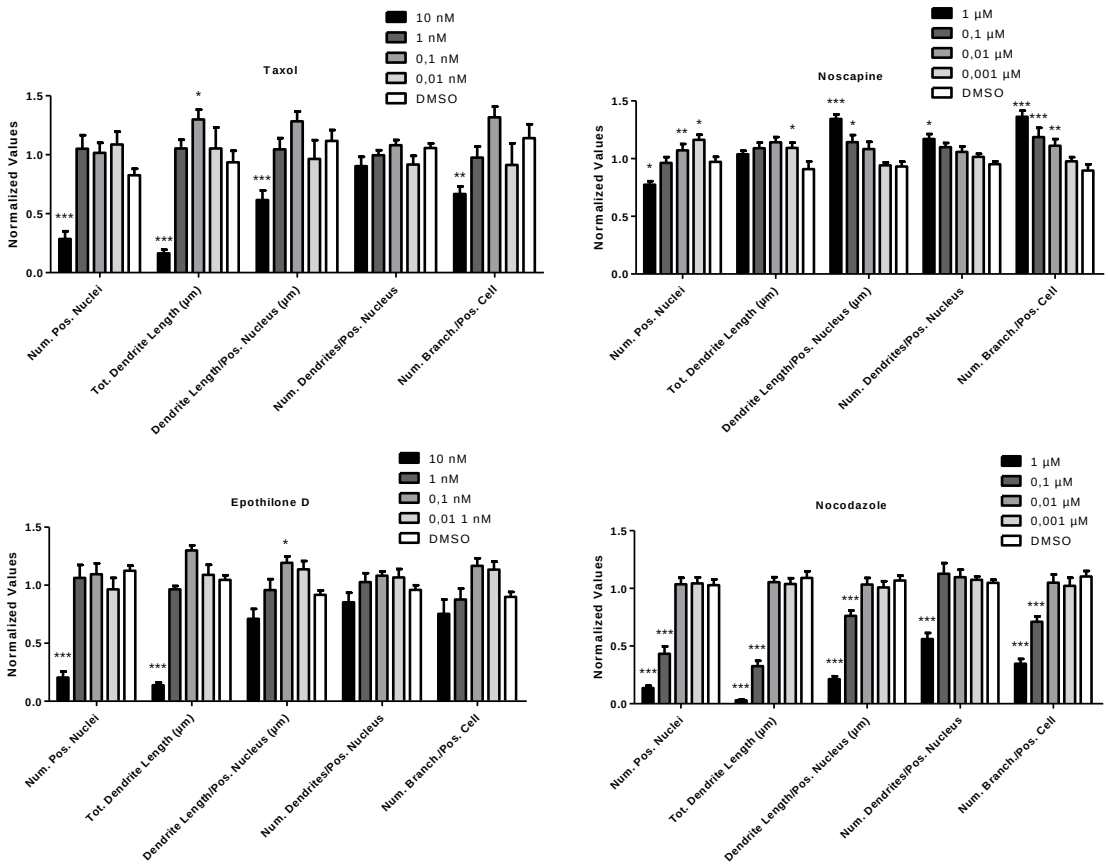


Figure 14 – For caption please refer to page 48.

3.5 Characterization of microtubule PTMs in an AD *in vitro* model

Here, we made use of In-Cell ELISA to quantitatively analyze microtubule PTMs in a tau-aggregation AD *in vitro* model aiming to understand if microtubule stability is compromised. Primary hippocampal neurons were virally transduced at 3 DIV with GFP (control) or human mutated P301L tau. At 7 DIV, pre-formed fibrils of K18P301L (truncated form of human tau containing only the 4 microtubule-binding domains, K18, with a P301L point mutation, K18P301L, prone to aggregation) were added to part of hP301L tau transduced neurons, while others remained without pre-formed tau fibrils. By 14 DIV all neurons were fixed and analyzed (AD *in vitro* model established in primary hippocampal neurons according to Calafate, S., "Tauopathy seeding models as a platform for tau aggregation and clearance study", Master Thesis, University of Coimbra, 2012). The addition of pre-formed tau fibrils possibly attracts free non-microtubule bound tau further promoting aggregation of hP301L tau and, by this means, should leave microtubules vulnerable to depolymerization and decrease microtubule stability (not tested before), as tau is an important MAP known to stabilize microtubules. In parallel, neurons where also transduced with GFP (control) or human WT tau at 3 DIV and fixed at 14 DIV without the addition of pre-formed tau fibrils. The overexpression of hWT tau should increase microtubule stability in opposition to the combined effect of hP301L tau with pre-formed tau fibrils.

Transduction of rat primary hippocampal neurons with hWT tau or hP301L tau increased human total tau expression levels by ~4 or ~6 times, respectively, compared to controls (Figure 15 A). Unexpectedly, neurons transduced with hWT tau did not show significant changes in the relative levels of total, acetylated or tyrosinated tubulin (Figure 15 Bi) suggesting absence of microtubule overstabilization effect by hWT tau overexpression. Neurons transduced with hP301L tau without pre-formed tau fibrils added also did not show variations regarding the relative levels of total, acetylated or tyrosinated tubulin (Figure 15 Bii). However, neurons with pre-formed tau fibrils added besides hP301L tau transduction showed a slight decrease (not statistically significant) in the relative levels of total and acetylated tubulin (Figure 15 Bii), suggesting that microtubules stability could be affected.

Figure 14 – Quantification of dendrite length, number of dendrites and branching alterations induced by Taxol, Epothilone D, Noscapine and Nocodazole by a high-content screening approach – Primary hippocampal neurons were either treated after 1 DIV for 6 days (**A**) or after 7 DIV for 7 days (**B**). Several images were acquired with a 20x objective in an automated microscope and the effect induced by these drugs on neuronal morphology alterations was analyzed by the software NEO. **A,B:** graphs showing the quantification of neuronal morphology alterations induced by the above mentioned drugs, normalized to the respective controls (mean ± SEM) for the respective treatment time points. Interestingly, here, in opposition to the previous experiment, where both axons and dendrites were assessed at the same time, Taxol, Epothilone D and Noscapine induced a moderate increase in the dendrite length/pos. nucleus and in the number of branches/pos. nucleus only when neurons were treated after 7 DIV for 7 days. Nocodazole, again as expected, decreased the dendrite length/pos. nucleus, the number of dendrites/pos. nucleus and the number of branches/pos. nucleus in both treatment time points. Statistical analysis by Two-way ANOVA + Bonferroni post-tests, n=2.

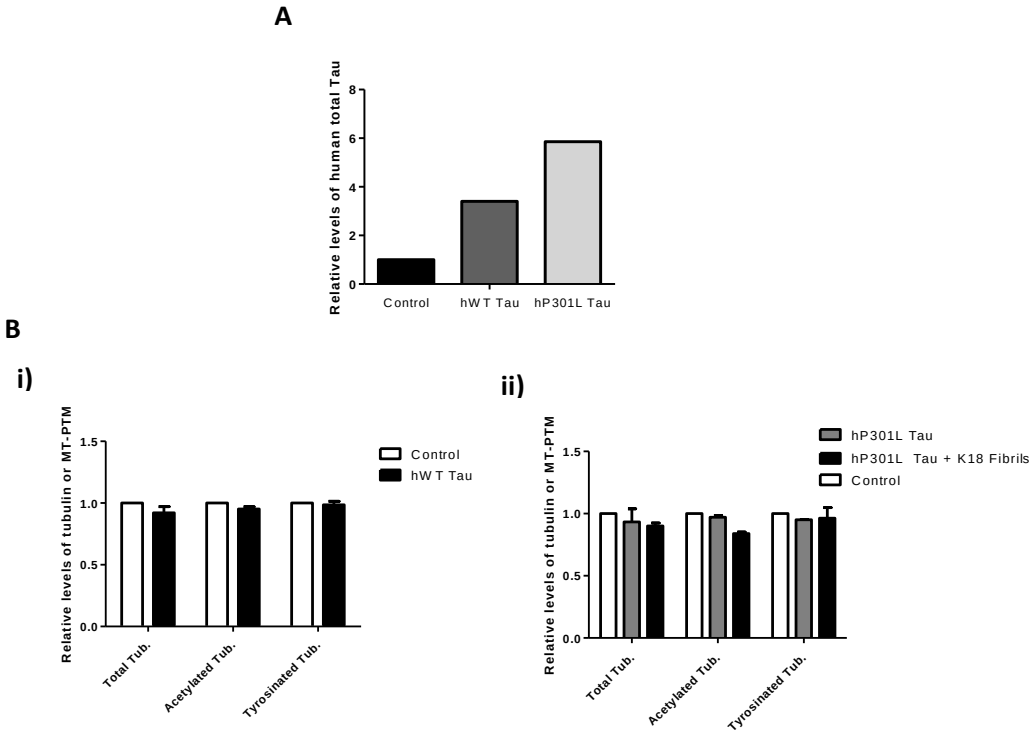


Figure 15 – Quantification of microtubule PTMs alterations in a tau-aggregation Alzheimer's disease *in vitro* model – Hippocampal neurons transduced with hP301L tau and with pre-formed tau fibrils added or not after transduction were fixed at 14 DIV. In parallel, neurons were transduced with hWT tau and fixed at 14 DIV. After fixation, the relative levels of total polymerized tubulin as well as of acetylated and tyrosinated tubulin were quantified by In-Cell ELISA as previously, in order to infer about microtubule stability. **A –** Graphs showing the quantification of the total human tau relative levels after transduction (n=1). Expression of hWT tau and hP301L tau is working as the relative levels of human total tau increase ~4 and ~6 times respectively. **B –** Quantification of microtubule PTMs (mean ± SEM) in the case of overexpression of hWT tau (n=2) (**i**) and in a tau-aggregation AD *in vitro* model (n=2) (**ii**). Neurons transduced with hWT tau did not show significant changes in the relative levels of total, acetylated or tyrosinated tubulin (absence of microtubule overstabilization), while neurons transduced with hP301L tau and pre-formed tau fibrils added showed a slight decrease in the relative levels of total and acetylated tubulin suggesting that microtubule stability decreased also. Statistical analysis by Two-way ANOVA + Bonferroni post-tests.

4. Discussion and Conclusion

4.1 Discussion

Several neurodegenerative diseases (Brunden et al., 2009, Sudo and Baas, 2011, Franker and Hoogenraad, 2013, Hinckelmann et al., 2013, Millecamps and Julien, 2013, Esteves et al., 2014, Smith et al., 2014) are known to report microtubule instability and consequent neurite degeneration. Microtubules are essential to neurons as they support intracellular transport of a vast number of different cargos to places spread all over the extensive neuronal area (Kapitein and Hoogenraad, 2011). Furthermore, it was recently discovered that microtubules have an important role regarding the development and maintenance of dendritic spines and therefore, contribute to the proper functioning of synaptic connectivity (Gu and Zheng, 2009, Jaworski et al., 2009). This reinforces the importance of microtubules in neurons and shows why these particular cells are vulnerable to microtubule instability. For that reason, it is important to study microtubule stability in neurodegenerative diseases in order to deepen our knowledge on possible disease-inducing mechanisms, always having in mind new valuable therapeutic targets. Particularly in AD and other tauopathies, Tau, a MAP, is compromised and is not able to fulfill one of its main functions: to organize and stabilize microtubules (Brunden et al., 2009). Therefore, the use of MTA to compensate for tau loss-of-function has been a hot topic among AD therapeutic strategies. Additionally, microtubule PTMs are known to generally occur in tubulin polymerized long enough to accumulate PTMs. Therefore, modified microtubules are associated with long-lived microtubules, generally considered stable (Hammond et al., 2008, Baas and Ahmad, 2013). For that reason, one way of studying microtubule stability is by looking at their PTMs.

First, we showed the presence of acetylated tubulin, associated with stable microtubules, in axons but also in dendritic shafts, except for their most distal parts. Interestingly, and by opposition, tyrosinated tubulin, freshly polymerized tubulin associated with dynamic microtubules, was shown to be present in growth cones but also in the extremities of dendrites.

Additionally, here we showed that Taxol, Epothilone D and Noscapine, all MTA, have the ability to increase the relative levels of polymerized tubulin as well as the relative levels of the acetylated tubulin. In addition, these drugs induced changes in neuronal morphology by boosting neurite extension and ramification during initial stages of neuronal development. Furthermore a tau-aggregation AD *in vitro* model showed a slight decrease in the total amount of polymerized tubulin but also in the acetylated tubulin. This means that microtubule stability was compromised and that, probably, Taxol, Epothilone D or Noscapine could revert this effect.

4.1.1 Acetylated tubulin localizes to axons and dendritic shafts in opposition to tyrosinated tubulin, mainly present in growth cones and dendritic tips

Microtubule PTMs are not equally distributed along the neuronal microtubule network but instead show a specific pattern where microtubules from different subcellular compartments have different PTMs types and levels (Verhey and Gaertig, 2007, Janke and Kneussel, 2010). This pattern is thought to have an important role regarding the sorting of cargos from the soma to distant places in the neuron (Janke and Kneussel 2010). The presence of stable microtubules in axons and dendritic shafts as in opposition to the

presence of dynamic microtubules in growth cones and distal parts of the dendrites (Kollins et al., 2009) is in agreement with the function microtubules support in these subcellular compartments. It makes sense that in axons and dendritic shafts microtubules are stable to preserve the transport tracks that intracellular transport rely on, sometimes to deliver cargos to distant sites in the neuron (Kapitein and Hoogenraad, 2011). Whereas dynamic microtubules, with high growing/shrinking rates, are able to support axon guidance in growth cones but also deliver the plasticity needed by dendritic spines regarding their morphology when trying to find pre-synaptic terminals in order to establish and maintain synaptic connections (Tanaka et al., 1995, Jaworski et al., 2009, Kapitein et al., 2010).

4.1.2 Changes in microtubule PTMs induced by Taxol, Epothilone D and Noscapine during initial stages of neuronal development suggest a microtubule-stabilizing effect

MTA bind to the microtubule lattice consequently changing its conformation. This interaction is capable of facilitating polymerization, depolymerization or even stabilization of the microtubule polymer length within a certain range, depending on the agent. Furthermore it is known that microtubule PTMs concentrate in long-lived microtubules, probably because they are more time exposed to the enzymes responsible for PTMs, comparing to recently polymerized microtubules (Hammond et al., 2010). So, one way of looking into the overall microtubule network stability in a cell is by quantifying its microtubule PTMs.

Only neurons treated at 1 DIV by MSA were susceptible to alterations to the total polymerized tubulin or PTMs. Probably, because at this age neurons are in the beginning of the differentiation process, are more "plastic" and so more prone to drug-induced alterations in microtubule PTMs or morphology. As we confirmed, Taxol, Epothilone D and Noscapine promote microtubule stabilization reported by the increase in the relative levels of acetylated tubulin. However, they do it by different mechanisms. It is known that Taxol and Epothilone D bind tubulin monomers and are able to promote microtubule polymerization from already existent polymers or even the nucleation of new microtubules, here confirmed by the increase in the relative levels of the total polymerized tubulin. Conversely, Noscapine binds already polymerized tubulin, stabilizes microtubule length within a certain range without facilitating polymerization or depolymerization. Accordingly, it was unable to promote tubulin polymerization, confirmed by the absence of an increase in the relative levels of total polymerized tubulin. However, there was a moderate increase induced by Noscapine in the relative levels of acetylated tubulin, probably due to the prolonged exposure of these microtubules to the modifying enzyme responsible for tubulin acetylation, as Noscapine stabilized microtubule length. Furthermore, the difference between these drugs is further accentuated regarding the effect on the relative levels of tyrosinated tubulin (recently polymerized tubulin): Taxol and Epothilone D decrease them, while Noscapine does not change these levels. This suggests that Taxol and Epothilone D stabilize microtubules at the expense of a negative effect on dynamic microtubules for high concentrations (overstabilization), while Noscapine just keeps the microtubules in a steady-state (does not promote polymerization nor depolymerization) and therefore dynamic instability of microtubules is conserved. This overall difference in microtubule-related effects induced by Taxol and Epothilone D in comparison to Noscapine could be useful for future drug screening purposes regarding MTA: an increase in the total and acetylated tubulin in a dose-response manner while at the same time decreasing the tyrosinated tubulin could identify drugs with a Taxol-like mechanism.

Differently, an increase in the acetylated tubulin in a non dose-response manner without increasing total tubulin nor decreasing tyrosinated tubulin could help identify drugs with a Noscapine-like mechanism. The separation of Taxol-like drugs and Noscapine-like drugs could be important in the selection of MTA because Noscapine did not show toxic effect for high concentrations used comparing to Taxol and Epothilone D, probably because tubulin polymerization is not exacerbated and dynamic microtubules are conserved with Noscapine.

Interestingly, the effect of Taxol and Epothilone D in the relative levels of total, acetylated and tyrosinated tubulin showed a dose-dependent response, while Noscapine effect reached a "plateau" regarding the moderate increase in the relative levels of acetylated tubulin. It makes sense that, as Taxol and Epothilone D bind tubulin monomers and promote polymerization, increasing molecules of these drugs induce a proportional increase in the polymerized tubulin (and consequently in the acetylated tubulin as more tubulin is prone to be acetylated), as long as free tubulin is available. Noscapine binds the already polymerized tubulin in a different tubulin site, favoring the dynamic steady-state of microtubules and does not promote polymerization (Landen et al., 2002, Landen et al., 2004). Therefore, it makes sense that increasing concentrations of Noscapine do not proportionally increase the relative levels of acetylated tubulin once microtubule length remains more or less the same.

In neurons treated only at 7 DIV, Taxol, Epothilone D and Noscapine could not show any significant effect. In order to make sure it was not a technical problem as the signal could be saturated (no difference between values for different drug concentrations) due to the use of incorrect antibody dilutions, we checked the effect of Taxol regarding total, acetylated and tyrosinated tubulin for treatments starting at 1, 3 or 7 DIV with higher dilutions of the respective antibodies. We observed that the effect induced by Taxol at 1 DIV, decreases at 3 DIV and completely fades away at 7 DIV (data not shown). Therefore, the reason why Taxol, Epothilone D and Noscapine are not capable of inducing their effect after 7 DIV is probably biological and not due to experiment-related technical issues. It is possible that at that age neurons cannot be easily forced to change their microtubule network as they are already mature and less "plastic". This is in accordance with the fact that at 7 DIV only Nocodazole (microtubule-depolymerizing drug) is capable of showing its negative effects, decreasing the total, acetylated, and tyrosinated tubulin.

Finally, no drug used here was capable of inducing an effect in the relative levels of detyrosinated tubulin, not even Nocodazole, confirming the existence of a population of microtubules resistant to Nocodazole (Conde and Caceres, 2009). In agreement, detyrosinated tubulin is known to be found in the stable microtubules (Hammond et al., 2008, Hammond et al., 2010). However, if Taxol, Epothilone D and Noscapine were proved to enhance microtubule stability, for example increasing acetylated tubulin, why cannot they increase the levels of detyrosinated tubulin? It is possible that this PTM occurs only in a defined space along microtubules, and the increase in length and stability of microtubules by these drugs does not influence the intensity of its occurrence as it did with acetylated tubulin (Hammond et al., 2010). Moreover, this PTM probably localizes to microtubule positions far away from the growing tip, once Nocodazole, although capable of decreasing the total amount of polymerized tubulin, did not decrease the relative levels of detyrosinated tubulin, demonstrating as a result that this microtubule PTM is resistant to Nocodazole action.

4.1.3 Taxol, Epothilone D and Noscapine induce morphological changes in initial stages of neuronal development

Taxol and Epothilone D were able to increase the relative levels of polymerized tubulin as well as the relative levels of the acetylated tubulin, associated with stable microtubules. Noscapine could also increase the relative levels of acetylated tubulin although not so robustly as the previous drugs. Here, we tried to understand if these drugs were capable of inducing neurite outgrowth, as wells as formation of new neurites and branching of existing ones by a high-content screening approach, once they were able to induce microtubule polymerization and or stabilization, important processes for neurons to develop (Sakakibara et al., 2013).

We observed that the MTA induced a moderate increase in neurite length/pos. nucleus and in the number of branches/pos. nucleus in neurons treated at 1 DIV but not the ones treated at 7 DIV. These results are consistent with the In-Cell ELISA quantifications of microtubule PTM where treatments after 7 DIV with the same drugs were unable to increase the relative levels of the total, acetylated and tyrosinated tubulin. Accordingly, it seems like neuronal microtubules can be forced to polymerize, and neurites induced to extend and branch at 1 DIV, but not at 7 DIV, probably because, as already discussed, at this stage of development neurons start to differentiate and be less "plastic", consolidating their morphology and so do not respond to drug treatment, where only toxic concentrations promote the already stated negative effects (Chuckowree and Vickers, 2003). Also, inhibitory contacts between neurons as the neuronal network becomes bigger could hinder further neurite extension. Differently, microtubules can serve as a substrate for Nocodazole at both 1 and 7 DIV as expected, since depolymerization is possible as long as microtubules are present, and consequently neurites retract due to microtubule instability. Importantly, Taxol and Epothilone D exhibited a similar profile regarding their effect on the morphological parameters studied, as already did for the effects in microtubule PTM.

Until this point, we sought to find out the influence of Taxol, Epothilone D and Noscapine on neurite outgrowth and branching. We used β-III Tubulin to label microtubules all over the neuron with the purpose of tracking neurites, including both dendrites and axons. This approach (using β-III Tubulin as a marker) is useful to get general morphological information of the neuron. However, information regarding specific neuronal subcompartments such as dendrites and axons is not given separately. These two subcompartments are morphologically different, where axons normally extend for bigger distances and only one axon exists per neuron, while on the other hand several dendrites are normally present per neuron and they are more branched than the axon. Besides, microtubule polarity is different between these two compartments: in axons the majority of microtubules have the growing plus-end oriented towards the growth cone while in dendrites microtubules present mixed polarity (Baas and Lin, 2011). With that in mind we tried to understand if dendrites alone could be affected by the same drugs using MAP2 as a marker of neurites instead of β-III Tubulin, as this MAP is mainly present in dendrites.

Intriguingly, the results of this experiment were the reverse of the previous experiment: here drugs were able to induce an effect after 7 DIV but not after 1 DIV. This could mean that only after 7 DIV dendrites are prone to morphological changes facilitated by these drugs, and probably this effect was diluted by the absence of effect in axons after 7 DIV in the first experiment, using β-III Tubulin (axons cover bigger distances than dendrites representing a higher percentage of the total neurite length). In conclusion, considering the fact that in the experiment with β-III Tubulin these drugs could only show their effect when treatment started after 1 DIV, it could be that axons are more prone to morphological changes

after 1 DIV during initial stages of development and dendrites after 7 DIV, when neurons are already in mid stages of development.

4.1.4 Tau-aggregation AD *in vitro* model shows a moderate decrease in microtubule stability

On one hand, we expected the overexpression of hWT tau in neurons to induce microtubule stabilization reported by an increase in the total and acetylated tubulin relative levels. On the other hand, we expected the opposite effect to be observed when hP301L tau is overexpressed and pre-formed tau fibrils added once hP301L tau is mutated in the microtubule-binding domain, which reduces tau affinity for microtubules (Guo and Lee, 2011), thereby probably destabilizing microtubules. In addition, pre-formed tau fibrils are able to attract both exogenous and endogenous tau further promoting tau loss-of-function (Guo and Lee, 2011, 2013).

Although the overexpression of hWT tau resulted in an increment of the exogenous protein by ~4 times, microtubule stability remained unchanged, suggested by the lack of effect on the total, acetylated or tyrosinated tubulin. Interestingly, as transduction is done at 3 DIV, and exogenous protein peak expression in the neuron occurs only at 7 DIV, it could be that, as suggested before for neurons treated at 7 DIV with Taxol and Epothilone D, by this age neurons cannot be forced to further stabilize their microtubule network or extend their axons beyond a limit. In opposition, it should be possible to destabilize microtubules and induce neurite retraction. However, neurons transduced with hP301L tau alone did not report any alterations to microtubule stability neither. In this case, it could be that endogenous functional rat tau is able to compensate for dysfunctional hP301L tau, maintaining microtubule integrity. Conversely, the neuronal microtubule network could be destabilized when the combined effect of hP301L tau transduction and pre-formed tau fibrils addition was applied to neurons and as a decreasing trend in the relative levels of total polymerized tubulin and acetylated tubulin was observed. It is possible that added pre-formed tau fibrils attracted not only hWT tau but also normal endogenous tau, thereby affecting microtubule stability. In another scenario, the presence of pre-formed tau fibrils could simply provoke physical disturbance, or promote a neuronal stress response, thereby affecting the normal structure and function of microtubules.

4.2 Conclusion

Overall, the results shown here encourage the use of MTA to tackle microtubule-related deficiencies in neurodegenerative diseases. Taxol, Epothilone D, and Noscapine, known MTA, increased the levels of polymerized tubulin as well as acetylated tubulin in primary hippocampal neurons. Moreover, these drugs could also induce changes in neuronal morphology increasing neurite length and branching. Importantly, they did so by different mechanisms where Noscapine did not show toxicity for the concentrations used unlike Taxol or Epothilone D, and thus, Noscapine-like drugs (MMA) should be highly considered regarding microtubule-stabilization focused therapies in neurodegenerative disease. Additionally, the tau-aggregation AD *in vitro* model used here displayed modest microtubule instability by showing a slight decrease in the levels of total and acetylated tubulin. An interesting next step would be to analyze

neuronal morphology, as we did here, but instead of normal neurons, using this model. Moreover, it is known that dendritic spine numbers decrease in AD (Penzes et al., 2011) and by direct consequence synaptic function is compromised too. So, it would also be interesting to quantify the number of synapses and respective morphology in the same model. Actually, we started to optimize a protocol to automatically quantify synapses in normal neurons, using synapsin as a synaptic marker, with same software used here to analyze neuronal morphology. However, still many rearrangements are needed to improve the quality of the analysis. Finally, the ultimate goal is to use MTA in diseased neurons and try to understand if they can revert negative effects like microtubule instability, neurite degeneration and synaptic loss. However, other AD *in vitro* models presenting a more pronounced damage to microtubules, and reported damage to synapses, could be used in the future in order to validate the effect induced by these or other MTA (Wagner et al., 1996, Qiang et al., 2006, Stoppelkamp et al., 2011, Zempel and Mandelkow, 2012).

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