UNIVERSITÀ DEGLI STUDI DI MILANO

PhD Course in Molecular and Cellular Biology

XXXV Cycle

INVESTIGATION OF α-SYNUCLEIN/TUBULIN INTERACTION IN PRESYNAPTIC MICROTUBULE DYNAMICS IN DOPAMINERGIC NEURONS

Alessandro Comincini

PhD Thesis

Scientific tutor: Prof. Graziella Cappelletti

Academic year: 2021-2022

SSD: BIO/11

Thesis performed at the Department of Biosciences, Università degli Studi di Milano and at the Department of Pathology and Cell Biology, Columbia University.

“An unexamined life is not worth living”

Socrates

Table of Contents

PART II

Published paper 1 (Amadeo et al., *Int. J. Mol. Sci.*, 2021)

"The association between α-synuclein and α-tubulin in brain synapses"

Published review 1 (Basellini, Kothuis, Comincini et al., *Front. Biosci (Landmark Ed).*, 2023)

"Pathological pathways and α-synuclein in Parkinson's disease: a view from the periphery"

PART III

SIDE RESEARCHS:

Published paper 2 (Mazzetti et al., *CNS Neuroscience & Therapeutics*, 2022)

"A protective astrocyte subtype expresses Vitamin D-activating enzyme in Parkinson's disease"

Abstract

The pathological mechanism underlying the degeneration and loss of the dopaminergic (DA) neurons of the *substantia nigra pars compacta* (SNpc) in Parkinson's disease (PD) are still unknown. Among them, α-synuclein aggregation, mitochondrial impairment, increase oxidative stress and synaptic dysfunction are often indicated as culprits, although it is unclear whether these events are primary or secondary insults in PD neurodegeneration. Unlike other neurodegenerative diseases where cytoskeletal alterations are linked with mutations in key proteins involved in the progression of the pathology, PD lacks these findings. Nonetheless, microtubule (MT) dysfunction has recently emerged as a putative cause of the selective damage of the striatal DA synapses and consequent reduction in dopamine release that characterize PD clinical symptoms.

It was only recently when dynamic MTs were shown to enter the pre- and post-synapse as regulatory elements required for proper neurotransmitter release and synaptic plasticity. In pyramidal neurons, excitatory presynaptic sites are hotspots for MT nucleation, a process that regulates neurotransmission by providing the tracks for targeted bidirectional delivery of a rate-limiting supply of synaptic vesicles (SVs) to sites of stimulated release. Notably, α-synuclein, a small synaptic protein that aggregates in PD becoming the main component of Lewy bodies (LBs) and Lewy neurites (LNs), was recently discovered to promote MT assembly *in vitro*. α-synuclein-mutated PD forms lose this ability, suggesting a possible new connection between synaptic dysfunction and α-synuclein pathology through a tubulin-mediated mechanism.

Here, I investigated whether the α-synuclein/tubulin interaction is a feature of brain synapses and whether α-synuclein is a regulator of MT dynamics in primary DA neurons. I also reported a novel role for α-synuclein in activity-evoked presynaptic MT nucleation at *en passant* boutons, suggesting that α-synuclein may sustain synaptic transmission also by regulating MT nucleation at sites of release.

Overall, these data point to α-synuclein as a novel regulator of MT dynamics in primary neurons and reinforce the idea that MT dysfunction may represent an early insult in the sequence of events leading to DA synapse impairment.

PART I

1. Introduction

1.1 Neuronal microtubules: structure and function

The ability of eukaryotic cells to acquire a plethora of different morphologies and to undertake coordinated and oriented movements relies on a complicated network of protein filaments, known as the cytoskeleton, that spans throughout the cytoplasm. This system is finely designed to help the cell to resiliently adapt to changes in surroundings or signalling in order to maintain cellular homeostasis. To achieve this purpose, the cytoskeleton, rather than being a fixed scaffold, is required to be dynamic and able to constantly reorganize according to responses to the environment that the cell has to undertake such as, for example, differentiation or cellular division. The cytoskeleton is mainly composed of three different types of protein filaments: microfilaments, intermediate filaments and MTs. Each of them is composed of a different protein subunit: actin for microfilaments, a family of related fibrous proteins for intermediate filaments, and dimers of α- and β-tubulin for MTs.

Microfilaments are double-stranded helical polymers composed of actin and they have an average diameter of 7 ± 2 nm. They are polarized and dynamic structures that normally exist in bundles and their major role consists in providing an actin rich layer below the plasma membrane in order to control shape and surface movements of eukaryotic cells. In neurons, the most abundant isoform is β-actin, which plays critical roles in the modulation of the growth cone and is required for additional pivotal mechanisms such as controlling dendritic spine plasticity, signalling and synaptic homeostasis [1–3]. Actin filaments interact with a variety of actin-binding proteins as well as communicating with MTs by interacting with MT-associated proteins (MAPs) (Fig. 1.1).

Intermediate filaments are ubiquitous rope-like stress-resistant components of the cytoskeleton and are composed of two-stranded coiled coils of fibrous polypeptides with an extended α-helical conformation and a diameter ranging from 8 to 10 nm [4] (Fig.1.1). A wide array of filament proteins, which form a large, heterogeneous family, are currently known and all share a structural and/or stretch-resistant function within the cell. Examples of these proteins are vimentin, keratins, desmin, neurofilaments (NFs), and glial fibrillary acidic protein. Neurons contain a specific subset of intermediate filaments, differently expressed throughout the central (CNS) and peripheric nervous system (PNS) at different stages of neuronal development. Among them, the most abundant and well-known are the NFs, which are the major component of large, myelinated axons, and are responsible of determining the axonal caliber and providing mechanical stability. In mammals, three NF proteins

were identified: NF-L, NF-M, and NF-H, for low (70 kDa), middle (160 kDa), and high (200 kDa) molecular weight, respectively. All these three structural subunits are usually present in NFs. NFs in axons are linked side by side by their carboxyl-terminal tail domains to provide a continuous rope of filaments that can be a meter or more in length [5,6].

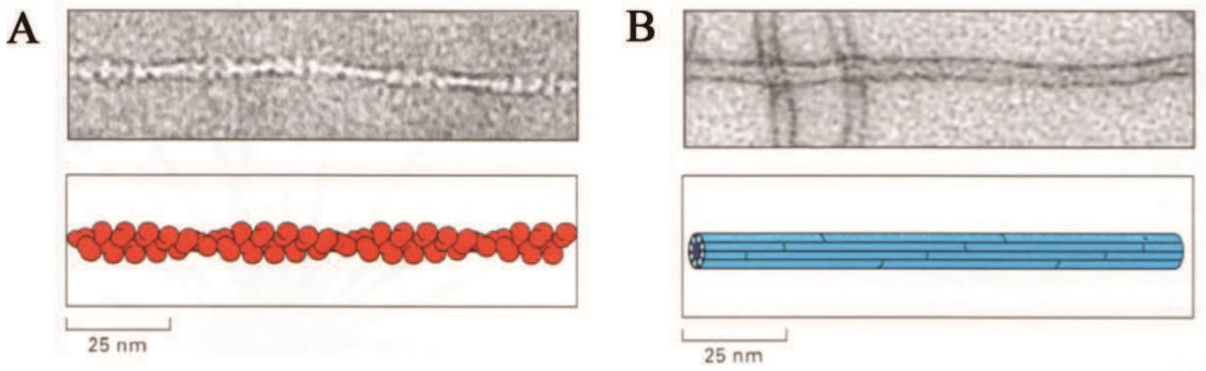

Figure 1.1. Structure of microfilaments and intermediate filaments. Ultrastructure of (A) actin (or microfilaments) and (B) intermediate filaments by electron microscopy. (modified from Alberts et al., 2008)

MTs are non-covalent cytoskeletal polymers that are involved in intracellular transport, mitosis, cell motility, and maintenance of cell polarization and shape. They are polarized structures composed of α- and β-tubulin heterodimers assembled in a head-to-tail fashion. The sequence and structure of tubulins have been highly conserved throughout the evolution, with molecular weight of 50 kDa for each monomer that is linked to one another in a non-covalent manner. Together, α/β heterodimers form linear protofilaments, and 10-15 protofilaments (usually 13 in mammalian cells [7]) associate in parallel to form the MT wall, creating a 25 nm wide hollow cylinder. The resulting polymer has a defined polarity, with two ends that are structurally and functionally distinct. In each protofilament, the α/β heterodimers are oriented with their β-tubulin monomer pointing towards the faster-growing end (plus end) and their α-tubulin monomer exposed at the slower-growing end (minus end). The lateral interaction between subunits of adjacent protofilaments has been described for 13 protofilaments MTs as a perfectly straight B-type lattice with a seam, whereas MTs with an even number of protofilaments have a A-type lattice without a seam and are truly helical with a very long pitch. In the cell, the minus end is often anchored at MT organizing centers (MTOCs), whereas the plus end is free in the cytoplasm or attached to a specific target such as the kinetochore [8].

The assembly of the α/β tubulin heterodimers into MTs, similarly to other cytoskeletal filaments, shows a particular time course. MT polymerization and depolymerization are complex and specific mechanisms with important biological roles. The kinetic of pure tubulin polymerization into MTs

was followed *in vitro* at 37°C in presence of Mg^{2+} and GTP by light scattering. The resulting sigmoidal curve highlights three main phases: a first initial lag phase (nucleation) that represents the time required for the MT to nucleate, second phase (elongation) where the newly formed MT rapidly grows, and a third phase (steady state) where a plateau level of polymerization is reached (Fig 1.2). Specifically, the lag phase represents the time required for single heterodimers to overcome the

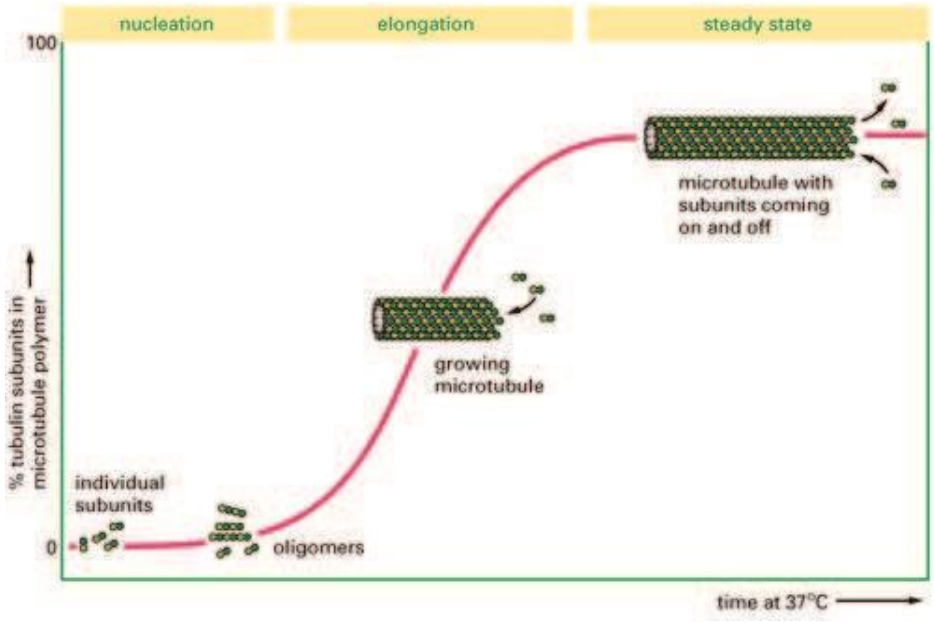

Figure 1.2. Tubulin polymerization curve *in vitro*. Tubulin polymerization starts with a lag phase, when individual tubulin dimers nucleate into oligomers and small protofilaments. This is followed by a rapid growth (elongation) that stops, reaching a plateau that represents the condition in which most of the free tubulin is polymerized into MTs and polymerization and depolymerization rate are equal (modified from Alberts et al., 2008).

kinetic hedge and slowly associate in metastable aggregates that act as nucleation spots. After this event follows the elongation phase, where GTP-tubulin subunits are rapidly added at the plus end of the newly formed MTs until the curve gets to its plateau. Since tubulin polymerization is proportional to the concentration of free tubulin, when the steady state is reached and almost all the free tubulin in the system is polymerized into MTs, polymerization and depolymerization rate are comparable. In the plateau phase, the concentration of free tubulin is below the critical concentration for tubulin polymerization, so that for each tubulin subunit added to the plus end another compensates depolymerizing from a shrinking MT (Fig. 1.2).

In mammalian cells, another isoform of tubulin, γ-tubulin, provides the template for MT assembly at the minus end, considerably reducing the time required for nucleation. γ-Tubulin is one of the main components of MTOCs, the cytoplasmic structures from where MTs emerge. MTOCs play an important role in the generation of the mitotic spindle at the centrosome and in the formation of basal bodies associated with cilia and flagella. Furthermore, post-mitotic neurons, along with other cells, are able to nucleate MTs from non-centrosomal MTOCs. In detail, neuronal MTs nucleate from the γ-tubulin template, that is a key component of a protein complex called γ-TURC. These non-centrosomal spots for MT nucleation are responsible for controlling axonal and dendritic polarity, cargos and organelles delivery along the neuron and neurotransmission and they are often localized at Golgi outpost at branching points, in dendrites, and at the synapse, in the axon [9–11]. A peculiar feature of MTs is their ability to undergo repeated cycles of assembly-disassembly, known as dynamic instability [12]. Their capacity to rapidly grow and disassemble allows the cytoskeleton to adapt to specific cell necessities, rearranging its structure in order to meet changes in the surrounding environment [13]. Dynamic MTs are individually short-lived so arrays of MTs are continuously in the process of recreation. In a population of MTs, growing, shrinking, and paused MTs always coexist at the same time and, in addition, the same MT can go through different states multiple times during its life [8,14]. The switch from growth to shrinkage is called catastrophe, whereas the opposite, namely from shrinking to growth is called rescue (Fig. 1.3). MT dynamic instability refers to the equilibrium that exists between polymerization and depolymerization of tubulin during the cycles of MT assembly-disassembly. Both α-tubulin and β-tubulin bind to GTP, but only β-tubulin has a nucleotide exchangeable site (E-site). When GTP is hydrolized into GDP, the MT becomes unstable and more prone to depolymerization. Lateral interactions between GTP-tubulin provide the force that hold together the natural tendency of protofilaments, made of GDP-tubulin, to curve. The tubulin dimers close to the growing end of the MT are more likely to bind GTP, forming a growing stable GTP-cap that prevents the depolymerization of the MT shaft, which is instead mostly composed of GDP-tubulin (Fig. 1.3). On the other hand, during catastrophe the GTP cap is lost, GDP-tubulin protofilaments disassemble and the MT shrinks [14,15].

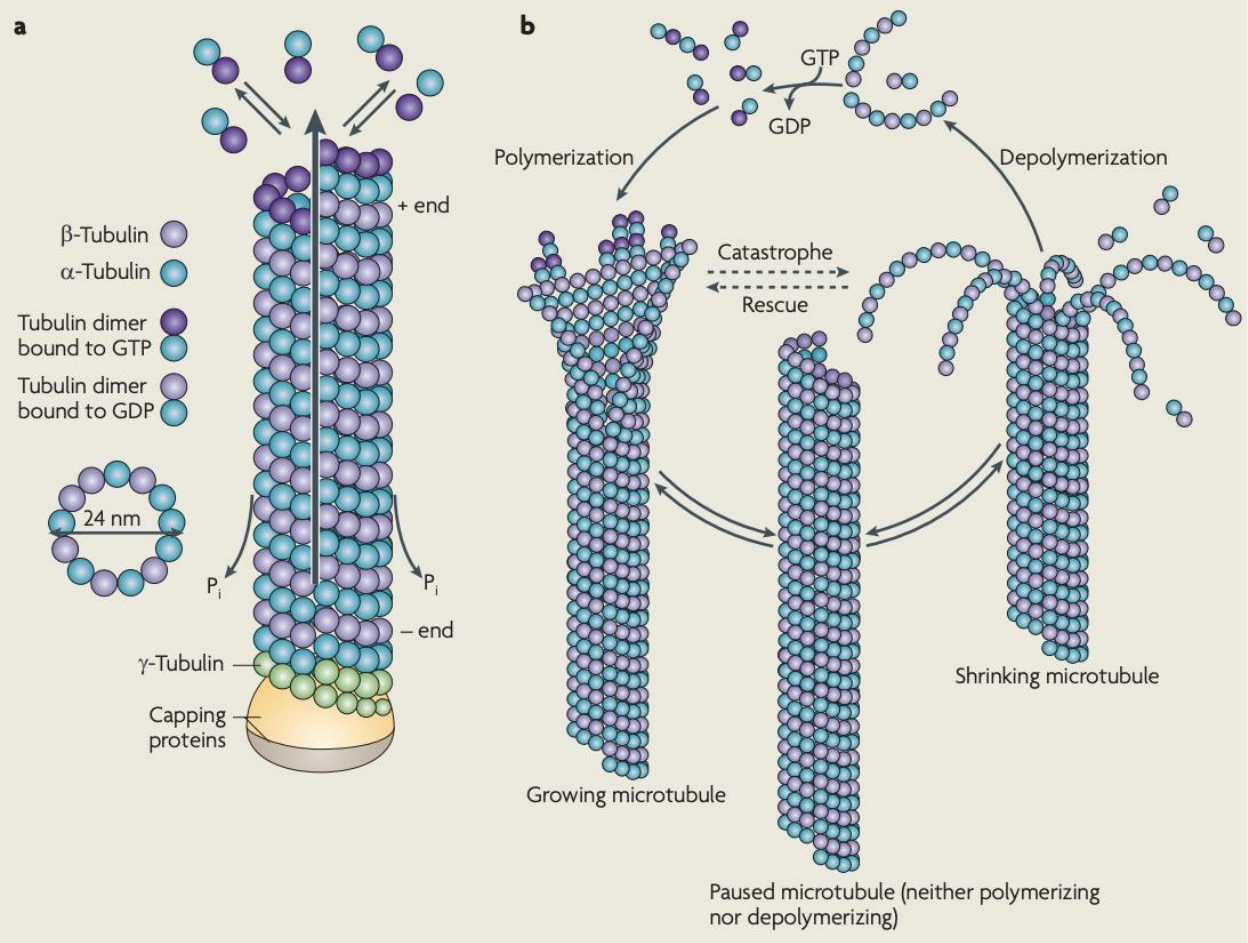

Figure 1.3. MT dynamic instability. a) Structure of a MT and **b)** schematic representation of the cycle of states that MTs can undergo due to their dynamic instability [11].

Unlike the cell-free systems, in the cells, MT dynamics are not only regulated by the concentration of free tubulin but are also controlled by the presence of MT associated proteins (MAPs). These proteins, such as EB1 or EB3, are responsible for stabilizing the MT GTP cap or they can stabilize bundles and regulate assembly, like tau or they can be responsible to cap the minus end, such as CAMSAP or Patronin [16–19]. Moreover, there are MT-severing enzymes, responsible for cutting MTs in presence of defects of the MT lattice or promoting their regrowth and increasing their number, such as katanin and spastin [20–22]. Additionally, there are motor proteins responsible to directionally

move cargos along the cell, using MTs as tracks to deliver vesicles and organelles in an anterograde (kinesin) or retrograde (dynein) fashion to and from specific cell sites [23–25].

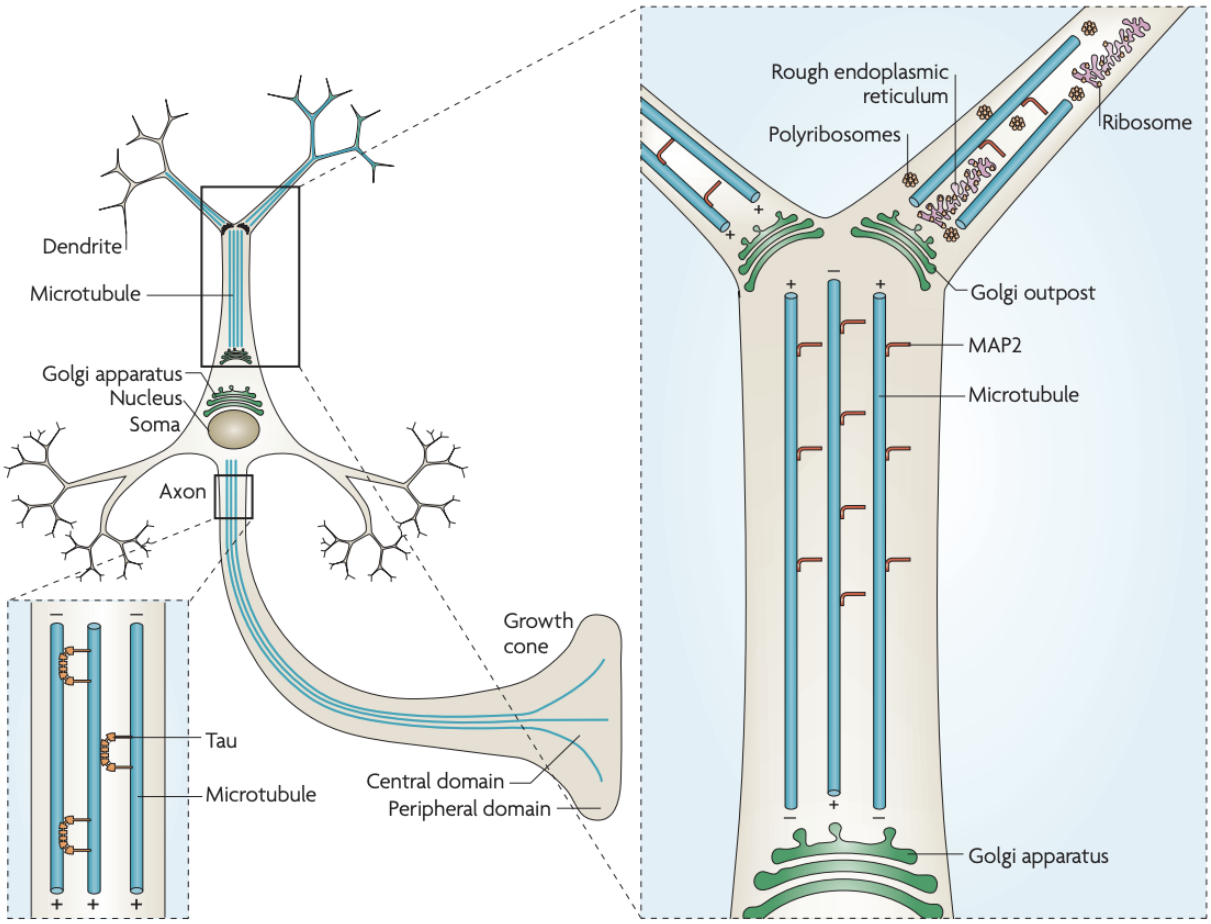

Figure 1.4. MT organization in the dendrites and axon. The axon shows a MT uniform polarity with all plus-end-out while dendrites exhibit a mixed polarity. The axonal MTs are stabilized by tau whereas MAP2 is mostly responsible for MT dynamic regulation in dendrites. Dendrites also contain organelles like rough endoplasmatic reticulum and Golgi outposts that usually anchor non-centrosomal MTOCs [11].

MTs are a key component of the neuronal cytoskeleton as they are essential for cellular and developmental processes such as neuronal migration and differentiation. Neurons are post-mitotic, highly specialized cells responsible for the transmission of the information in the CNS and PNS and for this reason they possess peculiar features such as a prominent polarization and excitability. Both of these characteristics rely on the proper assembly, organization and remodeling of the neuronal MT cytoskeleton [13]. Neurons have a peculiar morphology with a single long and thin axon emerging from the cell body, which is responsible to propagate the action potential to the presynaptic boutons, and

multiple thicker dendrites, that are often covered in spines, the postsynaptic sites that receive the inputs from axons. The ability of neurons to polarize is hence a pivotal component of their functioning and loss of polarity correlates with characteristic changes in MT organization and dynamics [26]. MTs are also involved in early neuronal development [27,28], provide tracks for intracellular and axonal trafficking [29,30], contribute to spine development and synaptic plasticity [31–33], anchor mitochondria [34] and control neurotransmission [10,35]. Although parallel bundles of MTs are required for neuronal development and maintenance in both axons and dendrites, MTs differ in these two neuronal compartments because axonal MTs are more stable and uniformly oriented with their plus end growing toward the distal tip of the axon whereas MTs have a mixed polarity in the dendrites [36] (Fig. 1.4). On the other hand, short and highly dynamic MTs can be found in the growth cone and their rapid and constant reorganization is required for its advance, axonal elongation and the recognition of guidance cues. Although the absence of an active centrosome in mature neurons is still a matter of debate [37], neurons mostly regulate MT nucleation and orientation through non-centrosomal MTs after complete development [38]. For example, axonal and dendritic branches develop during neuronal maturation due to *de novo* MT nucleation from non-centrosomal MTOCs (Fig. 1.4) and are oriented by specific subunits, such as HAUS, within this protein complex [39]. In addition, the activity of severing enzymes like spastin or katanin, which chop MTs at branching points [40], stimulate their polarized regrowth.

Due to their peculiar morphology and function, neurons are particularly susceptible to MT damages and the dysregulation of the MT cytoskeleton is a common feature in neurodegenerative diseases. Indeed, impairment of the MT defects caused by mutations in genes encoding for tubulins, MAPs, motor proteins, MT severing or modifying enzymes were strictly linked with neuronal dysfunction and neurodegeneration [41–44]. PD, Alzheimer's disease (AD), Amyotrophic Lateral Sclerosis (ALS), and other tauopathies were associated with a destabilization of the MT system, whereas other neurodegenerative diseases such as Hereditary Spastic Paraplegia (HSP) were associated with an MT hyper-stabilization [45]. This evidence suggested that the maintenance of this fine balance between stable and dynamic MTs is necessary for proper neuronal functioning and alterations of MT dynamics leads to disease progression [46,47]. Major examples of cytoskeleton defects that were associated with neurodevelopmental disorders include mutations in α or β tubulin isoform genes, called tubulinopathies, mostly characterized by cortical malformations and also linked to neurodegeneration [48–50]. All of these mutations disrupt the MT interaction with kinesin motor proteins [51], inducing the accumulation of kinesins in axon tips and perturbing the axonal transport of synaptic vesicles, mitochondria and other organelles in neurons [49]. Moreover, several findings have widely

demonstrated that dysfunctions in MAPs trigger neurodegeneration via defective regulation of MTs [52]. Among this family of neurodegenerative diseases are AD and other tauopathies that can be caused by mutations in the protein tau, which pushes the dynamic instability of MTs toward the destabilization of polymerized MTs [53,54] as well as deregulating axonal transport in neurons [55]. Since defects in the amount or in the structure of tau can affect its role as a MAP, this loss of function can lead to MT dysfunction and consequently to mislocalization of other subcellular structures like mitochondria or lysosomes, and, eventually, to neuronal impairment and death. In addition, tau phosphorylation has a double detrimental effect: i) it disrupts tau interaction with MTs and ii) hyperphosphorylated tau is more prone to aggregate becoming the essential component of the fibrillary tangles found in neurons of patients with AD [56,57]. Similarly, recent studies underline how α-synuclein, a small protein involved in synaptic neurotransmitter release [58] that aggregates in PD, is involved in the modulation of tubulin polymerization and that PD-related forms of α-synuclein lose this function [59]. α-Synuclein/α-tubulin interaction was mostly localized at the synapse [60], thus suggesting a possible role of this protein in the regulation of presynaptic MT dynamics.

1.1.1 Tubulin post-translational modifications

MT dynamic instability and their polarized structure can only partially provide the needed versatility that MT require to exert their functions within the cells. MTs are indeed highly conserved structures made of only two tubulin subunits but they are responsible for controlling a wide variety of neuronal mechanisms ranging from neurite development to axonal transport and neurotransmission [10,11,61] . To reach this functional heterogeneity, neurons exploit a tubulin "code" that, besides a first level of complexity obtained through the expression of different tubulin isoforms, relies also on post-translational modifications (PTMs) of both α- and β-tubulin [62,63]. Importantly, many different tubulin PTMs can coexist at the same time on the same MT, suggesting a potential role of tubulin PTMs in encoding cytoskeleton higher-order memory [63]. Tubulin can undergo these modifications as a soluble dimer, although modifying enzymes act preferentially on tubulin subunits that are already incorporated into MTs [11]. One of the preferential sites of tubulin PTM is the C-terminal domain of α-tubulin, whereas fewer modifications occur on the β-tubulin subunit (Fig. 1.5). Although many tubulin PTM functions remain unknown, it was observed that PTMs are not uniformly distributed along the MTs. Indeed, they create preferential tracks for motor proteins to deliver cargos [64], confer

special mechanic properties [65] or alter MT dynamics [66] or regulate the binding of MAPs according to specific cell needs.

The most studied tubulin PTMs is acetylation, which mostly occurs on α-tubulin lysine 40 and represents the only modification located in the MT lumen [67]. The only known enzyme responsible for tubulin acetylation is aTAT1, which is has a 100-fold higher catalytic affinity for polymerized MTs than soluble tubulin, although the mechanism of access to the intraluminal activity site still remains matter of debate. Acetylation was found to be correlated with MT stability and to reduce MT dynamics. Tubulin acetylation weakens lateral interactions between protofilaments, thus softening

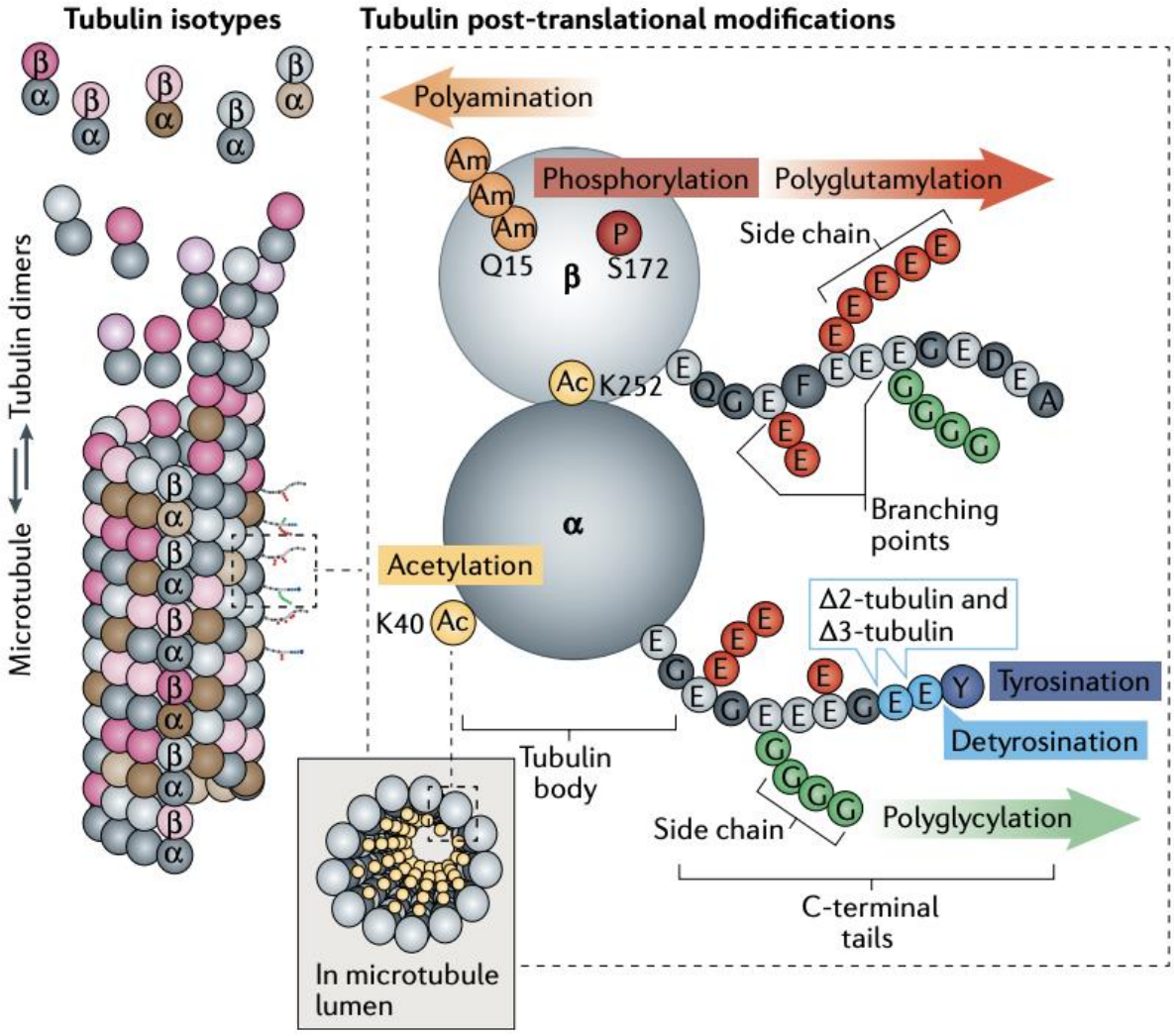

Figure 1.5. The components of the tubulin code. On the left, MT dynamic instability and, with colors, different tubulin isotypes. On the right, schematic representation of the α- and β-tubulin dimer and all the tubulin PTMs and the corresponding sites of the modification on the C-terminal tails [62].

MTs [68]. As MTs in living cells are frequently exposed to mechanical forces which can damage the lattice and subsequently result in MT depolymerization, an acetylation-induced increase in their flexibility would allow MTs to better resist mechanical stress, giving them more time to be repaired, consequently making acetylated MTs more long-lived [67,69]. Furthermore, MT acetylation was shown to be enriched in the middle of the dendritic shaft [70] and to promote kinesin-1 recruitment and transport, suggesting a possible effect of acetylated MTs on vesicular trafficking [71]. Unbalanced levels of acetylated tubulin were also associated with neurodegenerative diseases such as AD, ALS and PD [72–75]. Other important tubulin PTMs linked to neurodegeneration are tyrosination and detyrosination. The tyrosination/detyrosination cycle is regulated by the vasohibin-small vasohibin binding protein complexes (VASH1/2-SVBP) and MATCAP, which remove the terminal tyrosine from the α-tubulin C-terminal tail, while tubulin tyrosine ligase (TTL) re-adds it. VASH1/2-SVBP and MATCAP act on MTs whereas TTL acts on tubulin dimers [76]. While tyrosination was linked to dynamic and unstable MTs, detyrosination was related to stable and aged MTs. Indeed, α-tubulin tyrosination also affects MT dynamics, by regulation of the interactions between MTs and kinesin-13, determining a fast turnover (in the order of minutes) of tyrosinated MTs [66]. KIF2C, a motor-like protein that induces depolymerization of MT ends, has preferential activity for tyrosinated MTs, both *in vivo* and *in vitro* [77]. On the other hand, detyrosinated tubulin was linked to stable and long-lived MTs. Re-tyrosination deficiency was recently reported to be defective in AD neurons [78], leading to an unbalanced tyrosination/detyrosination cycle which also promotes increased acetylated tubulin levels [73]. When tubulin is detyrosinated, it exposes the last two glutamates of the C-terminal tail to an enzymatic reaction carried out by cytosolic carboxypeptidases (CCP). These enzymes of the deglutamylase family modify both lateral polyglutamate chains and primary tubulin chain [79]. The enzymatic reaction produces Δ2 and Δ3 tubulin, two irreversible PTMs that progressively accumulates in the neurons [80]. Since it cannot be re-tyrosinated by TTL, Δ2 tubulin influences MT dynamics by shifting it toward an hyper-stabilization of the cytoskeleton, and its accumulation was shown to be a hallmark of acute and slow-developing neurodegeneration in bortezomib-induced peripheral neuropathy [81]. Finally, there are other less known tubulin PTMs including polyglycylation, phosphorylation, and the recently discovered polyamination, whose functions still remain mostly unknown.

1.1.2 Dynamic microtubules at the synapse

In neurons, MTs are particularly important because they support complex and branching structures, like the dendritic tree and axonal arbors, while maintain segregation of functional compartments. In addition to providing structural support, MTs act as intracellular highways, creating tracks for motor proteins to deliver cargoes along the cell [13]. Axonal and dendritic MTs are composed by both a stable and a dynamic portion that often are present at the same time in the polymer [11,82]. Whereas the dynamic MT pool undergo constant passage from depolymerization to polymerization and vice versa [83], stable MTs remain relatively constant in their polymerized form. Stable MTs represent the majority of the neuronal MT mass and this stability is required to provide long-lasting support to the extensive

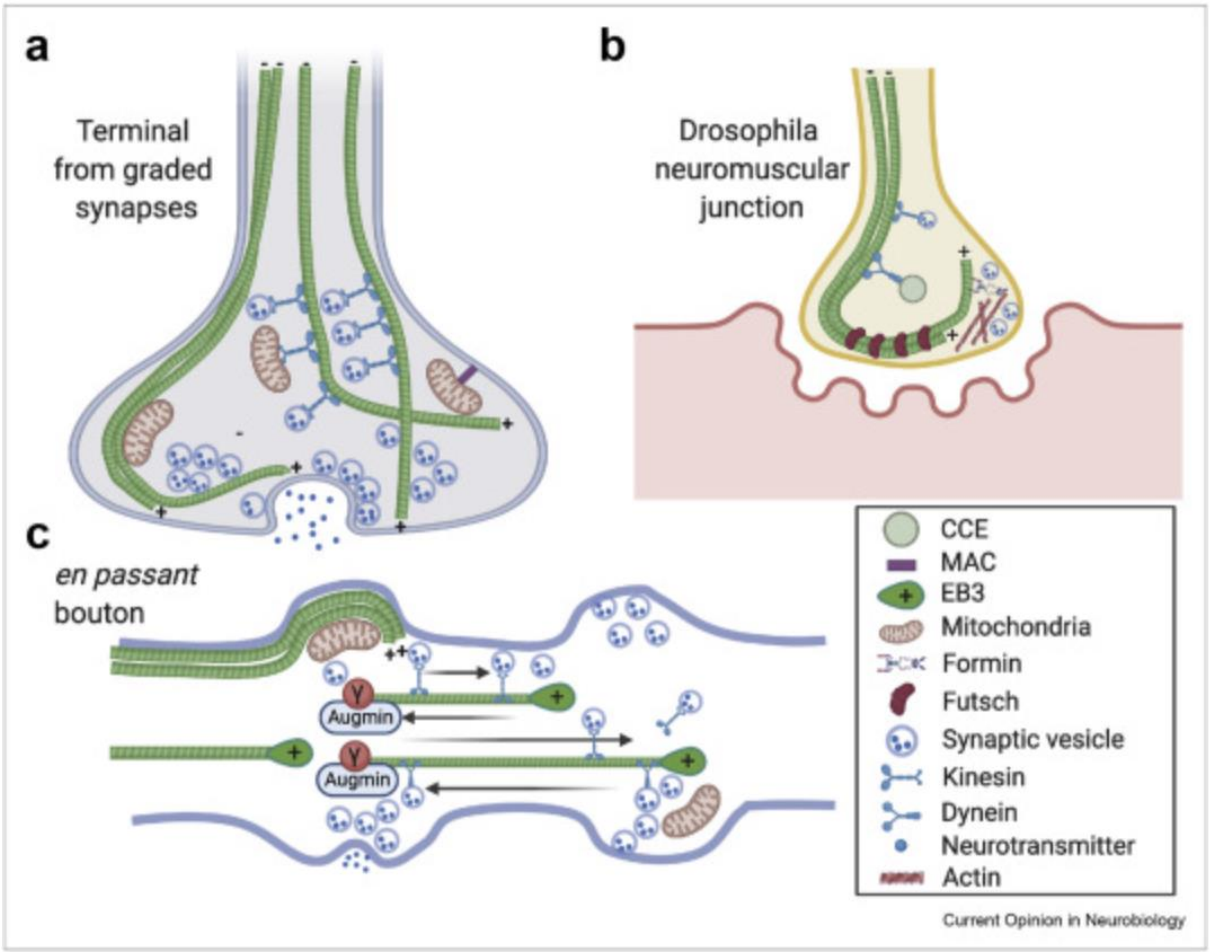

Figure 1.6. The dynamic MTs at the pre-synapse. a) At the presynaptic terminal of graded synapses, dynamic MTs controls high-frequency neurotransmission by restoring the synaptic vesicles releasable pool and providing energy supply by anchoring mitochondria. **b)** In the *Drosophila* neuromuscular junction, bundles of MTs enter the synapse assuring the delivery of kinesin-mediated synaptic vesicle delivery. **c)** In rat hippocampal neurons, γ-tubulin-dependent *de novo* MT nucleation at *en passant* boutons is required to control neurotransmission upon neuronal firing [35].

neuronal structures. MTs can be stabilized by the activity of MT end capping proteins localized at their plus growing end or by motor proteins and other MAPs along the shaft. Once MT are stabilized, they acquire sufficient longevity to be modified by enzymes that add PTMs, creating the tubulin code that provides the functional heterogenicity that allows the MT cytoskeleton to fulfil its many different functions. It was established only very recently that dynamic MTs play additional roles in the pre- and post-synaptic organization and function [32,35]. The first evidence of MTs entering the pre-synapse was obtained via electron microscopy. It was shown that MTs were attached to the synaptic membrane at the active zone and bound to SVs [84]. Synaptic MTs were isolated in synaptosomal fractions [85], and more recently, it was demonstrated that they directly enter highly active graded glutamatergic synapses. Dynamic MTs are responsible for anchoring mitochondria and replenishing the releasable pool of SVs, transporting them from the reserve pool to the active zone, thus sustaining high frequency neurotransmission in the Calyx of Held [86]. Bundles of MTs were also reported to be localized at the neuromuscular junction in sites that were associated with bouton branching and synaptic growth [87,88], proposing a role in synaptic development and plasticity. Additionally, Guedes-Dias et al. observed that, in cultured hippocampal neurons, dynamic MTs and their plus ends are enriched at *en passant* boutons and allow for the targeted SV precursors delivery and unloading by the kinesin-3 motor KIF1A, an axonal long-range kinesin that showed a lower affinity for GTP-tubulin [89]. In line with this study, in mammalian neurons, γ-tubulin-dependent *de novo* MT nucleation at *en passant* boutons was required to control exocytosis and SV transport between neighboring boutons upon neuronal firing [10] (Fig. 1.6). Besides their role in sustaining the proper functioning of the SV cycling machinery at the active zone, controlling axonal exocytosis, and providing a constant supply of ATP for the neurotransmitter release at the pre-synapse, dynamic MTs are also present at postsynaptic sites [32,35]. MTs are known to invade dendritic spines, controlling their shape and development. They were observed to interact with the postsynaptic scaffolding proteins PSD-93 and PSD-95, probably sustaining spine plasticity through targeted delivery of these pivotal proteins at excitatory synapses. Furthermore, dynamic MTs were revealed to anchor Gephyrin, the major scaffolding protein that organizes the postsynaptic density of inhibitory synapses by anchoring glycine and GABA receptors at inhibitory post-synapses [32].

Together, this evidence supports that an impairment in the MT dynamicity in neurons can have detrimental effects in pre- and post-synaptic activity and development, making them a possible target for pharmacological regulation in preventing neurodegenerative diseases such as PD and AD.

1.2 Parkinson's disease: an overview

PD is the second most common neurodegenerative disorder affecting 1% of the population over 65 years old and up to 5% of the population by the age of 85. It is a late-onset progressive neuropathological condition whereas, in some early-onset cases, symptoms appear before the age of 50 [90]. PD is clinically characterized by motor impairment, namely resting tremors, bradykinesia, postural instability and general rigidity. Motor deficits are often accompanied by a wide array of

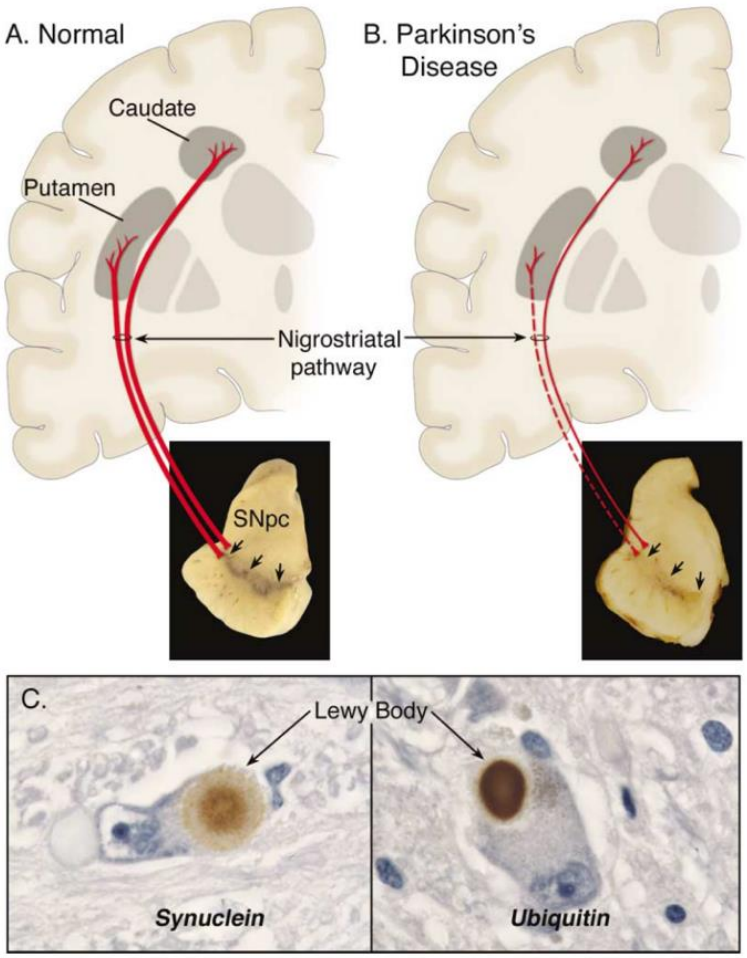

Figure 1.7. The neuropathology of PD. A) Schematic representation of the nigrostriatal pathway (in red). DA neurons cell bodies located in the SNpc project (thick solid red lines) to the basal ganglia and form synapses in the striatum (i.e., putamen and caudate nucleus). The photograph shows the normal pigmentation of the SNpc, produced by neuromelanin within the DA neurons. **B)** Schematic representation of the diseased nigrostriatal pathway. In PD, the nigrostriatal pathway degenerates and there is a consistent loss of DA neurons that project to the putamen (dashed line). The photograph demonstrates depigmentation of the SNpc due to the loss of DA neurons. **C)** Immunolabeling of α-synuclein in a LB in a SNpc DA neuron. On the right, immunostaining with an antibody against ubiquitin within the Lewy body.

non-motor symptoms including mood, sleep and cognitive disorders [91]. Although the pathological changes occur in different regions of the brain, these cardinal manifestations are mostly due to a depletion in the DA neuronal subpopulation of the SNpc. This structure is a basal ganglia structure located in the midbrain that projects to the *corpus striatum*, establishing the so-called nigro-striatal pathway, a trait crucially involved in the circuits that ensure control and modulation of movement [92]. A more modest neuronal loss occurs in the mesolimbic DA neurons, where the cell bodies in the

ventral tegmental area project their terminals to the caudate. The PD pathology clinically manifests when the disease has reached a very advanced stage, with 60% of DA neuronal loss in the SNpc and a massive dopamine depletion, around 80% [91,93]. Besides the SNpc degeneration, PD is always accompanied by extensive extranigral pathology with other brain regions implicated in PD neurodegeneration in later stages of the disease. Indeed, the dorsal motor nucleus of the vagus, the Raphe nuclei, the thalamus and the subthalamic nucleus exhibit neuronal death, while the cortex degenerates only in very advanced stages of the pathology [93]. PD hallmarks are proteinaceous inclusion within neuronal soma and neuropil, known as Lewy bodies (LB) and Lewy neurites (LN), respectively, whose main component was found to be α-synuclein [94]. According to the Braak stages, in the least affected brains (stage 1-2) the only neurons displaying the presence of PD-related inclusion bodies are in the dorsal motor nucleus and a few areas where it projects. As the pathology evolves (stage 3-4), the inclusions expand to vulnerable subnuclei of the substantia nigra and the thalamus, where an extensive death of DA neurons occur, causing in the motor symptoms. In the latest phases of the disease (stage 5-6) LB and LN severely invade the brain by spreading throughout the midbrain and reaching the neocortex.

To date, the etiology of PD remains mostly unknown and idiopathic PD still represents the large majority of the diagnosed cases. It is believed that both environmental and genetic factors contribute to the disease onset and progression although aging is still considered the major risk factor. Small molecules have been associated with the onset of motor symptoms, mitochondrial damage, oxidative stress increase, selective SNpc DA neurons loss and aggregation of α-synuclein. For these reasons they were extensively used in animal models to mimic PD pathology. Similarly, α-synuclein oligomers were shown to preferentially accumulate and bind to mitochondria, interfering with the respiratory chain and mitophagy as well as interfering with tubulin polymerization [95–97]. Hence, environmental toxins can mimic the detrimental effect induced by unregulated protein aggregation in PD, albeit no toxin-induced model simulates all PD pathology features.

In contrast, the discovery of mutations in proteins involved in PD neurodegeneration helped to better understand the pathogenesis of the disease and to underline genetic risk factors that contribute to the onset and the progression of the pathology. Familial PD was correlated with a number of genes mutated in patients and among the most relevant we find SNCA (PARK1), LRRK2 (PARK8), PARK2, PINK1 and DJ1 (PARK7) encoding for α-synuclein, dardarin, Parkin, PARK6-PTEN-induced kinase 1 and DJ1, respectively. While α-synuclein and LRRK2 mutations were associated with early onset autosomal dominant PD, Parkin, PINK1 and DJ1 mutations are linked to recessive form of the disease. Duplications and triplications of the SNCA gene as well as conformational

changes in α-synuclein structure due to point mutations such as A53T, A30P and E46K were shown to lead to an accelerated α-synuclein aggregation into toxic oligomers and amyloid fibrils compared to the WT and, therefore, to early synaptic dysfunction [98–101]. Parkin, LRRK2, PINK1, DJ1 and other related PD proteins, are identified to participate in different processes, including mitochondrial homeostasis, ubiquitin proteosome pathway and, interestingly, also in the regulation of MT system. Their altered function was correlated with the degeneration and death of SNpc DA neurons although the majority of the mechanism of induction of PD pathology remain unknown.

1.2.1 The synaptic function of α-synuclein

α-Synuclein is a protein enriched at the synapse which is pathologically connected to PD and other neurodegenerative disorders that are collectively known as "synucleinopathies". LBs and LNs are currently considered the main hallmark of PD, and since the discovery that they are mainly composed of α-synuclein [94], an intensive research has focused the attention on the mechanism that leads α-synuclein to acquire an aberrant conformation and then aggregate into amyloid fibrils. Relatively little is known about its physiological function at the synapse. α-Synuclein is a small protein of 140 amino acids (~14 kDa) that is ubiquitously and mostly expressed in vertebrate brain. There are other two isoforms, β- and γ-synuclein, whose function is still not well clarified. Additionally, α-synuclein expression, to a lesser extent, is found in other tissues in the body such as in skin cells, at the neuromuscular junctions in muscle cells, red blood cells, lymphocytes and platelets, suggesting a general cellular function in addition to its activity in the brain [102–106]. α-Synuclein levels increase during development and remain high in mature neurons, and once produced in the soma, it reaches the synapses due to its high affinity for SVs [58].

α-Synuclein structure is composed of three domains: an amphipathic, lipid binding domain (1-59) which mediates its affinity with lipid membranes; a central non-amyloid component (NAC) involved in β-sheet fibril aggregation (60-95) and an unstructured negatively charged C-terminal tail (96-140) (Fig. 1.8). α-Synuclein exists in a dynamic equilibrium between a soluble, natively unfolded state and a partial secondary structure state. Indeed, the interaction between α-synuclein and lipid membranes is believed to induce the N-terminus of the protein to form an α-helix, thus mediating its cellular activities [107–109]. α-Synuclein was observed *in vitro* to have a higher affinity to membrane with high curvatures such as small SVs. Currently, one of the known function for α-synuclein is at the synapse,

binding the SNARE-protein synaptobrevin-2 and chaperoning SNARE-complex assembly [110]. Indeed, triple synuclein KO mice show changes in synaptic structure and size and impaired survival [110,111]. Despite this evidence, different studies in α-synuclein KO indicated either an enhanced [111–113] or a decreased [114,115] release at the synapse, thus, α-synuclein effect on neurotransmitter release has been not completely clarified. It was reported that α-synuclein is able to directly or indirectly interact with a wide array of synaptic proteins including synapsin III [116], VMAT2 [117], DAT [118,119] and SERT [120] although there are no mechanistic insights on how these interactions are regulated at the

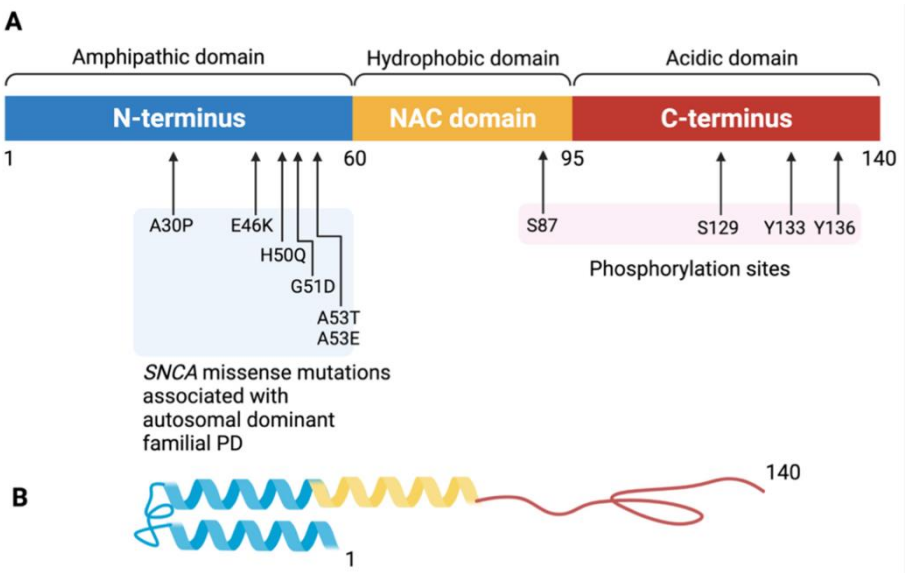

Figure 1.8. The structure of α-synuclein monomer. A) Schematic representation of α-synuclein structure. The 140-amino-acid protein can be divided into three distinct domains. The N-terminal amphipathic domain (in blue) can fold into an α-helix and is responsible for the membrane-binding properties. It also contains the amino acid residues affected by the main α-synuclein gene mutations (A30P, E46K, H50Q, G51D, A53T, A53E) associated with autosomal dominant PD. The hydrophobic non-amyloid β-component (NAC) domain (in yellow) is responsible for promoting aggregation into amyloids. The C-terminal domain (in red) forms an acidic tail. **B**) Tertiary structure of the α-synuclein monomer [121].

synapse. A recent study identified a role *in vivo* for α-synuclein both as a facilitator and suppressor of dopamine release in SNpc DA neurons, confirming its role as an enhancer of SV fusion and turnover. It was shown that α-synuclein facilitates SV fusion at the active zone when intense neuronal activity is stimulated, and suppresses release in longer firing intervals [122]. Accordingly, it was proposed that α-synuclein, due to its high affinity for high curvature membranes, localizes circumferentially around the SV fusion pore: this facilitates SV fusion pore closure and allows SVs to be more rapidly removed from presynaptic binding sites, thus providing faster refilling of the releasable SV pool and facilitating dopamine release during phasic neuronal firing [123,124].

Interestingly, α-synuclein was shown also to interact with tubulin assembly *in vitro*, acting as a "dynamase" promoting polymerization, catastrophe and nucleation of MTs [59]. Moreover, this interaction was identified to mostly occur at presynaptic sites in human and murine *striatum* [60], suggesting a possible control of dopamine release through an MT-mediated mechanism.

1.2.2 α-Synuclein aggregation and synaptic dysfunction

Protein aggregation and formation of inclusion bodies is a common feature of many neurodegenerative disorders including PD, AD, Huntington disease, prion disorders and ALS. These proteinaceous aggregates, called amyloid plaques, are mainly constituted of fibrils of aberrant proteins enriched in β-sheet structures [125]. Despite the discovery of dominant and recessive mutations that can trigger the misfolding of some of the proteins that compose these inclusions, the mechanisms that foster the appearance of these prone-to-aggregate secondary structures are unknown.

α-Synuclein can change its conformational state by switching from a physiological soluble natively unfolded or partial α-helical structure into a β-sheet secondary structure. As mentioned above, its aggregation into amyloid fibrils [126], was linked to pathological neurodegenerative conditions called "synucleinopathies", which include PD, dementia with Lewy bodies (DLB) and multiple system atrophy (MSA). Point mutations in the SNCA gene encoding for α-synuclein such as A53T, A30P and E46K as well as duplication and triplication of this gene can trigger α-synuclein polymerization and they were connected with early onset dominant familial PD [98–101]. Mutations or increased levels of α-synuclein, indeed, provoke misfolding and consequent nucleation into small β-sheet soluble oligomers (tetramers, pentamers and hexamers [127]). The fate of these oligomers can follow two different paths: i) degradation by lysosomes through activation of autophagy pathways or through ubiquitination and disassembling in the proteasome; ii) accumulation in the cytoplasm with aggregation into fibrils that eventually forms the LBs and LNs. Small α-synuclein aggregates such as oligomers and protofibrils were also described to self-propagate from cell to cell in a prion-like manner, although, the mechanism that provides the spreading is unclear. It was reported that α-synuclein is transmitted via exocytosis to adjacent neurons, forming aggregates that propagate to neighboring brain regions and provoke synaptic dysfunction and neuronal loss [128–130] (Fig. 1.9).

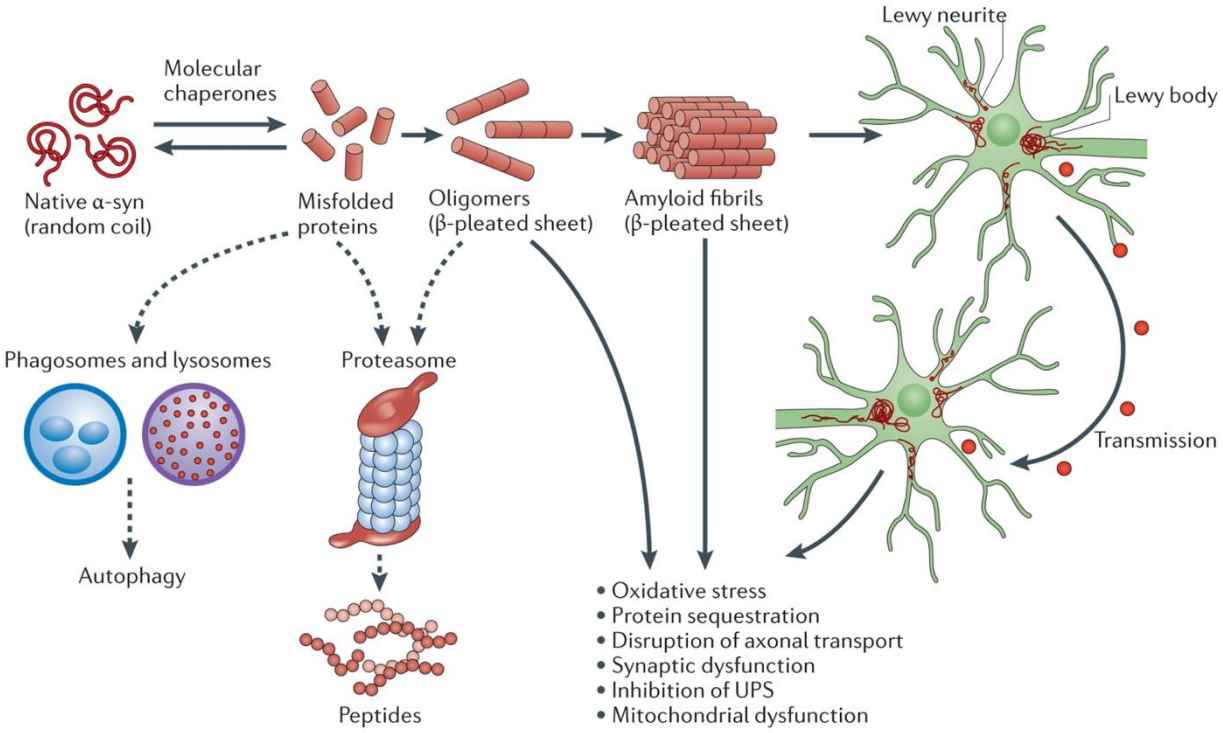

Figure 1.9. α-Synuclein aggregation and spread of the pathology in PD. α-Synuclein physiologically exists as a natively unfolded protein. Mutations or increased level of α-synuclein can trigger its misfolding into a β-sheet conformation that nucleates into small oligomers. These toxic soluble structures can be degraded following the autophagy or ubiquitin-proteasome pathway or they can further aggregate into insoluble amyloid fibrils provoking neuronal damage and death. This aberrant oligomers spread in a prion-like manner and can act as seeds for fibril formation in adjacent neurons [128].

Supporting this hypothesis, PD mouse models were created by injecting in the brain α-synuclein pre-formed short fibrils, that were shown to act as seeds for a rapid endogenous α-synuclein aggregation into LB-like inclusions which spread simulating α-synuclein pathology [131–133]. Furthermore, oligomers (smaller than 100 nm) are considered to be the active toxic species of α-synuclein aggregates and show a wide array of mechanism of cellular toxicity. They were reported to have a higher affinity for lipid double layers compared to monomeric α-synuclein. This was shown to be associated with the compromise of cell membrane integrity, leading to dysregulated ion influx in DA neurons and an increased inflammatory response [134,135]. Alterations in neuronal activity were also reported in presence of α-synuclein oligomers since they induce synaptic dysfunction and suppression of long-term potentiation by corrupting Ca^{2+} influx mechanisms in glutamatergic neurons [136]. Additionally, cellular respiration defects and decreased mitochondrial membrane potential were reported accompanied by impairment in cellular autophagy and protein degradation [137,138].

In PD, the DA neurons of the SNpc progressively die, causing the motor symptoms. Although α-synuclein is ubiquitously present in different synaptic populations and its aggregation manifests

throughout several brain regions, it is controversial why the DA neurons of the SNpc are particularly affected by α-synuclein pathology. Studies underlined the higher susceptibility of DA neurons to oxidative stress [139,140] and to mitochondrial damage [141] and the direct interaction between dopamine and α-synuclein as a driving force in α-synuclein aggregation [142] but a clear mechanism that explains the preponderant aggregation of α-synuclein in SNpc neurons is still missing.

1.2.3 Microtubules and Parkinson's disease

A growing number of studies report the involvement of cytoskeleton defects in neurodegenerative diseases. Different pathogenic molecular mechanisms such as mitochondrial dysfunction, oxidative stress and accumulation of α-synuclein aggregates have been implicated in the death of SNpc DA neurons [139–142], although it is not known whether these are primary or secondary insults in PD neurodegeneration. The concept that MT dysfunction, in terms of organization and dynamics, as well as failure of MT-dependent neuronal processes such as axonal transport, can participate to PD progression is still under investigation.

Evidence of the participation of the MT cytoskeleton in early PD neuronal dysfunction was first collected from studies on PD-neurotoxins. Among them, exposure to 1- methyl- 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was linked with mitochondrial and oxidative stress damage of the DA neurons of the SNpc. MPTP is selectively uptaken by DAT into the DA synapse where is converted to its active metabolite MPP^+, leading to synaptic dysfunction and neuronal damage. Interestingly, signs of axonal transport impairment and MT dysfunction are detected before death of DA neurons in PD mouse models injected with MPTP [143]. In addition, MT destabilization has been shown to precede mitochondrial transport deficit and neurite degeneration in MPP^+-treated cells [144]. Another PD-neurotoxin, rotenone, was reported to inhibit tubulin self-assembly *in vitro* [145] and to be selectively toxic for DA neurons through induction of MT depolymerization. Indeed, MT disruption provokes vesicular trafficking impairment and accumulation of dopamine vesicles whose leaking induces oxidative stress and profound neuronal damage [146]. Stabilization of MTs was also reported to be protective in DA neurons against rotenone toxicity [147].

It is also known that several proteins implicated in PD, such as Parkin, LRRK2, DJ1 and α-synuclein, were linked to MT alterations and/or interference with MT assembly. α-synuclein aggregation has been associated with a plethora of disruptive effects on the cytoskeleton. Deposits of α-synuclein colocalize with tubulin [148,149] and both neurofilaments and β-tubulin are found in close association to

phosphorylated Ser129 α-synuclein at the periphery of LBs [150] whereas three tubulin chains (α-4A, β-3, β-4) are preferential interactors of oligomeric α-synuclein [151]. In turn, α-synuclein oligomers were shown to impair neuronal MT-kinesin interplay in human iPSC-derived neurons and to induce a relocation of transport-regulating proteins such as Miro1 and Tau, suggesting that early axonal transport deficits may drive synaptic dysfunction and PD pathology [152]. Furthermore, α-synuclein was reported to induce tau hyperphosphorylation, thus strongly driving MT destabilization [153].

Parkin, a protein whose mutations are associated with most cases of juvenile parkinsonism, is an E3 ubiquitin ligase responsible for the transfer of activated ubiquitin to damaged or misfolded proteins that are degraded by the proteasome [154]. Parkin is well characterized in its role in the regulation of mitophagy but it has also been linked to modulation of the MT cytoskeleton. In 2005, Yang et al. reported that parkin acts a MT stabilizer, and parkin expression protects against PD-neurotoxins like rotenone and MT-depolymerizing agents [155]. Recent evidence supports a role for parkin in maintaining the morphological complexity of human midbrain derived-iPSC neurons through its MT-stabilizing effect while parkin PD-linked mutants reduce neuronal complexity and promote MT destabilization [156]. In line with these studies, our lab reported that parkin KO mice showed a rapid induction of MT acetylation both in DA neuron cell bodies and fibers localized in the SNpc and *corpus striatum*, respectively. Interestingly, these alterations were revealed to precede mitochondrial trafficking impairment. Additionally, loss of parkin in differentiated PC12 cells caused mitochondrial mobility damage and this effect was rescued by the MT-stabilizer taxol [157]. This demonstrated that the integrity of MT network is essential for the survival of DA neurons, which are particularly vulnerable to MT depolymerization because of their peculiar morphology and neurochemistry.

LRRK2 interacts preferentially with β tubulin isoforms and has been shown to modulate MT stability through regulation of the acetylated state of MTs, a marker of stable MTs. Fibroblasts from PD patients bearing mutations in LRRK2 and LRRK2 KO mice fibroblasts show increased MT acetylation [45,158,159]. Since LRRK2 is mostly localized at the growth cone and in proximity of dynamic MTs, when this protein carries PD-related mutations, it is possible that it partially loses its role in the regulation of MT dynamics, increasing the stable pool of acetylated MTs and fostering cytoskeleton hyper-stabilization. Finally, Sheng and colleagues demonstrate a novel role for DJ-1 as a MT dynamics regulation and proved that its deficiency leads to striatal neurite outgrowth impairment [160].

1.2.4 The α-synuclein/tubulin interaction

Unlike other neurodegenerative diseases such as AD in which the contribution of cytoskeletal defects to the progression of the pathology is well established, only circumstantial evidence links PD to MT alterations. Mutant α-synuclein was shown to drive DA neuron degeneration mostly by provoking mitochondrial distress, impaired Ca^{2+} influx and membrane disruption [134,136,138]. However, aggregated α-synuclein was also recently associated with PD pathology by altering MT dynamics. Firstly, it was shown to co-immunoprecipitate with tubulin and reported to affect MT assembly *in vitro* [149,161]. Secondly, evidence correlated the expression of wild type α-synuclein with MT disruption in HeLa cells [162]. Notwithstanding, it is controversial which region of the protein is responsible for the interaction with tubulin. While it was firstly indicated that the C-terminal region of α-synuclein was involved [161], a more recent study reported the α-synuclein NAC region to had remarkable tubulin binding capacity [162]. Our lab hypothesized that α-synuclein may act as a MT-dynamase based on its ability to fold upon interaction with tubulin, enhance tubulin polymerization, nucleation and MT catastrophe. Importantly, circular dichroism analysis of PD-mutants α-synuclein such as A53T, E46K and A30P showed that they all lose the ability to fold while interacting with tubulin [59].

Indirect proof of α-synuclein interaction with MTs also exists [163], supporting the notion that α-synuclein may play a role as a MAP and that its aggregation and/or mutation can drive PD neurodegeneration. Notably, increased levels of α-synuclein were able to partially restore dendritic transport in tau KO mice, whereas no rescue was observed in the case of other MAPs [164]. A MT-mediated mechanism for α-synuclein-mediated recruitment of DAT to the plasma membrane was also suggested in neurons [165].

We recently demonstrated that α-synuclein/tubulin interaction occurs in human and mouse brain and that this association mostly localizes to the presynaptic compartment in murine *corpus striatum*, where most of the DA synapses of the SNpc neurons are localized. Our observations suggest that α-synuclein might be a key player in the regulation of presynaptic MTs, a process recently demonstrated to be rate-limiting in the control of synaptic transmission [10].

This cumulative knowledge supports the idea that α-synuclein may act as a direct and/or indirect modulator of MT assembly. Further studies are needed to obtain mechanistic insight to clarify how α-synuclein interaction with tubulin regulates the neuronal MT cytoskeleton and whether impairment of this interaction contributes to PD neurodegeneration.

2. Aim of the Project

PD is one of the most common neurodegenerative disorders. The neuropathological features of PD are the selective and progressive loss of DA neurons in the SNpc, a midbrain region, deficiencies in striatal dopamine levels, and the presence of intraneuronal α-synuclein deposits known as LBs. Mutations of proteins that cause PD, including α-synuclein, induce cytoskeletal defects in the central nervous system and synaptic dysfunction. It remains unclear, however, which mechanisms lead to synaptic dysfunction in PD and whether alterations in cytoskeletal dynamics are primary or secondary events in the induction of neurodegeneration of SNpc DA neurons.

MTs are ubiquitous cytoskeletal elements that play critical functions in cell polarity, intracellular transport and structural support. While it has long been known that MTs support neuronal structures, however, their roles at synapses have been explored only over the past decade. It was reported that dynamic MTs enter into dendritic spines, and invasion by dynamic MTs regulates spine enlargement and synaptic strength [32, 35]. The role of MTs at mammalian presynaptic elements on the other hand has remained uncharted territory until very recently. In highly active synapses that require accurate, graded neurotransmitter release, presynaptic MTs are rate limiting for high-frequency neurotransmission and in anchoring mitochondria at sites of high metabolic demand. In pyramidal neurons excitatory presynaptic sites are hotspots for MT nucleation, a process regulating neurotransmission by providing the tracks for targeted bidirectional delivery of a rate-limiting supply of synaptic vesicles (SVs) to sites of stimulated release [10]. The role of presynaptic MTs in DA neurons remains unexplored.

Emerging data indicate that MTs also interact with α-synuclein. Physiologically, α-synuclein associates with membranes and is involved in the regulation of dopamine release, mobilization and docking of SVs. Interestingly, α-synuclein folds upon interaction with tubulin and pathogenic α-synuclein PD variants lose this function and promote tubulin aggregation [59]. However, its specific role in regulating neurotransmitter release through a presynaptic MT-mediated mechanism and whether loss of this activity contributes to pathology has not been elucidated yet.

The aim of my study was to interrogate, by using different experimental models, whether and how α-synuclein acts as a modulator of presynaptic MT dynamics in DA neurons. Firstly, I investigated the occurrence of a direct interaction between α-synuclein and α-tubulin in synaptic compartments of human and murine *corpus striatum*. Next, I analysed whether suppression of α-synuclein expression

fostered an accumulation of specific tubulin PTMs associated with MT stability and impacted on presynaptic MT dynamics. This work led to the first characterization of the dynamic MT cytoskeleton in DA neurons and to preliminary evidence supporting a role for α-synuclein as a MT "dynamase" in ventral midbrain neurons.

3. Main Results

3.1 α-Synuclein and α-tubulin interact in human brain and at the pre-synapse in murine corpus striatum

When the colocalization of α-synuclein and α-tubulin was revealed in the synaptic compartment [60], we decided to explore whether the two proteins associate at synapses *in situ* by using brightfield PLA in both murine and human *corpus striatum*. First, we showed that the interaction occurs in murine brain. Two negative controls were used to assess the specificity of the PLA signal: (i) omission of the anti-α-synuclein primary antibody during the PLA staining, and (ii) α-synuclein/α-tubulin PLA performed on sections of C57BL/6J OlaHsd mice carrying spontaneous deletion of the SNCA gene [166]. As expected, no PLA signal was detected in the samples with no primary antibody and in C57BL/6J OlaHsd mouse sections. Next, we found that α-synuclein/α-tubulin interaction occurs in the human brain of healthy subjects. We observed a diffused signal localized mostly in the *corpus striatum* and in the cerebral cortex, especially in layer V, which is characterized by a high synaptic density. As a positive PLA control, we analysed the distribution of α-tubulin/βIII-tubulin PLA signal in the human brain and found a pattern of staining that was more intense in the white matter, showing its specificity for regions that are rich in MTs such as the bundles of fibers of the *corpus striatum* or the apical dendrites of pyramidal neurons.

Based on the evidence that α-synuclein and α-tubulin directly interact in mouse and human brain, we examined whether this interaction occurs at synaptic terminals. To this end, we localized fluorescent α-synuclein/α-tubulin PLA signal with synaptophysin immunofluorescence to label striatal synapses. We found that α-synuclein/α-tubulin PLA signal widely colocalized with synaptophysin staining. We expressed the colocalization by using Manders' coefficients M1 and M2, parameters that quantify the overlap of the PLA signal on synaptophysin (M1) and viceversa (M2). Specifically, more than half of the fraction of PLA signal (65%, M1 = 0.65) colocalized with synaptophysin signal while only a small percentage (5%, M2 = 0.05) of striatal synapses revealed the association between the two proteins. Furthermore, analysis of the intensity profiles confirmed the complete superimposition of PLA staining with synaptophysin signal, confirming the localization of this interaction within the synaptic compartment in mouse *corpus striatum* [60].

To conclude, our data demonstrate a close proximity and a potential direct interaction between α-synuclein and α-tubulin not only in different neuronal compartments such as dendrites and axons, but also at the pre-synapse where their mutual interaction may be crucial for controlling SV transport and neurotransmitter release.

3.2 α-Synuclein colocalizes at both plus and minus ends of more dynamic MTs

Next, we investigated whether α-synuclein associates with α-tubulin at specific regions of the MT. Since we hypothesize that α-synuclein may directly be involved in the regulation MT polymerization and/or nucleation, we checked its localization in proximity of the MT plus or minus end. Furthermore, we analyzed whether α-synuclein localizes preferentially with selected tubulin post-translational modifications (PTMs). To this end, we examined the colocalization of α-synuclein with both acetylated and tyrosinated tubulins, markers of stable and dynamic MTs, respectively. To achieve this, we moved to a simpler cellular model, specifically PC12 rat pheocromocytoma cells, that we differentiated into a neuronal lineage for 2 or 5 days with human nerve growth factor (β-NGF). Upon differentiation, PC12 cells acquire a neuron-like phenotype, with neurites and growth cones and can be used as a simplified model for studying neuronal MTs. Since rat PC12 cells do not express α-synuclein, we transfected them with human α-synuclein-GFP 24-36 h before fixation. Cells were later immunostained with anti-human α-synuclein antibody and imaged using a 60x objective on a laser scanning Nikon confocal microscope.

Our analysis revealed that α-synuclein preferentially colocalizes with tyrosinated tubulin (38%, by Manders' coefficient, M1 = 0.38), whereas only 12% of α-synuclein signal overlaps with acetylated tubulin (by Manders' coefficients, M1 = 0.12) (Fig. 3.1). It also showed that α-synuclein displays a similar degree of colocalization with both EB3, a MT associated protein MAP that marks the MT plus end, and γ-tubulin, a key component of the γ-TURC that localizes at the MT minus end. Specifically, 18% of α-synuclein signal overlapped with EB3.

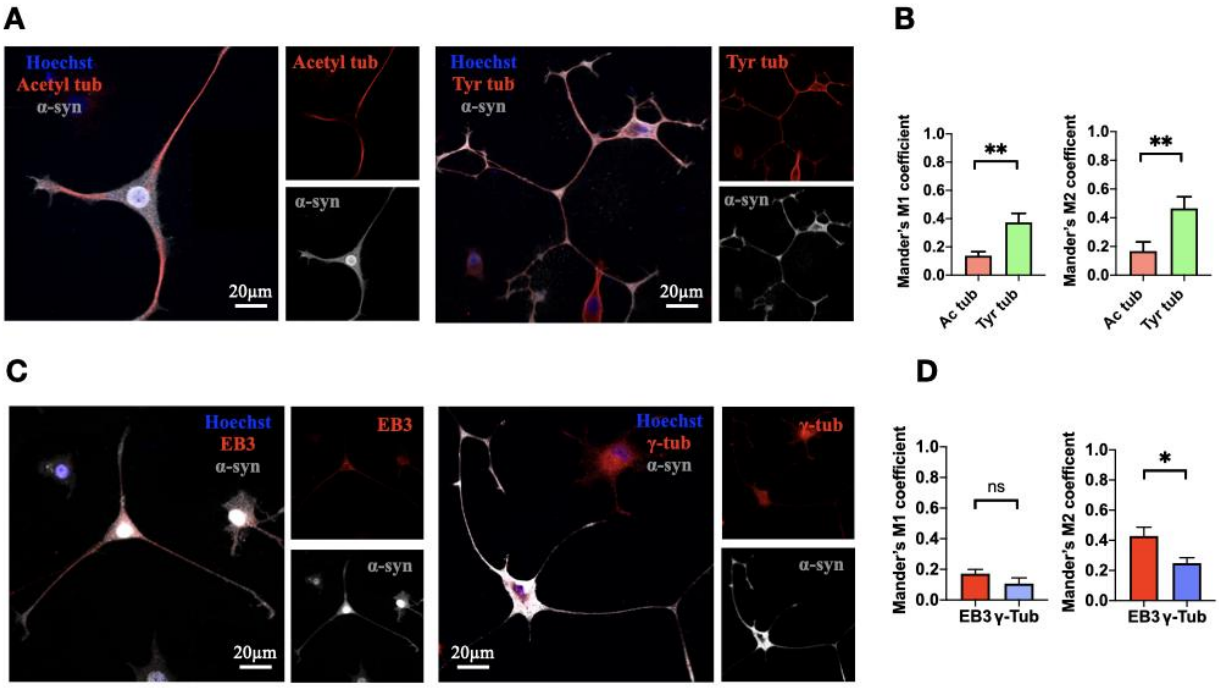

Figure 3.1: **Immunostaining of α-synuclein with acetylated and detyrosinated tubulin (up) and EB3 and γ-tubulin (down) in differentiated PC12** (**A**) Representative immunofluorescence images of acetylated tubulin (red, left) and tyrosinated tubulin (red, right) in 5 DIV differentiated rat PC12 cells overexpressing human α-synuclein-EGFP (grey). (**B**) Overlapping Mander's M1 coefficient of α-synuclein on acetylated tubulin and tyrosinated tubulin or overlapping Mander's M2 coefficient of acetylated tubulin and tyrosinated tubulin on α-synuclein. (**C**) Representative immunofluorescence images of EB3 (red, left) and γ-tubulin (red, right) in 5 DIV differentiated rat PC12 cells overexpressing human α-synuclein-EGFP (grey). (**D**) Overlapping Mander's M1 coefficient of α-synuclein on EB3 and γ-tubulin or overlapping Mander's M2 coefficient of EB3 and γ-tubulin on α-synuclein. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ by Mann-Whitney t test (B, D: N=15-25 cells).

(by Manders' coefficients, M1 = 0.18) and 12% with γ-tubulin, with not significant difference between the two (Fig. 3.1).

This evidence supports the notion that α-synuclein is involved in regulating MT dynamics by being a modulator of dynamic MTs. It further shows that α-synuclein does not preferentially associate with either the plus or the minus end of a MT, suggesting that it may act as a MT "dynamase" [59] by simultaneously regulating polymerization/catastrophe at the plus end and MT nucleation at the minus end.

3.3 α-Synuclein suppression induces accumulation of acetylated and detyrosinated tubulin

We hypothesized that α-synuclein could possibly be involved in the modulation of dynamic MTs as it preferentially colocalizes in proximity of tyrosinated tubulin, a marker of highly dynamic and unstable MTs. Thus, we tested whether α-synuclein suppression in primary neuronal cultures could lead to accumulation of specific tubulin PTMs, markers of long lived and more stable MTs. To this end, we silenced α-synuclein expression with lentiviral delivery of shRNA against rat α-synuclein (Vectorbuilder) in rat hippocampal primary neurons, 7 or 10 DIV, that were cultured up to 14 days to allow for synaptogenesis. Total levels of α-synuclein and selected tubulin PTMs were assessed via Western Blotting (Fig. 3.2). As shown in Fig. 3.2, α-synuclein suppression induced accumulation of detyrosinated tubulin and an increasing trend in acetylated tubulin after 4 and 7 days of knockdown. The total levels of α-tubulin were also increased in the absence of α-synuclein, although not to a significant degree. Finally, we could not detect any difference in the levels of polyglutamylated or Δ2 tubulin.

We concluded that the observed accumulation of acetylated or detyrosinated tubulins, two major indirect markers of MT stability, further supported our hypothesis that α-synuclein acts as an enhancer of MT dynamicity. When acutely suppressed, MTs may increase their lifetime and become

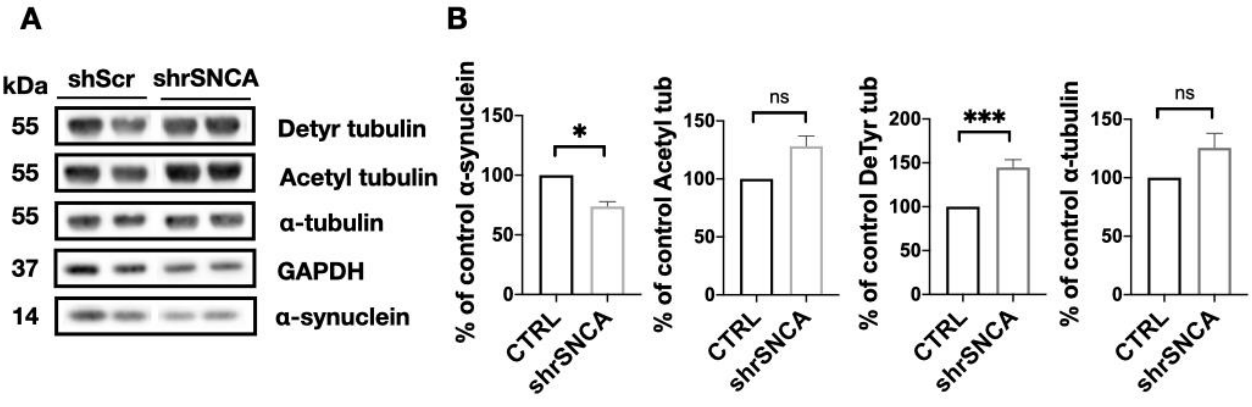

Figure 3.2: **Western blotting quantification of Acetylated and Detyrosinated tubulin in mature hippocampal neurons after α-synuclein knockdown after 7 days of infection with shSNCA or shScramble (CTRL) lentivirus**. Representative western blot bands (**A**) and quantification of the total levels (**B**) of α-synuclein, Acetylated tubulin, Detyrosinated tubulin and α-tubulin, from left to right. GAPDH was used as loading control. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ by Kruskal Wallis test with Dunn's multiple comparisons test (B: N=5 experiments).

more prone to be modified by the enzymes responsible for acetylation or detyrosination, tubulin PTMs that can further increase the stability properties of MTs by changing the mechanical properties of the lattice (acetylation) or inhibiting the binding of depolymerizing kinesins (detryrosination) [65,167].

3.4 α-Synuclein promotes activity-evoked MT nucleation at en passant boutons

Next, we investigated whether and how α-synuclein modulates presynaptic MT dynamics. Here we employed embryonic (E18) rat pyramidal glutamatergic neurons differentiated in culture for the following reasons: i) γ-tubulin is localized at pre-synaptic sites and γ-tubulin-mediated pre-synaptic MT nucleation controls neurotransmission in hippocampal neurons [10], ii) glutamatergic pyramidal neurons express high levels of α-synuclein [168] and iii) the hippocampus and the limbic system are regions involved in PD neurodegeneration at later stages of the disease [169].

For this experiment, α-synuclein expression was silenced by lentiviral delivery of shRNA against rat α-synuclein (Vectorbuilder) at 11-14 DIV for 7 days. 24-36 h before live imaging, neurons were transfected with EB3-EGFP and VGluT1-mCherry, markers of MT growing plus ends and *en passant* boutons, respectively. 4-6 h before imaging, neurons were exposed to 50 μM D-AP5 (NMDA inhibitor) to silence basal neuronal activity in excitatory glutamatergic neurons and, after AP5 washout, 20 μM Bicucullin ($GABA_A$ antagonist) was added to the imaging medium. Bicucullin

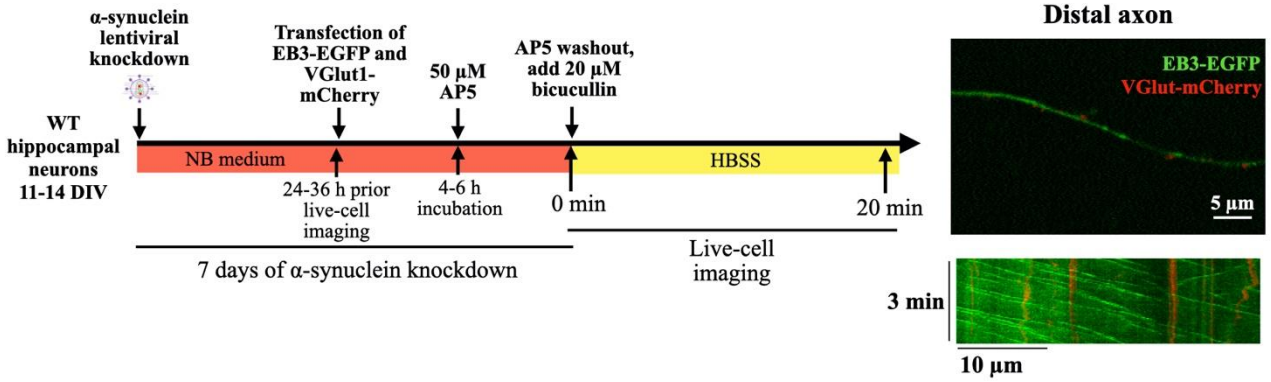

Figure 3.3: Schematic representation of the experimental timeline for the MT nucleation assay. Rat α-synuclein expression was knocked down by lentiviral delivery of shRNA prior to transfecting neurons with EB3-EGFP and VGluT1-mCherry to visualize MT plus end and *en passant* glutamatergic boutons, respectively. Neurons were pre-treated (4-6 h) with the NMDA inhibitor AP5 and live imaging was performed in the distal region of the axon (> 100 μm from the cell body) upon neuronal stimulation with the GABA receptor inhibitor Bicucullin.

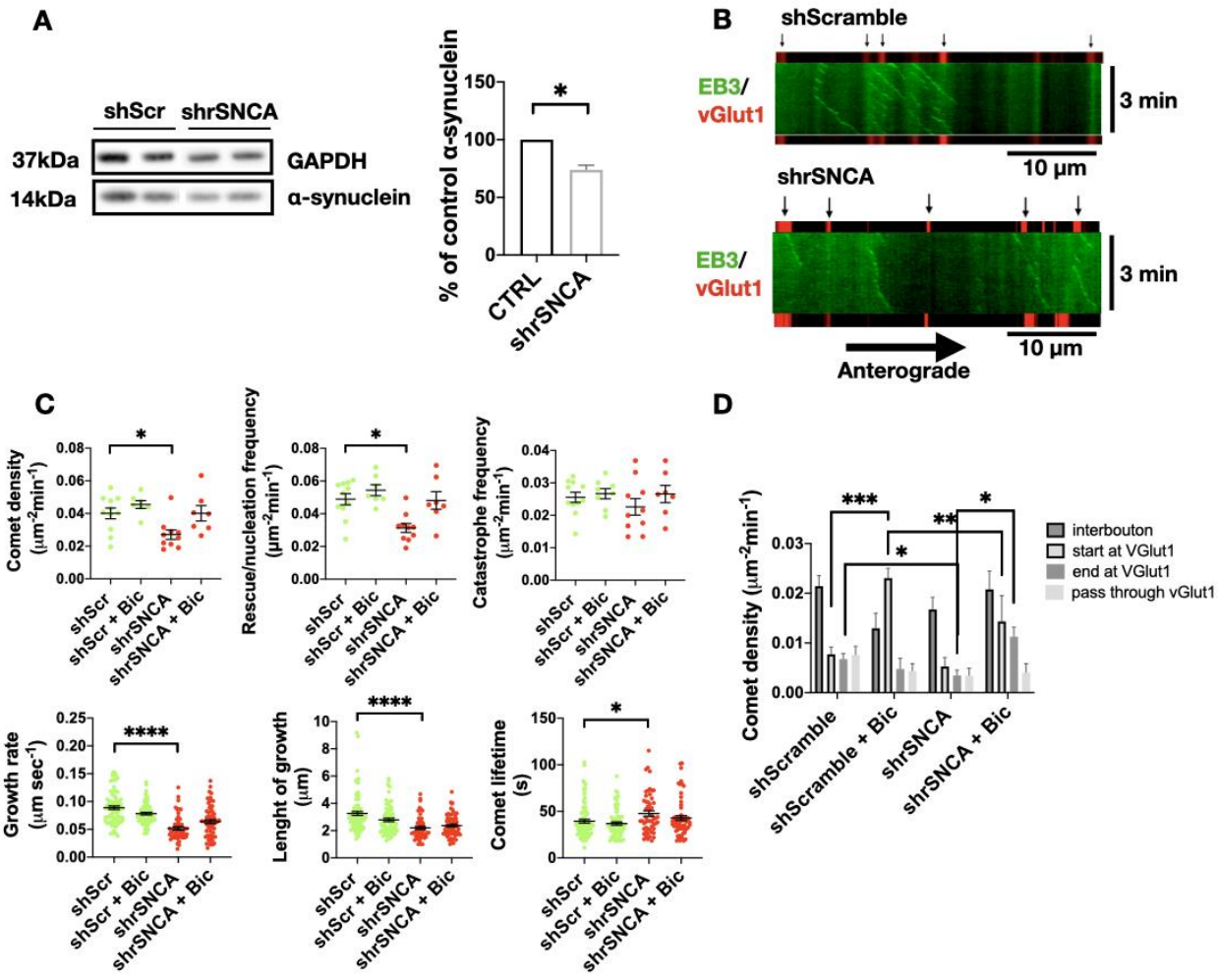

Figure 3.4: **MT nucleation assay and MT dynamics in α-synuclein knockdown hippocampal neurons.** (**A**) Quantitative Western blot analysis of α-synuclein levels in hippocampal neurons (18-21 DIV) infected with either scramble control (shScramble) or shRNA lentivirus to silence rat α-synuclein (shrSNCA) for 7 days. (B) Representative kymographs of EB3 comets in selected distal regions of the axons in hippocampal neurons treated as in A. For live-imaging of presynaptic MTs, neurons were transfected with EB3-EGFP and VGluT1-mCherry 24-36 h prior to live imaging and pretreated with AP5 for 4-6 h. (**C**) MT dynamics parameters from kymograph analysis of EB3 comets in axons of hippocampal neurons treated as in (**B**) or incubated with bicuculline for 1–20 min (+Bic) after washout. (**D**) Quantification of subclassified EB3 comet density relative to stable VGluT1+ puncta of hippocampal neurons treated as in (**C**). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ by Kruskal Wallis test with Dunn's multiple comparisons test (C: N=5 experiments; B: N=7-10 axons), Mann-Whitney t tests (D: N=7-10 axons).

suppresses the inhibitory stimuli coming from hippocampal GABAergic interneurons present in the culture, thus stimulating the rising of action potentials in pyramidal neurons. Live imaging recording of EB3 comet dynamics was performed with an epifluorescence microscope equipped with a 60x objective in the distal region of the axon (> 100 µm from the cell body) for no more than 20 min after AP5 washout with or without Bicucullin. MT dynamics parameters were calculated by measuring

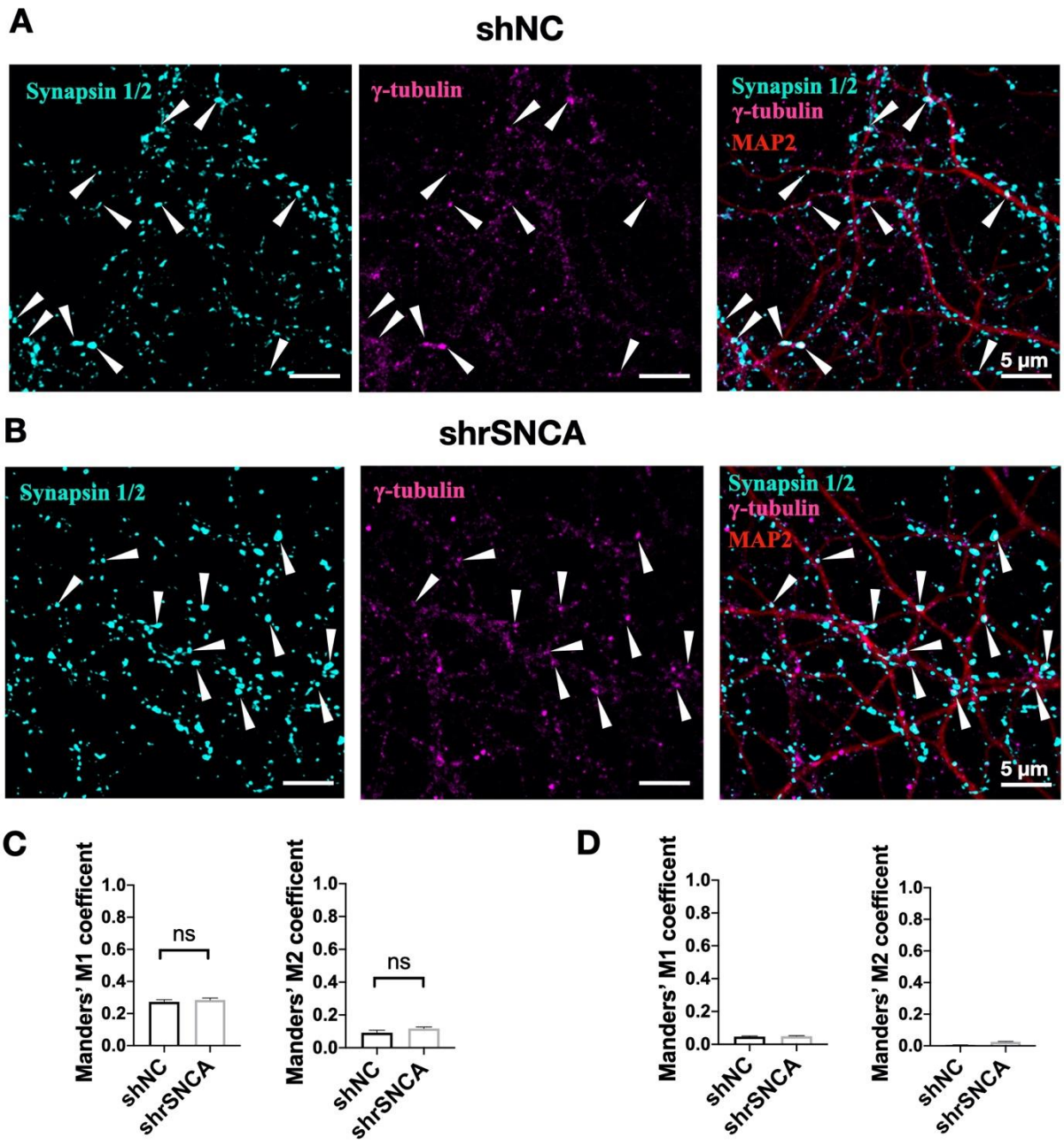

Figure 3.5: **Immunostaining of γ-tubulin and synapsin 1/2 in mature rat hippocampal neurons after infection with shRNA against α-synuclein or sh non-coding control for 7 days.** (**A**) Mature hippocampal neurons infected with shNC immunostained with synapsin 1/2 (cyan), γ-tubulin (magenta) and merge (pointed by white arrows) with MAP2 (red), from left to right. (**B**) Mature hippocampal neurons infected with shSNCA immunostained with synapsin 1/2 (cyan), γ-tubulin (magenta) and merge (pointed by white arrows) with MAP2 (red), from left to right. (**C**) Overlapping Mander's M1 coefficient of γ-tubulin on synapsin 1/2 or overlapping Mander's M2 coefficient of synapsin 1/2 on γ-tubulin. (**D**) Overlapping Mander's M1 and M2 coefficient obtained after +/- 90° rotation cross-correlation analysis of the same images analyzed in (**C**). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ by Mann-Whitney t test (B: N=20 fields of view).

EB3-EGFP movement as published [170]. Fast time-lapse imaging of EB3-labeled MT plus ends allows the quantitative assessment of MT dynamics at or away from individual glutamate release sites labeled with VGluT1-mCherry. Pre-synaptic dynamic MTs were distinguished based on their plus-end contact with VGluT1+ boutons and were defined as (1) *interbouton,* MTs that have no contact with the presynaptic markers, or (2) *intrabouton*, MTs that interact with the pre-synaptic SV marker at any point during their lifetime. Intrabouton MTs were further classified as (1) nucleating/rescuing (starting) at boutons, (2) undergoing catastrophe/pausing (ending) at the boutons, or (3) passing through the bouton (Fig. 3.4 D) [10]. For general MT dynamics interbouton and intrabouton MTs were pooled together (Fig. 3.4 C).

I observed that silencing α-synuclein expression caused steady-state reduction in the total density of growing MT ends and that neuronal firing restored this parameter to values comparable to the non-stimulated control (Fig. 3.4 C). We also analysed the effect of suppressed α-synuclein expression on pre-synaptic MT dynamics. Upon evoked-activity we observed a clear reduction in stimulated MT nucleation at pre-synaptic boutons compared to control neurons, indicating that α-synuclein acts as an enhancer of evoked MT nucleation at sites of neurotransmitter release. Interestingly, loss of α-synuclein also lead to an increase in the number of dynamic MTs reaching to a more distal bouton (Fig. 3.4 D) whereas no effect was measured in the density, size and interbouton distance. Moreover, we immunostained mature rat hippocampal neurons after lentiviral delivery of shRNA against α-synuclein in order to verify that α-synuclein suppression did not provoke a shift in the synaptic localization of the MT nucleator γ-tubulin [10]. No significant change in the overlapping of γ-tubulin (around 30%) on the synaptic marker synapsin 1/2 (M1) was seen nor in the total number of synapses containing γ-tubulin (around 15%) (M2). We established the reliability of our staining with a cross-correlation analysis by rotating the analyzed images +/- 90° and recalculating the to (Fig. 3.5 C). Manders' coefficient afterwards. As expected, the Manders's coefficients decreased to values close to zero (Fig. 3.5 D).

These results demonstrate that in excitatory hippocampal neurons α-synuclein is a positive regulator of MT dynamics upon activity-evoked MT nucleation at presynaptic sites, indicating that α-synuclein acts as an axonal "dynamase". They also suggest that, by preventing more comets from reaching a more distal bouton, α-synuclein may also act as an inhibitor of KIF1A-mediated SV precursor drop off at distal sites of release, a mechanism that sustains synaptic strength in hippocampal neurons by allowing high-precision motor detachment at the pre-synapse [171].

3.5 Dopaminergic neuron MT dynamics show features of other CNS neurons

Surprisingly, neither basal not activity-evoked MT dynamics has ever been described in DA neurons. To do this, we employed DAT-Td-tomato P1-P3 postnatal mouse ventral midbrain neurons that we obtained through our collaboration with Dr. David Sulzer's lab. These neurons express Td-tomato under a DAT promoter to allow for visualization of DA in culture. Differentiated DA neurons in culture (11 DIV to 14 DIV) were infected 36-48 h with EB3-EGFP lentivirus prior to live and post-fixation immunostaining with the axonal initial segment (AIS) marker ankyrin G was performed to relocate and distinguish axons from dendrites (Fig. 3.6 A). We found that in DA neurons MTs were more dynamic and with a higher nucleation/rescue rate in dendrites compared to the axons while no difference was observed in catastrophe rates or MT dynamicity in the proximal (<100 μm from cell body) and distal region of either axons or dendrites (Fig. 3.6 B). As is the case with pyramidal neurons, MT orientation was mixed in the dendrites with an increasing percentage of anterograde comets in the distal regions. In the axons (Fig. 3.6 C) all comets were mono-oriented toward the distal tip of the neurite.

Altogether, our data demonstrate that the comet density and rescue/nucleation frequency in axons and dendrites of DA neurons appear to be comparable with what was previously observed in hippocampal neurons [10]. Interestingly, the catastrophe frequency in DA neurons revealed to be consistently lower than in hippocampal neurons (almost 1.5 fold decrease) both in axons and dendrites [10] indicating that MTs are more stable in this neuronal subtype and less susceptible to shrinking.

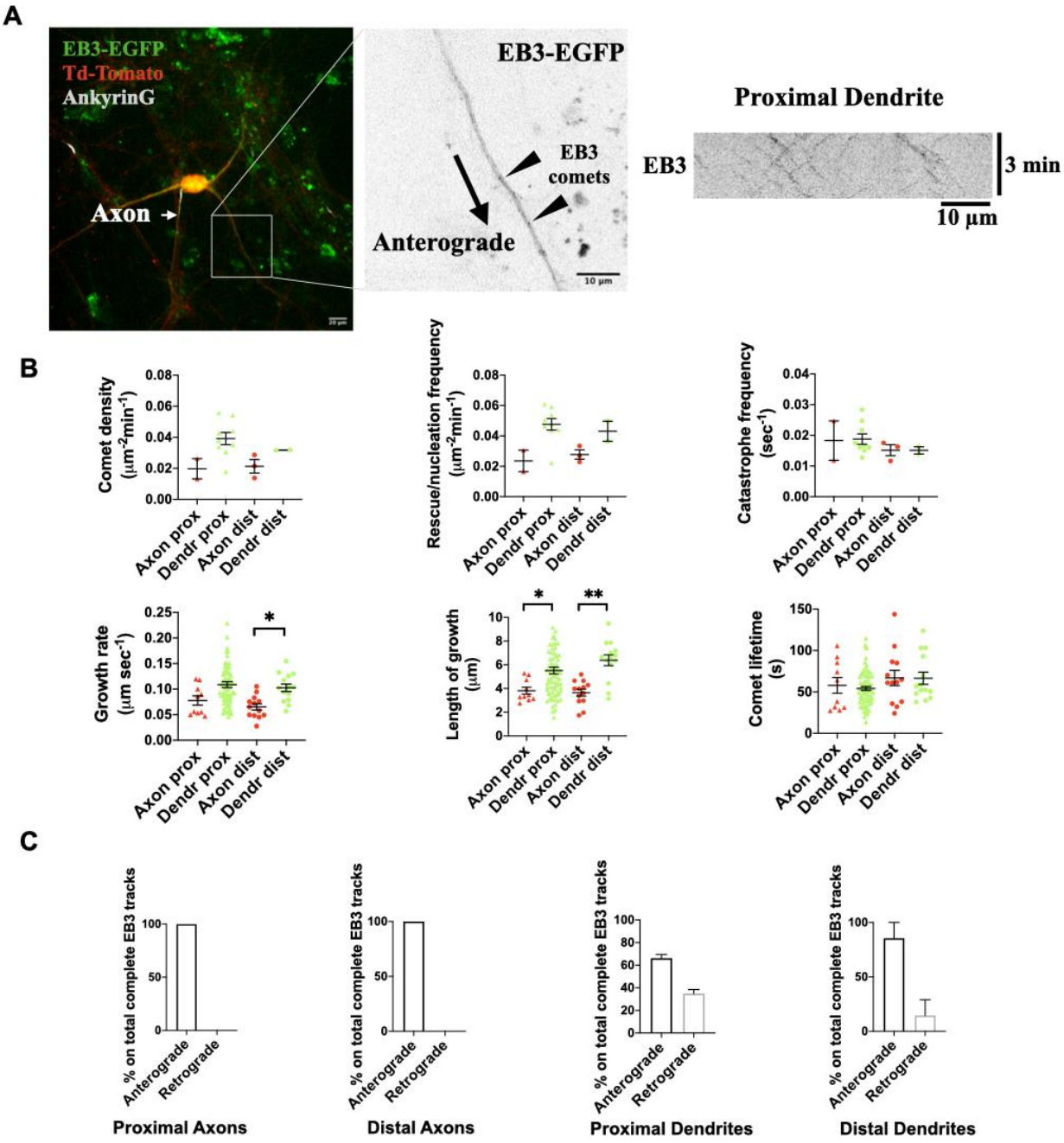

Figure 3.6: **MT dynamics in untreated mature mouse DA neurons.** (**A**) Representative single frame from a time-lapse movie and relative kymograph of a boxed region on a proximal dendrite of a DAT-Td-Tomato DA neuron (right). Post-fixation immunostaining with ankyrinG to highlight the axon (left). (**B**) Parameters of MT dynamics derived from kymograph analysis of EB3 comet movement in axons and dendrites of DAT-Td-Tomato DA neurons as in A. (**C**) EB3 comet orientation in proximal (< 100 µm) and distal (> 100 µm) regions of axons and dendrites. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ by Kruskal Wallis tests with Dunn's multiple comparisons tests (B, C: N=2-3 axons N=2-10 dendrites).

3.6 α-Synuclein positively regulates MT dynamics in ventral midbrain neurons

Next, we examined whether α-synuclein regulates MT dynamics also in ventral midbrain (VM) neurons. For this purpose, 36-48 h before live imaging we infected WT and α-synuclein KO VM cultures with lentiviral EB3-EGFP (Fig. 3.7 A). As we observed in hippocampal neurons, loss of α-synuclein reduced comet density, rescue/nucleation and catastrophe frequency in neurites (Fig. 3.7 B), suggesting that α-synuclein acts as a MT "dynamase" also in DA neurons by promoting MT nucleation, elongation and shrinking of MTs.

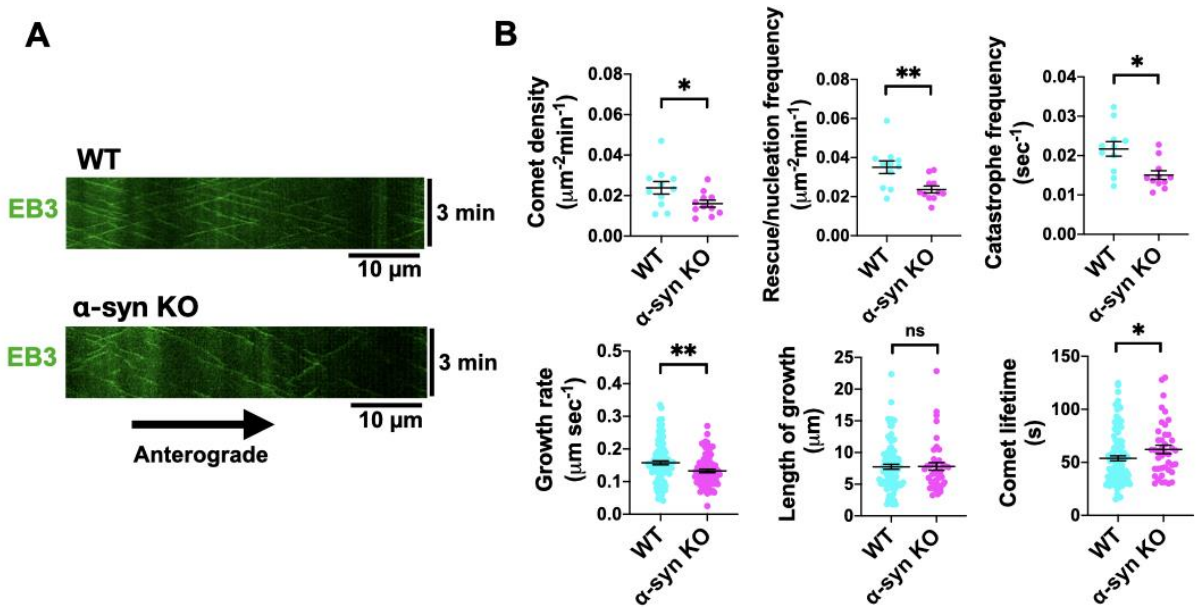

Figure 3.7: **MT dynamics in mouse ventral midbrain neurons in WT vs α-synuclein-KO** (**A**) Representative kymographs of EB3 comets in neurites of WT and α-synuclein KO mouse ventral midbrain neurons (11-14 DIV) infected with EB3-EGFP lentivirus 36-48 h prior to live imaging. (**B**) Parameters of MT dynamics derived from kymograph analysis of EB3 comet movement in WT and α-synuclein-KO mouse ventral midbrain mature neurons infected as in (**A**). * p<0.05; ** p<0.01; *** p<0.001 by Mann-Whitney t test (B: N=11 neurites).

4. Conclusions and Future Perspectives

The evidence reported in this study clearly demonstrate that, in mammalian neurons, a physiological interaction between α-synuclein and α-tubulin occurs at the pre-synapse and that α-synuclein is able to positively regulate MT dynamics as well as MT nucleation at sites of neurotransmitter release.

The direct α-synuclein/α-tubulin association was shown to be localized in human healthy subjects and WT murine striatum and to mostly occur within the synaptic compartment in physiological conditions [60]. Then, we tested whether α-synuclein was localized at the MT plus or minus end, and whether there was a preferential localization of α-synuclein for a particular subset of MTs. We found that α-synuclein colocalizes both with EB3 and γ-tubulin, marker of plus and minus end, respectively. We also detected that α-synuclein has also a preferential colocalization with tyrosinated tubulin, marker of highly dynamic MTs [172] compared to acetylated tubulin, marker instead of stable and mechanically resistant MTs [67,69]. Although we cannot rule out the presence of α-synuclein along the MT shaft, our results suggest that α-synuclein colocalizes with no specificity at sites of tubulin nucleation and polymerization as well as being more likely accumulated in proximity of dynamic MTs.

Next, we investigated whether and how α-synuclein modulates presynaptic MT dynamics upon neuronal firing. Fast time-lapse imaging of EB3-labeled MT growing ends showed that, in the distal region of hippocampal axons, α-synuclein suppression causes a steady-state reduction in the total density of growing MT ends and that neuronal firing restored this parameter to values comparable to the non-stimulated control. We also analysed the effect of α-synuclein silencing on presynaptic MTs. Upon evoked-activity we observed a clear reduction in stimulated MT nucleation at presynaptic boutons, indicating α-synuclein as positive regulator of synaptic MT nucleation in excitatory neurons. Interestingly, α-synuclein seems to decrease also the number of MTs reaching the next boutons suggesting that it may also act as an inhibitor of KIF1A-mediated SV drop off at more distal sites of release [89]. In support of our hypothesis, we performed western blotting analysis after suppression of α-synuclein expression in glutamatergic rat hippocampal neurons, finding that there was a consistent increase of the total levels of detyrosinated tubulin, thus indicating that silencing the effect of α-synuclein on MT dynamics leads to MT stabilization. In accordance with a previous study [173], an increasing trend in the amount of total tubulin was also revealed, suggesting an upregulation of tubulin expression due to a reduced tendency of MTs to depolymerize. When α-synuclein is acutely suppressed, MTs increase their lifetime and become more prone to be modified by the enzymes

responsible for acetylation or detyrosination, tubulin PTMs that can further increase the stability properties of MTs by enhancing their mechanical resistance (acetylation) and inhibiting the binding of depolymerizing kinesins (detryrosination) [167].

The finding that α-synuclein promotes MT dynamics in hippocampal neurons does not necessarily implicate that the same effect will be seen in DA neurons, since, as we showed, its effect is strictly related with firing. DA neurons, indeed, have a totally different pattern of neuronal activity showing a tonic response to stimuli but also a spontaneous pacemaker ability to evoke action potentials. Moreover, some varicosities in DA neurons are not synaptic, they have a consistent number of silent varicosities [174,175] and the active ones has revealed a volumetric type of release of the neurotransmitter [176,177]. These particular features might determine a different role of presynaptic MTs in this neuronal subtype. Indeed, the role of presynaptic MTs in tonically active DA neurons with a volumetric neurotransmitter release remains unexplored. Additionally, the extensive axonal arbor of DA neurons compared to other CNS neurons opens to the possibility that MT dynamics might be different to what was previously found in different types of neurons. To check this possibility, we characterized the MT dynamics of axons and dendrites in unstimulated DA neurons that, surprisingly, was never described before. We found that, overall, MTs were more dynamic in DA neurons while there was a consistency in the uniform orientation of the plus end toward the growth cone in axons and a mixed polarity in the dendrites, compared to hippocampal neurons [10]. Then, we investigated for the first time whether α-synuclein has a role in regulating MT dynamics in VM neurons. Our results reported that, in α-synuclein KO VM neurites, there was a consistent reduction of the nucleation/rescue as well as the catastrophe rate and a total decrease of the number of MT growing ends, thus suggesting that α-synuclein might be an MT-dynamase also in DA neurons, although the effect on presynaptic MTs has not been investigated yet.

Based on this collective evidence, we propose a model where α-synuclein directly affects tubulin polymerization, acting as an axonal enhancer of MT dynamics and whose effect is strictly related to neuronal activity. Although our cell culture system can only partially simulate the signalling network of the brain, in this study we are still considering a way greater level of complexity compared to the previous *in vitro* experiments [59] by considering the whole cellular machinery of the neuron. Since the cellular integrity and functioning are preserved, we cannot rule out that α-synuclein might partially exert its function indirectly, for example through modulation of MAPs activity, interacting with motor proteins or tubulin-modifying enzymes. Additionally, since this model is likely to be dependent on the pattern of neuronal firing, we cannot state yet that the positive effect on presynaptic nucleation can be translated also to DA neurons although its effect on MT polymerization seems to be retained. Indeed, our observation that α-synuclein regulates MT dynamics is in line with the

finding that it was shown to act as a "dynamase" [59] that induces polymerization of MTs *in vitro* system of purified tubulin [161] and that presynaptic boutons are enriched in MT plus ends [171]. Notwithstanding, our results differ from this last study since we observed that, i) only MT nucleation (initiation), but not catastrophe/pausing (termination), is preferentially regulated at boutons at basal levels and ii) we see an increase in the MT ending at the presynaptic boutons upon neuronal firing in absence of α-synuclein. This could be explained by the fact that α-synuclein may compete with KIF1A for the binding site to GTP-tubulin, thus inhibiting the mediated SV drop off at more distal sites of release.
In this context, we soon aim to dissect the effect of α-synuclein on presynaptic MT nucleation in DA neurons, in order to identify whether α-synuclein is involved in the regulation of dopamine release through an MT-mediated mechanism. Putative differences on how this mechanism sustains DA neurotransmission may shed light on the selective vulnerability of DA neurons of the SNpc to α-synuclein aggregation and synaptic impairment. Moreover, at present, whether dysregulation of tubulin modulating activity by α-synuclein PD variants contributes to the synaptic pathology in PD has not been elucidated. These findings pave the way for the exploration the putative corruption of the tubulin-regulating activity of PD-mutated forms of α-synuclein, thus correlating early PD synaptic dysfunction with α-synuclein mutations through a tubulin-mediated mechanism.

5. Materials and Methods

1.3 PC12 cells

Rat pheochromocytoma PC12 cells were seeded onto poly-L-lysine coated coverslips and differentiated for 2 or 5 days with 50 ng/ml of human β-NGF. Cells were then transfected with α-synuclein-GFP or GFP control vectors (Addgene) by using Lipofectamine 2000 (Invitrogen).

1.4 Primary hippocampal neurons

Primary hippocampal neuronal cultures were prepared as previously described [166]. Briefly, hippocampi were dissected from E18 rats, and neurons plated on 100 μg/mL poly-D-lysine-coated 12-well-plates at the density of $3x10^5$ cells/well for biochemistry assays, $7x10^4$ cells/dish for live imaging in the chamber of 35 mm MatTek dishes, or $4x10^4$ cells/coverslip on 18 mm coverslips for immunofluorescence. Primary neurons were maintained in Neurobasal medium (Invitrogen) with the supplement of 2% B-27 (Invitrogen) and 0.5 mM glutamine (Invitrogen) at 37°C, and 300 μl of the medium was changed every 7 days up to 3 weeks in culture.

1.5 Lentivirus preparation

Production of lentiviral particles was conducted using the 2nd generation packaging system as previously described [166]. Briefly, HEK293T were co-transfected with lentiviral shRNA constructs and the packaging vectors pLP1, pLP2, and pLP-VSV-G (Invitrogen) using calcium phosphate. 24 h, 36 h and 48 h after transfection, the virus was collected, filtered through 0.45 mm filter, and further concentrated with lentiviral precipitation solution (ALSTEM) as recommended by the manufacturer. Concentrated virus was aliquoted and stored at -80°C.

Lentiviral construct to knockdown rat α-synuclein was purchased from Vectorbuilder with the following DNA sense strand sequence 5'- GTGCTGTGAAATTTGTTAATA - 3' onto a mammalian shRNA knockdown lentivector. The scramble control lentiviral vector was purchased from Vectorbuilder. Lentiviral construct to knockdown mouse α-synuclein was purchased from Sigma

Aldrich (TRCN0000366591) with the following DNA sense strand sequence 5'-GATCCTGGCAGTGAGGCTTAT - 3' onto pLKO.5 lentivector. The pLKO.5 vector with noncoding (NC) sequence (SHC202) was used as control. Lentiviral EB3-EGFP was generated by subcloning EB3-EGFP (a gift from Franck Polleux) into pLVX lentivector by Afe1 and Not1 sequential digestions.

1.6 Immunofluorescence microscopy and analysis

For hippocampal primary neurons and PC12 cells staining, cells were fixed in 4% PFA + 4% sucrose diluted in PBS. Permeabilization was performed with 0.2% Triton X-100 for 10 min and the blocking step was carried out by incubating with 3% BSA for 30 min. Staining with primary antibodies was done overnight at 4°C followed by 2 h incubation with secondary antibodies. Mounted samples were observed, with IX83 Andor Revolution XD Spinning Disk Confocal, using a 60x objective. All images were analyzed by ImageJ. The degree of colocalization of different antigens was calculated by Manders' coefficients, computed with the ImageJ JACoP plug-in [167].

1.7 Live-cell imaging

Hippocampal neurons grown on MatTek dishes were co-transfected with EB3-EGFP using Lipofectamine 2000CD (Invitrogen), together with the presynaptic markers VGluT1-mCherry. Live cell imaging was performed 24-36 h after transfection in complete HBSS media (HBSS, 30 mM glucose, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 4 mM $NaHCO_3$, and 2.5 mM HEPES, pH 7.4) using epifluorescence microscope (Nikon Ti) equipped with an Orca II ER charge-coupled device (CCD) camera (Hamamatsu, Japan) and a temperature-controlled (37 °C) CO_2 incubator using a 60×/1.40NA objective. Movies were acquired at 2 s/frame for 3 min. Maximum projections of movies were performed and analysed in ImageJ and single plane images of VGluT1-labeled boutons were taken right before and after the time-lapse movie of EB3-labeled comets was acquired. Kymographs were generated by drawing a region in the distal (>100 μm from the cell body) axon and axon were selected based on morphology and anterograde movement of EB3-labeled comets. For pharmacological induction of neuronal activity, neurons were pre-treated with 50 μM D-AP5 for 4-6 h prior to live

imaging in complete neurobasal media. To induce neuronal activity, neurons were washed 3x with complete HBSS media and 20 μM bicuculline or DMSO control was added to complete HBSS media after the washes. Time-lapse movies were taken 20 min upon treatment. Presynaptic MTs were classified based on their plus end contacts with stable VGluT1 labeled boutons. In our measurements of EB3 tracks starting or ending at boutons, we also included those that start or end at a bouton and pass through the next distal bouton.

Postnatal ventral midbrain DA neurons were prepared in the Sulzer's lab according to their published protocol [168]. Neurons were infected with EB3-EGFP overexpression lentivirus prepared as described above. Live cell imaging was performed 36-48 h after infection in complete HBSS media using IX83 Andor Revolution XD Spinning Disk Confocal System. The microscope was equipped with a 60x oil UApo objective, a multi-axis stage controller (ASI MS-2000), and a controlled temperature and CO_2 incubator. Movies were acquired with an Andor iXon Ultra EMCCD camera and Andor iQ 3.6.2 live cell imaging software at 2 s/frame for 3 min. Maximum projections of movies were performed and analysed in ImageJ. Kymographs were generated by drawing a region in the proximal (<100 μm from the cell body) or the distal (>100 μm) axons and/or dendrites based on post-fixation immunostaining for ankyrin G as described above.

Parameters describing MT dynamics were defined as follows: *i)* rescue/nucleation frequency is the number of rescue or nucleation events per μm^2 per min; *ii)* catastrophe frequency is the number of full tracks/total duration of growth; *iii)* comet density is the number of comets per μm^2 per min; *iv)* growth length is the comet movement length in μm; *v)* comet lifetime is the duration of growth; *vi)* growth rate is the growth length/comet lifetime. Last, % of retrograde tracks indicates the number of retrograde tracks/number of total moving tracks x 100 [169].

1.8 Western blot

Cells were lysed in Laemmli sample buffer and boiled at 96°C for 5 min. Cell lysates were sonicated by a probe sonicator to sheer cellular debris and genomic DNA. Proteins were separated by 10% Bis-Tris gel (Invitrogen) and transferred onto nitrocellulose membrane. After blocking in 3% BSA/TBS, membranes were incubated with primary antibodies for 2 h at room temperature or at 4°C overnight prior to 1 h incubation with infrared secondary antibodies (LI-COR Biosciences). Image acquisition was performed with an Odyssey imaging system (LI-COR Biosciences) and analyzed with Odyssey software.

1.9 Quantification and statistical analysis

Data are shown as means ± standard error of the mean (SEM) and from at least 3 independent experiments. Image analysis was performed by ImageJ (Fiji), and Andor iQ3. Western blot analysis was performed by LI-COR Image Studio Software. Statistical analysis between two groups was performed using Mann-Whitney tests (Fig. 3.1 B, D; Fig. 3.4 D; Fig. 3.5 C, D; Fig. 3.7 B). Comparison among three or more groups was performed using the Kruskal-Wallis test with Dunn's multiple comparisons test for non-parametric unpaired one-way ANOVA tests (Fig. 3.2 B; Fig. 3.4 A, C; Fig. 3.6 B). Statistical analysis and graph generation was performed with GraphPad Prism 9.0.0 software. Statistical significance was set for $p < 0.05$.

References

1. Dent, E. W., Merriam, E. B. & Hu, X. The dynamic cytoskeleton: backbone of dendritic spine plasticity. *Curr. Opin. Neurobiol.* **21**, 175–181 (2011).
2. Letourneau, P. C. Actin in axons: Stable scaffolds and dynamic filaments. *Results Probl. Cell Differ.* **48**, 65–90 (2009).
3. Ladt, K., Ganguly, A. & Roy, S. Axonal actin in action: Imaging actin dynamics in neurons. *Methods Cell Biol.* **131**, 91 (2016).
4. Herrmann, H. & Aebi, U. Intermediate Filaments: Structure and Assembly. *Cold Spring Harb. Perspect. Biol.* **8**, (2016).
5. Lobsiger, C. S. & Cleveland, D. W. Neurofilaments: Organization and Function in Neurons. *Encycl. Neurosci.* 433–436 (2009) doi:10.1016/B978-008045046-9.00728-2.
6. Yuan, A., Rao, M. V., Veeranna & Nixon, R. A. Neurofilaments and Neurofilament Proteins in Health and Disease. *Cold Spring Harb. Perspect. Biol.* **9**, (2017).
7. Evans, L., Mitchison, T. & Kirschner, M. Influence of the centrosome on the structure of nucleated microtubules. *J. Cell Biol.* **100**, 1185 1191 (1985).
8. Desai, A. & Mitchison, T. J. MICROTUBULE POLYMERIZATION DYNAMICS. *Annu. Rev. Cell Dev. Biol* **13**, 83–117 (1997).
9. Sánchez-Huertas, C. *et al.* Non-centrosomal nucleation mediated by augmin organizes microtubules in post-mitotic neurons and controls axonal microtubule polarity. *Nat. Commun.* **7**, (2016).
10. Qu, X., Kumar, A., Blockus, H., Waites, C. & Bartolini, F. Activity-Dependent Nucleation of Dynamic Microtubules at Presynaptic Boutons Controls Neurotransmission. *Curr. Biol.* **29**, 4231-4240.e5 (2019).
11. Conde, C. & Cáceres, A. Microtubule assembly, organization and dynamics in axons and dendrites. *Nat. Rev. Neurosci. 2009 105* **10**, 319–332 (2009).
12. Mitchison, T. & Kirschner, M. Dynamic instability of microtubule growth. *Nat. 1984 3125991* **312**, 237–242 (1984).
13. Kapitein, L. C. & Hoogenraad, C. C. Building the Neuronal Microtubule Cytoskeleton. *Neuron* vol. 87 492–506 (2015).
14. Burbank, K. S. & Mitchison, T. J. Microtubule dynamic instability. *Curr. Biol.* **16**, R516–R517 (2006).
15. Nogales, E. A structural view of microtubule dynamics. *Cell. Mol. Life Sci.* **56**, 133–142 (1999).
16. Barbier, P. *et al.* Role of tau as a microtubule-associated protein: Structural and functional aspects. *Front. Aging Neurosci.* **10**, 204 (2019).
17. Akhmanova, A. & Hoogenraad, C. C. Microtubule minus-end-targeting proteins. *Curr. Biol.* **25**, R162–R171 (2015).
18. Roth, D., Fitton, B. P., Chmel, N. P., Wasiluk, N. & Straube, A. Spatial positioning of EB family proteins at microtubule tips involves distinct nucleotide-dependent binding properties. *J. Cell Sci.* **132**, (2019).
19. Mustyatsa, V. V. *et al.* Fine structure and dynamics of EB3 binding zones on microtubules in fibroblast cells. *Mol. Biol. Cell* **30**, 2105 (2019).
20. Kuo, Y. W., Trottier, O., Mahamdeh, M. & Howard, J. Spastin is a dual-function enzyme that severs microtubules and promotes their regrowth to increase the number and mass of microtubules. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 5533–5541 (2019).
21. Liu, Q., Zhang, G., Ji, Z. & Lin, H. Molecular and cellular mechanisms of spastin in neural development and disease (Review). *Int. J. Mol. Med.* **48**, 1–13 (2021).

22. Luptovčiak, I., Komis, G., Takáč, T., Ovečka, M. & Šamaj, J. Katanin: A sword cutting microtubules for cellular, developmental, and physiological purposes. *Front. Plant Sci.* **8**, 1982 (2017).
23. Hirokawa, N. & Noda, Y. Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics. *Physiol. Rev.* **88**, 1089–1118 (2008).
24. Guedes-Dias, P. *et al.* Kinesin-3 Responds to Local Microtubule Dynamics to Target Synaptic Cargo Delivery to the Presynapse. *Curr. Biol.* **29**, 268-282.e8 (2019).
25. Roberts, A. J., Kon, T., Knight, P. J., Sutoh, K. & Burgess, S. A. Functions and mechanics of dynein motor proteins. *Nat. Rev. Mol. Cell Biol. 2013 1411* **14**, 713–726 (2013).
26. Baas, P. W. Neuronal polarity: microtubules strike back. *Nat. Cell Biol. 2002 48* **4**, E194–E195 (2002).
27. Kuijpers, M. & Hoogenraad, C. C. Centrosomes, microtubules and neuronal development. *Mol. Cell. Neurosci.* **48**, 349–358 (2011).
28. Barnes, A. P. & Polleux, F. Establishment of Axon-Dendrite Polarity in Developing Neurons. *Annu. Rev. Neurosci.* **32**, 347 (2009).
29. Burute, M. & Kapitein, L. C. Annual Review of Cell and Developmental Biology Cellular Logistics: Unraveling the Interplay Between Microtubule Organization and Intracellular Transport. (2019) doi:10.1146/annurev-cellbio-100818.
30. Maday, S., Twelvetrees, A. E., Moughamian, A. J. & Holzbaur, E. L. F. Axonal Transport: Cargo-Specific Mechanisms of Motility and Regulation. *Neuron* **84**, 292–309 (2014).
31. Penazzi, L., Bakota, L. & Brandt, R. Microtubule Dynamics in Neuronal Development, Plasticity, and Neurodegeneration. in *International Review of Cell and Molecular Biology* vol. 321 89–169 (Elsevier Inc., 2016).
32. Parato, J. & Bartolini, F. The microtubule cytoskeleton at the synapse. *Neurosci. Lett.* **753**, (2021).
33. Gu, J., Firestein, B. L. & Zheng, J. Q. Microtubules in Dendritic Spine Development. *J. Neurosci.* **28**, 12120–12124 (2008).
34. Sheng, Z. H. Mitochondrial trafficking and anchoring in neurons: New insight and implications. *J. Cell Biol.* **204**, 1087–1098 (2014).
35. Waites, C., Qu, X. & Bartolini, F. The synaptic life of microtubules. *Curr. Opin. Neurobiol.* **69**, 113–123 (2021).
36. Yau, K. W. *et al.* Dendrites In Vitro and In Vivo Contain Microtubules of Opposite Polarity and Axon Formation Correlates with Uniform Plus-End-Out Microtubule Orientation. *J. Neurosci.* **36**, 1071–1085 (2016).
37. Meka, D. P., Scharrenberg, R. & Calderon de Anda, F. Emerging roles of the centrosome in neuronal development. *Cytoskeleton (Hoboken).* **77**, 84–96 (2020).
38. Nguyen, M. M., Stone, M. C. & Rolls, M. M. Microtubules are organized independently of the centrosome in Drosophila neurons. *Neural Dev.* **6**, 1–16 (2011).
39. Cunha-Ferreira, I. *et al.* The HAUS Complex Is a Key Regulator of Non-centrosomal Microtubule Organization during Neuronal Development. *Cell Rep.* **24**, 791–800 (2018).
40. Yu, W. *et al.* The microtubule-severing proteins spastin and katanin participate differently in the formation of axonal branches. *Mol. Biol. Cell* **19**, 1485–1498 (2008).
41. Bahi-Buisson, N. & Maillard, C. Tubulinopathies Overview. *GeneReviews®* (2021).
42. Solowska, J. M. *et al.* Pathogenic Mutation of Spastin Has Gain-of-Function Effects on Microtubule Dynamics. *J. Neurosci.* **34**, 1856 (2014).
43. Brady, S. T. & Morfini, G. A. Regulation of motor proteins, axonal transport deficits and adult-onset neurodegenerative diseases. *Neurobiol. Dis.* **105**, 273–282 (2017).
44. Strang, K. H., Golde, T. E. & Giasson, B. I. MAPT mutations, tauopathy, and mechanisms of neurodegeneration. *Lab. Invest.* **99**, 912–928 (2019).
45. Cartelli, D. & Cappelletti, G. Microtubule Destabilization Paves the Way to Parkinson's

Disease. *Molecular Neurobiology* vol. 54 6762–6774 (2017).
46. Sferra, A., Nicita, F. & Bertini, E. Microtubule dysfunction: A common feature of neurodegenerative diseases. *International Journal of Molecular Sciences* vol. 21 1–36 (2020).
47. Dubey, J., Ratnakaran, N. & Koushika, S. P. Neurodegeneration and microtubule dynamics: death by a thousand cuts. *Front. Cell. Neurosci.* **9**, 343 (2015).
48. Simons, C. *et al.* A de novo mutation in the β-tubulin gene TUBB4A results in the leukoencephalopathy hypomyelination with atrophy of the basal ganglia and cerebellum. *Am. J. Hum. Genet.* **92**, 767–773 (2013).
49. Tischfield, M. A. *et al.* Human TUBB3 mutations perturb microtubule dynamics, kinesin interactions, and axon guidance. *Cell* **140**, 74–87 (2010).
50. Smith, B. N. *et al.* Exome-wide Rare Variant Analysis Identifies TUBA4A Mutations Associated with Familial ALS. *Neuron* **84**, 324–331 (2014).
51. Niwa, S., Takahashi, H. & Hirokawa, N. β-Tubulin mutations that cause severe neuropathies disrupt axonal transport. *EMBO J.* **32**, 1352–1364 (2013).
52. Pellegrini, L., Wetzel, A., Grannó, S., Heaton, G. & Harvey, K. Back to the tubule: microtubule dynamics in Parkinson's disease. *Cellular and Molecular Life Sciences* vol. 74 409–434 (2017).
53. Drechsel, D. N., Hyman, A. A., Cobb, M. H. & Kirschner, M. W. Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. *https://doi.org/10.1091/mbc.3.10.1141* **3**, 1141–1154 (2017).
54. Drubin, D. G. & Kirschner, M. W. Tau protein function in living cells. *J. Cell Biol.* **103**, 2739 (1986).
55. Terwel, D., Dewachter, I. & Van Leuven, F. Axonal transport, tau protein, and neurodegeneration in Alzheimer's disease. *Neuromolecular Med.* **2**, 151–165 (2002).
56. Šimić, G. *et al.* Tau Protein Hyperphosphorylation and Aggregation in Alzheimer's Disease and Other Tauopathies, and Possible Neuroprotective Strategies. *Biomolecules* **6**, 2–28 (2016).
57. Alonso, A. D. *et al.* Hyperphosphorylation of Tau Associates with changes in its function beyond microtubule stability. *Front. Cell. Neurosci.* **12**, 338 (2018).
58. Burré, J. The synaptic function of α-synuclein. *Journal of Parkinson's Disease* vol. 5 699–713 (2015).
59. Cartelli, D. *et al.* α-Synuclein is a Novel Microtubule Dynamase. *Sci. Rep.* **6**, 1–13 (2016).
60. Amadeo, A. *et al.* The Association between α-Synuclein and α-Tubulin in Brain Synapses. *Int. J. Mol. Sci. 2021, Vol. 22, Page 9153* **22**, 9153 (2021).
61. Sainath, R. & Gallo, G. Cytoskeletal and signaling mechanisms of neurite formation. *Cell Tissue Res.* **359**, 267–278 (2015).
62. Janke, C. & Magiera, M. M. The tubulin code and its role in controlling microtubule properties and functions. *Nat. Rev. Mol. Cell Biol. 2020 216* **21**, 307–326 (2020).
63. Janke, C. & Kneussel, M. Tubulin post-translational modifications: Encoding functions on the neuronal microtubule cytoskeleton. *Trends Neurosci.* **33**, 362–372 (2010).
64. Balabanian, L., Berger, C. L. & Hendricks, A. G. Acetylated Microtubules Are Preferentially Bundled Leading to Enhanced Kinesin-1 Motility. *Biophys. J.* **113**, 1551–1560 (2017).
65. Janke, C. & Montagnac, G. Causes and Consequences of Microtubule Acetylation. *Curr. Biol.* **27**, R1287–R1292 (2017).
66. Wloga, D., Joachimiak, E. & Fabczak, H. Tubulin post-translational modifications and microtubule dynamics. *International Journal of Molecular Sciences* vol. 18 (2017).
67. Eshun-Wilson, L. *et al.* Effects of α-tubulin acetylation on microtubule structure and stability. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 10366–10371 (2019).
68. Portran, D., Schaedel, L., Xu, Z., Théry, M. & Nachury, M. V. Tubulin acetylation protects

long-lived microtubules against mechanical ageing. *Nat. Cell Biol.* **19**, 391–398 (2017).
69. Xu, Z. *et al.* Microtubules acquire resistance from mechanical breakage through intralumenal acetylation. *Science (80-.).* **356**, 328–332 (2017).
70. Katrukha, E. A., Jurriens, D., Pastene, D. S. & Kapitein, L. C. Quantitative mapping of dense microtubule arrays in mammalian neurons. *Elife* **10**, (2021).
71. Reed, N. A. *et al.* Microtubule Acetylation Promotes Kinesin-1 Binding and Transport. *Curr. Biol.* **16**, 2166–2172 (2006).
72. Mazzetti, S. *et al.* Linking acetylated α-Tubulin redistribution to α-Synuclein pathology in brain of Parkinson's disease patients. *bioRxiv* 2022.12.29.522226 (2022) doi:10.1101/2022.12.29.522226.
73. Martínez-Hernández, J. *et al.* Crosstalk between acetylation and the tyrosination/detyrosination cycle of α-tubulin in Alzheimer's disease. *Front. Cell Dev. Biol.* **10**, (2022).
74. Esteves, A. R. *et al.* Acetylation as a major determinant to microtubule-dependent autophagy: Relevance to Alzheimer's and Parkinson disease pathology. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1865**, 2008–2023 (2019).
75. Cappelletti, G., Calogero, A. M. & Rolando, C. Microtubule acetylation: A reading key to neural physiology and degeneration. *Neurosci. Lett.* **755**, (2021).
76. Prota, A. E. *et al.* Structural basis of tubulin tyrosination by tubulin tyrosine ligase. *J. Cell Biol.* **200**, 259–270 (2013).
77. Howard, J. & Hyman, A. A. Microtubule polymerases and depolymerases. *Curr. Opin. Cell Biol.* **19**, 31–35 (2007).
78. Peris, L. *et al.* Tubulin tyrosination regulates synaptic function and is disrupted in Alzheimer's disease. *Brain* **145**, 2486–2506 (2022).
79. Sanyal, C. *et al.* The detyrosination/re-tyrosination cycle of tubulin and its role and dysfunction in neurons and cardiomyocytes. *Semin. Cell Dev. Biol.* **137**, (2023).
80. Paturle-Lafanechère, L. *et al.* Accumulation of delta 2-tubulin, a major tubulin variant that cannot be tyrosinated, in neuronal tissues and in stable microtubule assemblies. *J. Cell Sci.* **107**, (1994).
81. Pero, M. E. *et al.* Pathogenic role of delta 2 tubulin in bortezomib-induced peripheral neuropathy. *Proc. Natl. Acad. Sci. U. S. A.* **118**, e2012685118 (2021).
82. Baas, P. W., Rao, A. N., Matamoros, A. J. & Leo, L. Stability properties of neuronal microtubules. *Cytoskeleton (Hoboken).* **73**, 442 (2016).
83. Brouhard, G. J. & Rice, L. M. Microtubule dynamics: an interplay of biochemistry and mechanics. *Nat. Rev. Mol. Cell Biol.* **19**, 451–463 (2018).
84. Gray, E. Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *J. Anat.* (1959).
85. Chan, K. Y. & Bunt, A. H. An association between mitochondria and microtubules in synaptosomes and axon terminals of cerebral cortex. *J. Neurocytol.* **7**, 137–143 (1978).
86. Babu, L. P. A., Wang, H. Y., Eguchi, K., Guillaud, L. & Takahashi, T. Microtubule and actin differentially regulate synaptic vesicle cycling to maintain high-frequency neurotransmission. *J. Neurosci.* **40**, 131–142 (2020).
87. Ruiz-Cañada, C. & Budnik, V. Synaptic cytoskeleton at the neuromuscular junction. *Int. Rev. Neurobiol.* **75**, 217–236 (2006).
88. Roos, J., Hummel, T., Ng, N., Klämbt, C. & Davis, G. W. Drosophila Futsch regulates synaptic microtubule organization and is necessary for synaptic growth. *Neuron* **26**, 371–382 (2000).
89. Guedes-Dias, P. *et al.* Kinesin-3 Responds to Local Microtubule Dynamics to Target Synaptic Cargo Delivery to the Presynapse. *Curr. Biol.* **29**, 268-282.e8 (2019).
90. Forman, M. S., Trojanowski, J. Q. & Lee, V. M. Y. Neurodegenerative diseases: A decade of

discoveries paves the way for therapeutic breakthroughs. *Nat. Med.* **10**, 1055–1063 (2004).
91. Dauer, W. & Przedborski, S. Parkinson's disease: Mechanisms and models. *Neuron* **39**, 889–909 (2003).
92. Dickson, D. W. Parkinson's Disease and Parkinsonism: Neuropathology. *Cold Spring Harb. Perspect. Med.* **2**, (2012).
93. Braak, H. *et al.* Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol. Aging* **24**, 197–211 (2003).
94. Spillantini, M. G. *et al.* α-synuclein in Lewy bodies [8]. *Nature* vol. 388 839–840 (1997).
95. Chen, L. *et al.* Oligomeric α-synuclein inhibits tubulin polymerization. *Biochem. Biophys. Res. Commun.* **356**, 548–553 (2007).
96. Liu, W. *et al.* PINK1 defect causes mitochondrial dysfunction, proteasomal deficit and alpha-synuclein aggregation in cell culture models of Parkinson's disease. *PLoS One* **4**, (2009).
97. Wang, X. *et al.* Pathogenic alpha-synuclein aggregates preferentially bind to mitochondria and affect cellular respiration. *Acta Neuropathol. Commun.* **7**, 41 (2019).
98. Konno, T., Ross, O. A., Puschmann, A., Dickson, D. W. & Wszolek, Z. K. Autosomal dominant Parkinson's disease caused by SNCA duplications. *Parkinsonism Relat. Disord.* **22 Suppl 1**, S1–S6 (2016).
99. Fredenburg, R. A. *et al.* The impact of the E46K mutation on the properties of α-synuclein in its monomelic and oligomeric states. *Biochemistry* **46**, 7107–7118 (2007).
100. Li, J., Uversky, V. N. & Fink, A. L. Conformational behavior of human alpha-synuclein is modulated by familial Parkinson's disease point mutations A30P and A53T. *Neurotoxicology* **23**, 553–567 (2002).
101. Conway, K. A. *et al.* Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 571–576 (2000).
102. Mazzetti, S. *et al.* α-Synuclein oligomers in skin biopsy of idiopathic and monozygotic twin patients with Parkinson's disease. *Brain* **143**, 920–931 (2020).
103. Tamo, W. *et al.* Expression of α-synuclein, the precursor of non-amyloid β component of Alzheimer's disease amyloid, in human cerebral blood vessels. *Neurosci. Lett.* **326**, 5–8 (2002).
104. Kim, S. *et al.* Alpha-synuclein induces apoptosis by altered expression in human peripheral lymphocyte in Parkinson's disease. *FASEB J.* (2004).
105. Askanas, V., Engel, W. K., Alvarez, R. B., McFerrin, J. & Broccolini, A. Novel immunolocalization of alpha-synuclein in human muscle of inclusion-body myositis, regenerating and necrotic muscle fibers, and at neuromuscular junctions. *J. Neuropathol. Exp. Neurol.* **59**, 592–598 (2000).
106. Michell, A. W., Luheshi, L. M. & Barker, R. A. Skin and platelet α-synuclein as peripheral biomarkers of Parkinson's disease. *Neurosci. Lett.* **381**, 294–298 (2005).
107. Davidson, W. S., Jonas, A., Clayton, D. F. & George, J. M. Stabilization of α-Synuclein secondary structure upon binding to synthetic membranes. *J. Biol. Chem.* **273**, 9443–9449 (1998).
108. Liu, C. *et al.* The Membrane Interaction of Alpha-Synuclein. *Front. Cell. Neurosci.* **15**, 633727 (2021).
109. Burré, J. *et al.* Properties of native brain α-synuclein. *Nature* **498**, E4 (2013).
110. Burré, J. *et al.* α-Synuclein promotes SNARE-complex assembly in vivo and in vitro. *Science (80-.).* **329**, 1663–1667 (2010).
111. Greten-Harrison, B. *et al.* αβγ-Synuclein triple knockout mice revealage-dependent neuronal dysfunction. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 19573–19578 (2010).
112. Liu, S. *et al.* alpha-Synuclein produces a long-lasting increase in neurotransmitter release. *EMBO J.* **23**, 4506–4516 (2004).

113. Gureviciene, I., Gurevicius, K. & Tanila, H. Role of alpha-synuclein in synaptic glutamate release. *Neurobiol. Dis.* **28**, 83–89 (2007).
114. Cabin, D. E. *et al.* Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. *J. Neurosci.* **22**, 8797–8807 (2002).
115. Abeliovich, A. *et al.* Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* **25**, 239–252 (2000).
116. Zaltieri, M. *et al.* α-synuclein and synapsin III cooperatively regulate synaptic function in dopamine neurons. *J. Cell Sci.* **128**, 2231–2243 (2015).
117. Guo, J. T. *et al.* Inhibition of vesicular monoamine transporter-2 activity in α-synuclein stably transfected SH-SY5Y cells. *Cell. Mol. Neurobiol.* **28**, 35–47 (2008).
118. Bellucci, A. *et al.* Redistribution of DAT/α-Synuclein Complexes Visualized by "In Situ" Proximity Ligation Assay in Transgenic Mice Modelling Early Parkinson's Disease. *PLoS One* **6**, e27959 (2011).
119. Butler, B. *et al.* Dopamine Transporter Activity Is Modulated by α-Synuclein. *J. Biol. Chem.* **290**, 29542 (2015).
120. Wersinger, C., Rusnak, M. & Sidhu, A. Modulation of the trafficking of the human serotonin transporter by human alpha-synuclein. *Eur. J. Neurosci.* **24**, 55–64 (2006).
121. Fan, T. S., Liu, S. C. H. & Wu, R. M. Alpha-Synuclein and Cognitive Decline in Parkinson Disease. *Life 2021, Vol. 11, Page 1239* **11**, 1239 (2021).
122. Somayaji, M. *et al.* A dual role for α-synuclein in facilitation and depression of dopamine release from substantia nigra neurons in vivo. *Proc. Natl. Acad. Sci.* 202013652 (2020) doi:10.1073/pnas.2013652117.
123. Logan, T., Bendor, J., Toupin, C., Thorn, K. & Edwards, R. H. α-Synuclein promotes dilation of the exocytotic fusion pore. *Nat. Neurosci. 2017 205* **20**, 681–689 (2017).
124. Sulzer, D. & Edwards, R. H. The physiological role of α-synuclein and its relationship to Parkinson's Disease. *J. Neurochem.* **150**, 475–486 (2019).
125. Srinivasan, E. *et al.* Alpha-Synuclein Aggregation in Parkinson's Disease. *Front. Med.* **8**, (2021).
126. Li, Y. *et al.* Amyloid fibril structure of α-synuclein determined by cryo-electron microscopy. *Cell Res.* **28**, 897–903 (2018).
127. Pountney, D. L., Voelcker, N. H. & Gai, W. P. Annular alpha-synuclein oligomers are potentially toxic agents in alpha-synucleinopathy. Hypothesis. *Neurotox. Res.* **7**, 59–67 (2005).
128. Irwin, D. J., Lee, V. M. Y. & Trojanowski, J. Q. Parkinson's disease dementia: convergence of α-synuclein, tau and amyloid-β pathologies. *Nat. Rev. Neurosci. 2013 149* **14**, 626–636 (2013).
129. Recasens, A. & Dehay, B. Alpha-synuclein spreading in Parkinson's disease. *Front. Neuroanat.* **8**, (2014).
130. Desplats, P. *et al.* Inclusion Formation and Neuronal Cell Death through Neuron-to-Neuron Transmission of α-Synuclein. *Proceedings of the National Academy of Sciences of the United States of America* vol. 106 13010–1301.
131. Chung, H. K., Ho, H.-A., Pérez-Acuña, D. & Lee, S.-J. Modeling α-Synuclein Propagation with Preformed Fibril Injections. *J. Mov. Disord.* **12**, 139–151 (2019).
132. Zhang, B. *et al.* Stereotaxic targeting of alpha-synuclein pathology in mouse brain using preformed fibrils. *Methods Mol. Biol.* **1948**, 45–57 (2019).
133. Luk, K. C. *et al.* Pathological α-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. *Science (80-.).* **338**, 949–953 (2012).
134. Winner, B. *et al.* In vivo demonstration that α-synuclein oligomers are toxic. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 4194–4199 (2011).

135. Emin, D. *et al.* Small soluble α-synuclein aggregates are the toxic species in Parkinson's disease. *Nat. Commun.* **13**, (2022).
136. Diógenes, M. J. *et al.* Extracellular Alpha-Synuclein Oligomers Modulate Synaptic Transmission and Impair LTP Via NMDA-Receptor Activation. *J. Neurosci.* **32**, 11750–11762 (2012).
137. Klucken, J. *et al.* Alpha-synuclein aggregation involves a bafilomycin A 1-sensitive autophagy pathway. *Autophagy* **8**, 754–766 (2012).
138. Parihar, M. S., Parihar, A., Fujita, M., Hashimoto, M. & Ghafourifar, P. Alpha-synuclein overexpression and aggregation exacerbates impairment of mitochondrial functions by augmenting oxidative stress in human neuroblastoma cells. *Int. J. Biochem. Cell Biol.* **41**, 2015–2024 (2009).
139. Guo, C., Sun, L., Chen, X. & Zhang, D. Oxidative stress, mitochondrial damage and neurodegenerative diseases. *Neural Regen. Res.* **8**, 2003–2014 (2013).
140. Guo, J. D., Zhao, X., Li, Y., Li, G. R. & Liu, X. L. Damage to dopaminergic neurons by oxidative stress in Parkinson's disease (Review). *Int. J. Mol. Med.* **41**, 1817–1825 (2018).
141. Haddad, D. & Nakamura, K. Understanding the susceptibility of dopamine neurons to mitochondrial stressors in Parkinson's disease. *FEBS Lett.* **589**, 3702 (2015).
142. Dickson, D. W. Linking selective vulnerability to cell death mechanisms in Parkinson's disease. *Am. J. Pathol.* **170**, 16–19 (2007).
143. Cartelli, D. *et al.* Microtubule alterations occur early in experimental parkinsonism and the microtubule stabilizer Epothilone D is neuroprotective. *Sci. Rep.* **3**, 1–10 (2013).
144. Cartelli, D. *et al.* Microtubule dysfunction precedes transport impairment and mitochondria damage in MPP+-induced neurodegeneration. *J. Neurochem.* **115**, 247–258 (2010).
145. Marshall, L. E. & Himes, R. H. Rotenone inhibition of tubulin self-assembly. *Biochim. Biophys. Acta - Gen. Subj.* **543**, 590–594 (1978).
146. Ren, Y., Liu, W., Jiang, H., Jiang, Q. & Feng, J. Selective vulnerability of dopaminergic neurons to microtubule depolymerization. *J. Biol. Chem.* **280**, 34105–34112 (2005).
147. Jiang, Q., Yan, Z. & Feng, J. Neurotrophic factors stabilize microtubules and protect against rotenone toxicity on dopaminergic neurons. *J. Biol. Chem.* **281**, 29391–29400 (2006).
148. Shults, C. W. Lewy bodies. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 1661–1668 (2006).
149. Alim, M. A. *et al.* Tubulin seeds α-synuclein fibril formation. *J. Biol. Chem.* **277**, 2112–2117 (2002).
150. Moors, T. E. *et al.* Subcellular orchestration of alpha-synuclein variants in Parkinson's disease brains revealed by 3D multicolor STED microscopy. *bioRxiv* 470476 (2019) doi:10.1101/470476.
151. Betzer, C. *et al.* Identification of Synaptosomal Proteins Binding to Monomeric and Oligomeric α-Synuclein. *PLoS One* **10**, e0116473 (2015).
152. Prots, I. *et al.* α-synuclein oligomers impair neuronal microtubule-kinesin interplay. *J. Biol. Chem.* **288**, 21742–21754 (2013).
153. Duka, T., Duka, V., Joyce, J. N. & Sidhu, A. α-Synuclein contributes to GSK-3β-catalyzed Tau phosphorylation in Parkinson's disease models. *FASEB J.* **23**, 2820 (2009).
154. Seirafi, M., Kozlov, G. & Gehring, K. Parkin structure and function. *Febs J.* **282**, 2076 (2015).
155. Feng, J. Microtubule: a common target for parkin and Parkinson's disease toxins. *Neuroscientist* **12**, 469–476 (2006).
156. Ren, Y. *et al.* Parkin Mutations Reduce the Complexity of Neuronal Processes in iPSC-derived Human Neurons. *Stem Cells* **33**, 68 (2015).
157. Cartelli, D. *et al.* Parkin absence accelerates microtubule aging in dopaminergic neurons. *Neurobiol. Aging* **61**, 66–74 (2018).
158. Cartelli, D., Goldwurm, S., Casagrande, F., Pezzoli, G. & Cappelletti, G. Microtubule

Destabilization Is Shared by Genetic and Idiopathic Parkinson's Disease Patient Fibroblasts. *PLoS One* **7**, e37467 (2012).
159. Law, B. M. H. *et al.* A direct interaction between leucine-rich repeat kinase 2 and specific β-Tubulin isoforms regulates tubulin acetylation. *J. Biol. Chem.* **289**, 895–908 (2014).
160. Sheng, C. *et al.* DJ-1 deficiency perturbs microtubule dynamics and impairs striatal neurite outgrowth. *Neurobiol. Aging* **34**, 489–498 (2013).
161. Alim, M. A. *et al.* Demonstration of a role for α-synuclein as a functional microtubule-associated protein. *J. Alzheimer's Dis.* **6**, 435–442 (2004).
162. Zhou, R. M. *et al.* Molecular interaction of α-synuclein with tubulin influences on the polymerization of microtubule in vitro and structure of microtubule in cells. *Mol. Biol. Rep.* **37**, 3183–3192 (2010).
163. Carnwath, T., Mohammed, R. & Tsiang, D. The direct and indirect effects of α-synuclein on microtubule stability in the pathogenesis of parkinson's disease. *Neuropsychiatric Disease and Treatment* vol. 14 1685–1695 (2018).
164. Rierola, M. *et al.* Tau and α-synuclein shape microtubule organization and microtubule-dependent transport in neuronal dendrites. *bioRxiv* 2022.06.09.495530 (2022) doi:10.1101/2022.06.09.495530.
165. Wersinger, C. & Sidhu, A. Disruption of the interaction of alpha-synuclein with microtubules enhances cell surface recruitment of the dopamine transporter. *Biochemistry* **44**, 13612–13624 (2005).
166. Specht, C. G. & Schoepfer, R. Deletion of the alpha-synuclein locus in a subpopulation of C57BL/6J inbred mice. *BMC Neurosci.* **2**, (2001).
167. Peris, L. *et al.* Motor-dependent microtubule disassembly driven by tubulin tyrosination. *J. Cell Biol.* **185**, 1159 (2009).
168. Courte, J. *et al.* The expression level of alpha-synuclein in different neuronal populations is the primary determinant of its prion-like seeding. *Sci. Rep.* **10**, (2020).
169. Banwinkler, M., Theis, H., Prange, S. & van Eimeren, T. Imaging the Limbic System in Parkinson's Disease—A Review of Limbic Pathology and Clinical Symptoms. *Brain Sci.* **12**, (2022).
170. Stepanova, T. *et al.* Visualization of Microtubule Growth in Cultured Neurons via the Use of EB3-GFP (End-Binding Protein 3-Green Fluorescent Protein). *J. Neurosci.* **23**, 2655–2664 (2003).
171. Guedes-Dias, P. *et al.* Kinesin-3 Responds to Local Microtubule Dynamics to Target Synaptic Cargo Delivery to the Presynapse. *Curr. Biol.* **29**, 268-282.e8 (2019).
172. Moutin, M. J., Bosc, C., Peris, L. & Andrieux, A. Tubulin post-translational modifications control neuronal development and functions. *Dev. Neurobiol.* **81**, 253–272 (2021).
173. Brazdis, R. M. *et al.* Demonstration of brain region-specific neuronal vulnerability in human iPSC-based model of familial Parkinson's disease. *Hum. Mol. Genet.* **29**, 1180–1191 (2020).
174. Ducrot, C. *et al.* Dopaminergic neurons establish a distinctive axonal arbor with a majority of non-synaptic terminals. *FASEB J.* **35**, (2021).
175. Elizarova, S. *et al.* A fluorescent nanosensor paint detects dopamine release at axonal varicosities with high spatiotemporal resolution. *Proc. Natl. Acad. Sci. U. S. A.* **119**, (2022).
176. Liu, C. & Kaeser, P. S. Mechanisms and regulation of dopamine release. *Curr. Opin. Neurobiol.* **57**, 46–53 (2019).
177. Sulzer, D., Cragg, S. J. & Rice, M. E. Striatal dopamine neurotransmission: Regulation of release and uptake. *Basal Ganglia* **6**, 123–148 (2016).

Acknowledgments

Some of the results shown in this work are included in the paper Amadeo *et al*, submitted and accepted in 2021 by *Int. J. Mol. Sci*.. I wish to thank Prof. Graziella Cappelletti, supervisor of this work and guide during my whole PhD project; Prof. Francesca Bartolini, in whose laboratory at Columbia University Medical Center I spent the last year of my PhD program, which was a mentor and an important leader in this project, who helped to create a successful scientific collaboration between our laboratories; Prof. David Sulzer that kindly helped me as a co-mentor during my last year and all the people in his lab who I collaborated with. I also want to thank Dr. Samanta Mazzetti, as a friend and colleague, whose collaboration was fundamental to carry out the majority of my work at Università degli Studi di Milano.

This study was partially funded in collaboration with Fondazione Grigioni per il Morbo di Parkinson.

A great thanks goes to all my colleagues in the Cappelletti's Lab, in particular to Simone, Elena and my master student Daniela for their support and friendship. A huge thanks to my colleagues Maria Elena, Julie and Ryan of the Bartolini's lab, for helping, teaching and mentoring me during my time spent at Columbia University. Among them, a special thanks goes to Caterina, trusted friend during my whole time in NYC.

My final thought goes to my family, my friends and all my beloved ones, both in Italy and in NYC, whose support has been irreplaceable for me during this time.

PART II

Table of Contents

Published paper 1

Alida Amadeo †, Sara Pizzi, **Alessandro Comincini**, Debora Modena, Alessandra Maria Calogero, Laura Madaschi, Gaia Faustini, Chiara Rolando, Arianna Bellucci, Gianni Pezzoli, Samanta Mazzetti and Graziella Cappelletti.
"The association between α-synuclein and α-tubulin in brain synapses." *Int. J. Mol. Sci.*, **2021**; 22(17), 9153.

Published review 1

Milo Jarno Basellini †, Josine Marie Kothuis †, **Alessandro Comincini** †, Gianni Pezzoli, Graziella Cappelletti and Samanta Mazzetti.
"Pathological pathways and α-synuclein in Parkinson's disease: a view from the periphery." *Front. Biosci. (Landmark Ed)*, **2023**; 28(2): 33.

International Journal of
Molecular Sciences

MDPI

Article

The Association between α-Synuclein and α-Tubulin in Brain Synapses

Alida Amadeo [1,2,*,†], Sara Pizzi [1,†], Alessandro Comincini [1], Debora Modena [1], Alessandra Maria Calogero [1], Laura Madaschi [3], Gaia Faustini [4], Chiara Rolando [1], Arianna Bellucci [4], Gianni Pezzoli [5], Samanta Mazzetti [1,5,‡] and Graziella Cappelletti [1,2,*,‡]

[1] Department of Biosciences, University of Milan, Via Celoria 26, 20126 Milano, Italy; sara.pizzi1@unimi.it (S.P.); alessandro.comincini@unimi.it (A.C.); debora.modena@unimi.it (D.M.); alessandra.calogero@unimi.it (A.M.C.); chiara.rolando@unimi.it (C.R.); samanta.mazzetti@gmail.com (S.M.)
[2] Center of Excellence on Neurodegenerative Diseases, University of Milan, Via Celoria 26, 20126 Milano, Italy
[3] UNITECH NOLIMITS, University of Milan, Via Celoria 26, 20133 Milan, Italy; laura.madaschi@unimi.it
[4] Division of Pharmacology, Department of Molecular and Translational Medicine, University of Brescia, Viale Europa 11, 25123 Brescia, Italy; gaia.faustini@unibs.it (G.F.); arianna.bellucci@unibs.it (A.B.)
[5] Fondazione Grigioni per il Morbo di Parkinson, Via Zuretti 35, 20125 Milano, Italy; pezzoli@parkinson.it
* Correspondence: alida.amadeo@unimi.it (A.A.); graziella.cappelletti@unimi.it (G.C.); Tel.: +39-025-031-4885 (A.A.); +39-025-031-4752 (G.C.)
† These authors contributed equally to this work.
‡ Co-last authors.

Abstract: α-synuclein is a small protein that is mainly expressed in the synaptic terminals of nervous tissue. Although its implication in neurodegeneration is well established, the physiological role of α-synuclein remains elusive. Given its involvement in the modulation of synaptic transmission and the emerging role of microtubules at the synapse, the current study aimed at investigating whether α-synuclein becomes involved with this cytoskeletal component at the presynapse. We first analyzed the expression of α-synuclein and its colocalization with α-tubulin in murine brain. Differences were found between cortical and striatal/midbrain areas, with *substantia nigra pars compacta* and *corpus striatum* showing the lowest levels of colocalization. Using a proximity ligation assay, we revealed the direct interaction of α-synuclein with α-tubulin in murine and in human brain. Finally, the previously unexplored interaction of the two proteins in vivo at the synapse was disclosed in murine striatal presynaptic boutons through multiple approaches, from confocal spinning disk to electron microscopy. Collectively, our data strongly suggest that the association with tubulin/microtubules might actually be an important physiological function for α-synuclein in the synapse, thus suggesting its potential role in a neuropathological context.

Keywords: microtubules; α-synuclein; presynaptic bouton; central nervous system; *striatum*; mouse; human brain; interaction; PLA; electron microscopy

check for updates

Citation: Amadeo, A.; Pizzi, S.; Comincini, A.; Modena, D.; Calogero, A.M.; Madaschi, L.; Faustini, G.; Rolando, C.; Bellucci, A.; Pezzoli, G.; et al. The Association between α-Synuclein and α-Tubulin in Brain Synapses. *Int. J. Mol. Sci.* **2021**, *22*, 9153. https://doi.org/10.3390/ijms22179153

Academic Editors: Fabio Cavaliere, Benjamin Dehay and Federico N. Soria

Received: 28 June 2021
Accepted: 19 August 2021
Published: 25 August 2021

1. Introduction

α-synuclein is widely expressed in vertebrate brain and strongly implicated in various neurodegenerative disorders [1]. It started to be interesting for the scientific community in 1997, when it was disclosed as major constituent of histopathological lesions in Parkinson's disease (PD) [2], and a point mutation in the *SNCA* gene was discovered in families with the hereditary form of this disease [3]. In healthy neurons, α-synuclein was detected in neuronal somata, dendrites and synaptic terminals of several brain regions in different species, and regional diversity in its expression was broadly reported [4–10], even if the use of different antibodies gave contradictory mappings and subcellular localizations [11,12]. Nevertheless, α-synuclein is mainly localized in presynaptic terminals, mostly the asymmetric excitatory glutamatergic ones [8,9], suggesting that its altered synaptic expression

could be a predisposing factor for impaired high-frequency neurotransmission and for the development of several pathologies [1,13–17].

α-synuclein is a soluble intrinsically unfolded protein that is able to adopt multiple conformations and to interact with several partners, thus, in turn, its role in a plethora of neuronal functions is emerging [18]. Starting from the discovery that α-synuclein interacts with synaptic vesicles [19] and promotes the assembly of the vesicular SNARE complex [20], many studies added pieces to the role of α-synuclein at the synapse over time and disclosed its implication in synaptic vesicle function and recycling. In detail, α-synuclein promotes clustering of synaptic vesicles [21,22], inhibits docking [23], and regulates the activity of dopamine transporters [24]. In addition, α-synuclein interacts with the vesicular monoamine transporter VMAT-2 [25] and synapsin III [26], regulating synaptic function in dopamine neurons. However, the contribution of α-synuclein to neurotransmission under physiologically relevant conditions proved to be elusive, even if a very recent paper strongly demonstrated that α-synuclein plays a dual role in both the facilitation and depression of dopamine release in vivo [27].

The cytoskeleton was reported to be a cellular partner of α-synuclein. It is a matter of debate as to whether and how this interplay could be pivotal for synaptic function. Focusing on the actin cytoskeleton, evidence exists that α-synuclein binds actin and regulates its dynamics [28], possibly tuning the vesicle release process [29]. The interplay with tubulin is actually more controversial [30,31]. The binding of α-synuclein to tubulin and the impact on tubulin polymerization, although unclear, was disclosed in vitro by multiple approaches including co-immunoprecipitation [32,33], fibrillogenesis and immunoelectron microscopy [34], colocalization [35], microtubule assembly assays [36] and live-cell imaging [37]. This interaction was also underlined in vivo by colocalization and immunoelectron microscopy [6,33,37], and by electrophysiological recordings and pharmacological treatments [15,16]. Nevertheless, there are several hints highlighting that α-synuclein might have a role in modulating the microtubular dynamics. Recently, we found that α-synuclein folds upon interaction with tubulin and pointed out its novel role in regulating multiple steps of microtubule dynamics in cell-free systems and in neuronal cells [38]. Despite this, to date, there have been no investigations aimed at elucidating the potential interaction of α-synuclein and tubulin in vivo at the synapse. One major doubt regarding the relevance of the interplay of α-synuclein with tubulin for synaptic function comes from the old view that synaptic vesicles in presynaptic terminals are not in contact with microtubules. Indeed, the presence of tubulin/microtubules in synaptic terminals was reported not only in vitro [39–41], but also in vivo [42–46], both in invertebrate and rodent models. Importantly, it is now clear that microtubule dynamics play a fundamental role in neurotransmission, controlling vesicle motility at presynaptic boutons [47] and regulating synaptic vesicle cycling [48,49].

Given this emerging role of microtubules at the synapse, unravelling the role of the interaction between α-synuclein and tubulin in this neuronal compartment remains a crucial challenge. Here, we firstly focused on α-synuclein expression in specific areas of wild-type murine brain, using biochemical and immunohistochemical methods, to give an overview of its distribution in specific brain areas. Afterwards, we deeply investigated the interplay between α-synuclein and α-tubulin using high-level and original morphological approaches, including the Proximity Ligation Assay (PLA) and ultrastructural analysis, with a focus on *corpus striatum* synapses. Moreover, we also validated their relationship in post-mortem human brain.

2. Results

2.1. α-Synuclein Distribution Changes in Different Areas of Murine Brain

We first evaluated the expression of α-synuclein protein in the forebrain and midbrain areas of adult wild-type (WT) mice, at postnatal day 60 (P60), by Western Blotting (Figure 1A) and densitometric analysis (Figure 1B). Although the differences were not significant, we found that the maximal expression of α-synuclein was observed in *cor-*

pus striatum, while the entorhinal cortex showed the lowest amount, suggesting that α-synuclein expression could be area-specific in the murine brain. Thus, we proceeded to investigate, in detail, the distribution of α-synuclein through an immunohistochemical approach. According to the prevalent localization at the presynaptic site reported in the literature [5,8], staining for α-synuclein appeared mainly in the neuropil and scarcely ever in neuronal cell bodies. However, in the *substantia nigra pars compacta*, the labelled puncta were sparse and the product of the reaction was mainly detected in the soma of some neurons (Figure 1C). Conversely, the *striatum* (Figure 1D) and somatosensory/entorhinal cortices (Figure S1A,D) showed a broadly diffuse and punctiform staining surrounding negative neuronal cell bodies. Interestingly, an intense labelled band between the granule cell and molecular layers of dentate gyrus (Figure S1B), and between the pyramidal cell and molecular layers of CA3 (*Cornu Ammonis*, region 3) *stratum oriens* (Figure S1C), was observed. The densitometric analysis performed on these brain areas strongly suggests a region-dependent distribution of α-synuclein. As shown in Figure 1E,F, some striking differences among areas were detectable. In detail, the expression level of the protein in the *substantia nigra pars compacta* appeared significantly lower than in the hippocampus (CA3 and dentate girus) and all the cortical regions we analyzed (Figure 1F).

To also check the subcellular distribution of α-synuclein, we performed an ultrastructural analysis on the prefrontal cortex, *corpus striatum* and *substantia nigra pars compacta* (Figure S2), confirming the predominant presynaptic localization of the protein in accordance with previous ultrastructural studies [5,6]. No glial structures showed α-synuclein staining (Figure S2). The subcellular localization via the immunoperoxidase ultrastructural method was confirmed with a single immunogold reaction during pre-embedding (not shown), as fully illustrated by the double immunogold experiments reported below.

2.2. α-Synuclein and α-Tubulin Colocalize in the Brain at the Presynapse

Given the emerging data on the interplay between α-synuclein and α-tubulin in vitro [32,34,37,38], we wondered whether this interaction occurs in vivo. Firstly, colocalization analysis on double immunofluorescence was carried out in those representative murine brain areas (*corpus striatum*, *substantia nigra pars compacta and reticulata*, entorhinal and prefrontal cortices) that are homologous to regions prone to α-synuclein aggregation and Lewy body formation in humans [50]. We observed the overall colocalization of α-synuclein and α-tubulin in neuronal cell bodies, processes and puncta (Figure 2A–C). The degree of overlap was defined by the M1 Manders'coefficient, which represents a good indicator of the α-synuclein fraction coincident with α-tubulin. As reported in Figure 2D, the different brain regions displayed several degrees of colocalization, all of them lower than 50% of the total α-synuclein. Statistically significant differences were observed between prefrontal and entorhinal cortices versus subcortical areas including both the *substantia nigra pars compacta* and *striatum* (Figure 2D). We observed that the degree of α-synuclein/α-tubulin colocalization followed the same trend of α-synuclein levels in the examined areas. Basically, they were both higher in the cortical areas and lower in the nigrostriatal system.

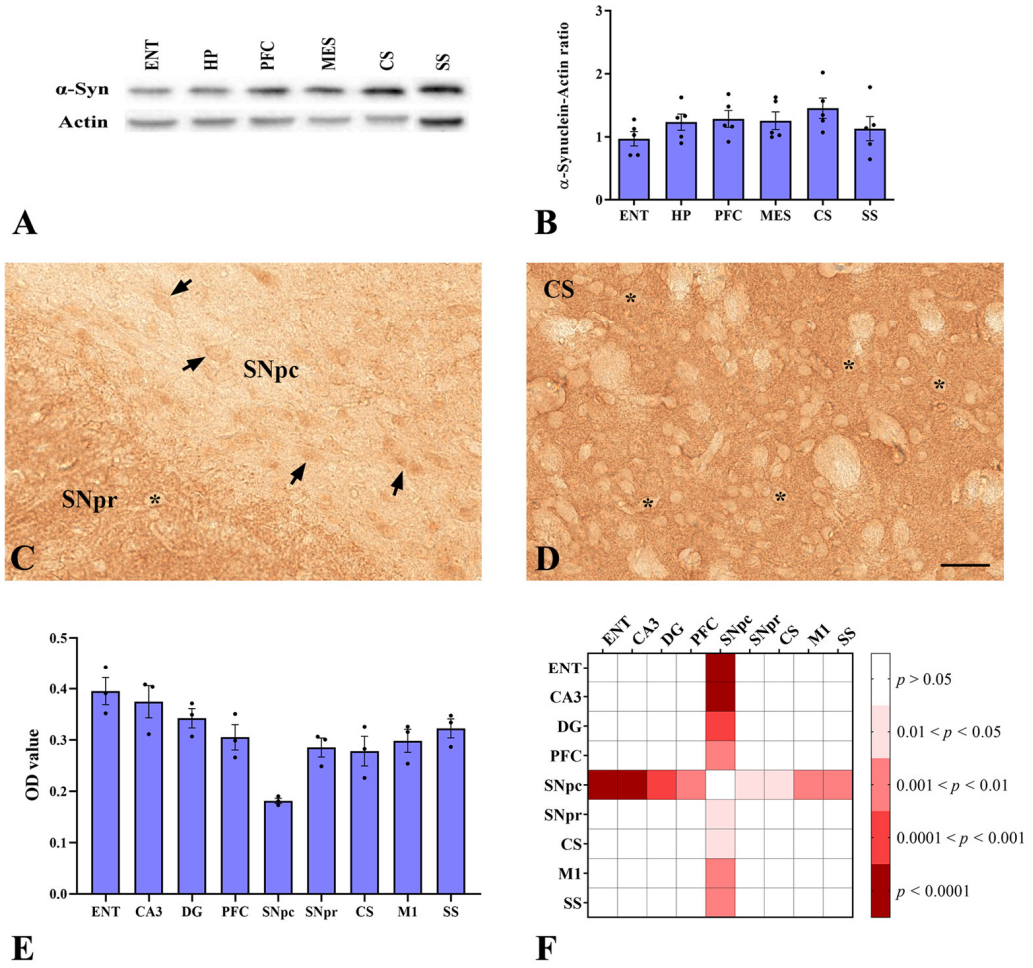

Figure 1. α-synuclein expression in WT murine brain. (**A**,**B**): Western blot analysis of α-synuclein levels in total lysates obtained from different cerebral regions. (**A**): Representative western blot showing α-synuclein levels in total lysates of entorhinal cortex (ENT), hippocampal formation (HP), prefrontal cortex (PFC), ventral mesencephalon (MES), *corpus striatum* (CS), and somatosensory cortex (SS). Actin is used as loading reference. (**B**): Quantification of α-synuclein protein levels normalized to actin. Data are mean ± SEM (n = 5 mice); One-way ANOVA was used for statistical analysis followed by Bonferroni's multiple comparisons test. (**C**,**D**): α-synuclein immunoperoxidase staining in coronal sections of WT mice brain. Light microscope images of α-synuclein immunolabeling in the *substantia nigra pars compacta* (SNpc) and *reticulata* (SNpr; (**C**)) and *corpus striatum* (CS; (**D**)). α-synuclein staining is punctiform and mainly neuropilar. The asterisks showed that the cell bodies are not labelled, except in the SNpc (arrows). Scale bar: 40 µm. (**E**): Densitometric analysis of α-synuclein immunoperoxidase staining in different areas of WT mice brain. Data are shown as mean optical density ± SEM (n = 3 mice). One-way ANOVA was used for statistical analysis followed by Bonferroni's multiple comparison test as described in the Materials and Methods section. p values are graphically represented in the heat-map in (**F**) (P60, n = 3). CA3: *Cornu Ammonis* region 3; DG: dentate gyrus; M1: primary motor cortex.

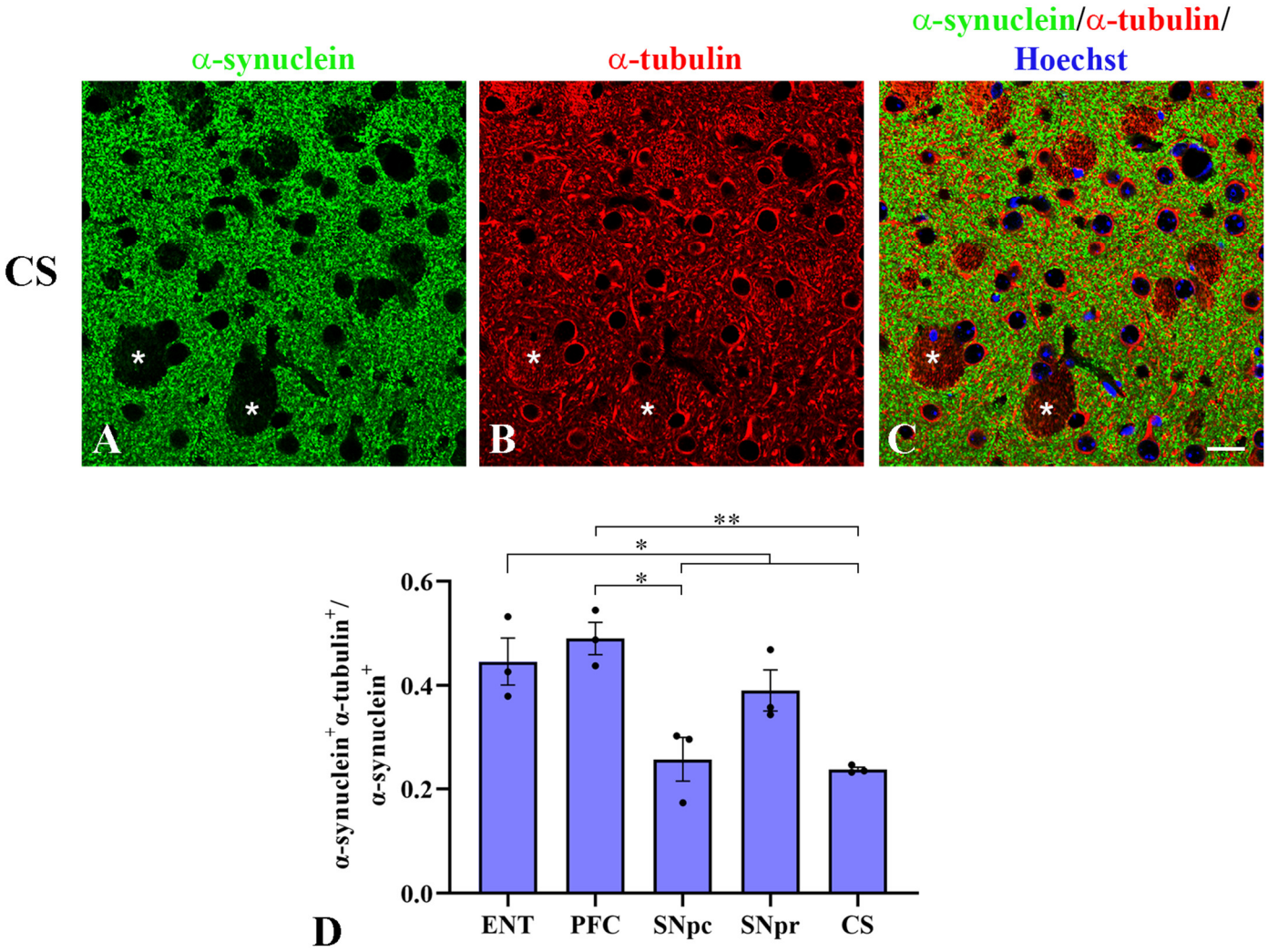

Figure 2. α-synuclein and α-tubulin colocalization in different brain areas of WT mice. (**A**–**C**): Double immunofluorescence in *corpus striatum* (CS) of WT mice for (**A**) α-synuclein (α-syn, green) and (**B**) α-tubulin (α-tub, red), and (**C**) the relative merge with Hoechst in blue as nuclear staining. Asterisks indicate bundles of fibers. (**D**): Colocalization analysis between α-synuclein and α-tubulin. The bars represent the Manders' coefficient 1 (M1) computed for each brain area; data are shown as mean ± SEM (n = 3 mice); one-way ANOVA was used for statistical analysis followed by Bonferroni's multiple comparison test as described in the Materials and Methods section; * $p \leq 0.05$, ** $p \leq 0.01$. ENT: entorhinal cortex; PFC: prefrontal cortex; SNpc: *substantia nigra pars compacta*; SNpr: *substantia nigra pars reticulata*; CS: *corpus striatum*. Scale bars: 20 µm.

Then, we focused on synapses to obtain an insight into the neuronal compartment where the interplay between α-synuclein and α-tubulin might occur. The analysis was carried out on *corpus striatum*, where a huge amount of synaptic terminals arising from different sources interacts in a neurochemically complex neuropil [51,52]. Notably, this region is involved in the signal transmission from the neocortex and receives afferent pathways from the *substantia nigra*, giving it a crucial role in studies concerning PD. To achieve our goal, we firstly confirmed the presence of α-tubulin in striatal synapses by ultrastructural analysis after pre-embedding immunogold staining (Figure S3). Then, we carried out a triple immunofluorescence for α-synuclein, α-tubulin and synaptophysin, which is a marker of the pre-synaptic compartment (Figure 3A–D,G). We evaluated α-synuclein/α-tubulin colocalization (Figure 3E,H) and combined α-synuclein/α-tubulin colocalization with synaptophysin (Figure 3F,I) in *corpus striatum*. To obtain quantitative outcomes from this analysis, we selected α-synuclein/α-tubulin colocalization signal (white in Figure 3E,H), overlapped this image mask (green in Figure 3F,I) with synapto-

physin, and performed colocalization analysis between the three antigens. A mean M2 Manders' coefficient of 0.168 revealed that almost 17% of the total synapses in *corpus striatum* contained both α-synuclein and α-tubulin (yellow signal in Figure 3F,I). Taken together, these results demonstrated a considerable colocalization of α-synuclein/α-tubulin that varies in selected forebrain and brainstem areas, and revealed that such a colocalization between the two proteins occurs in a subset of synaptic terminals in *corpus striatum*.

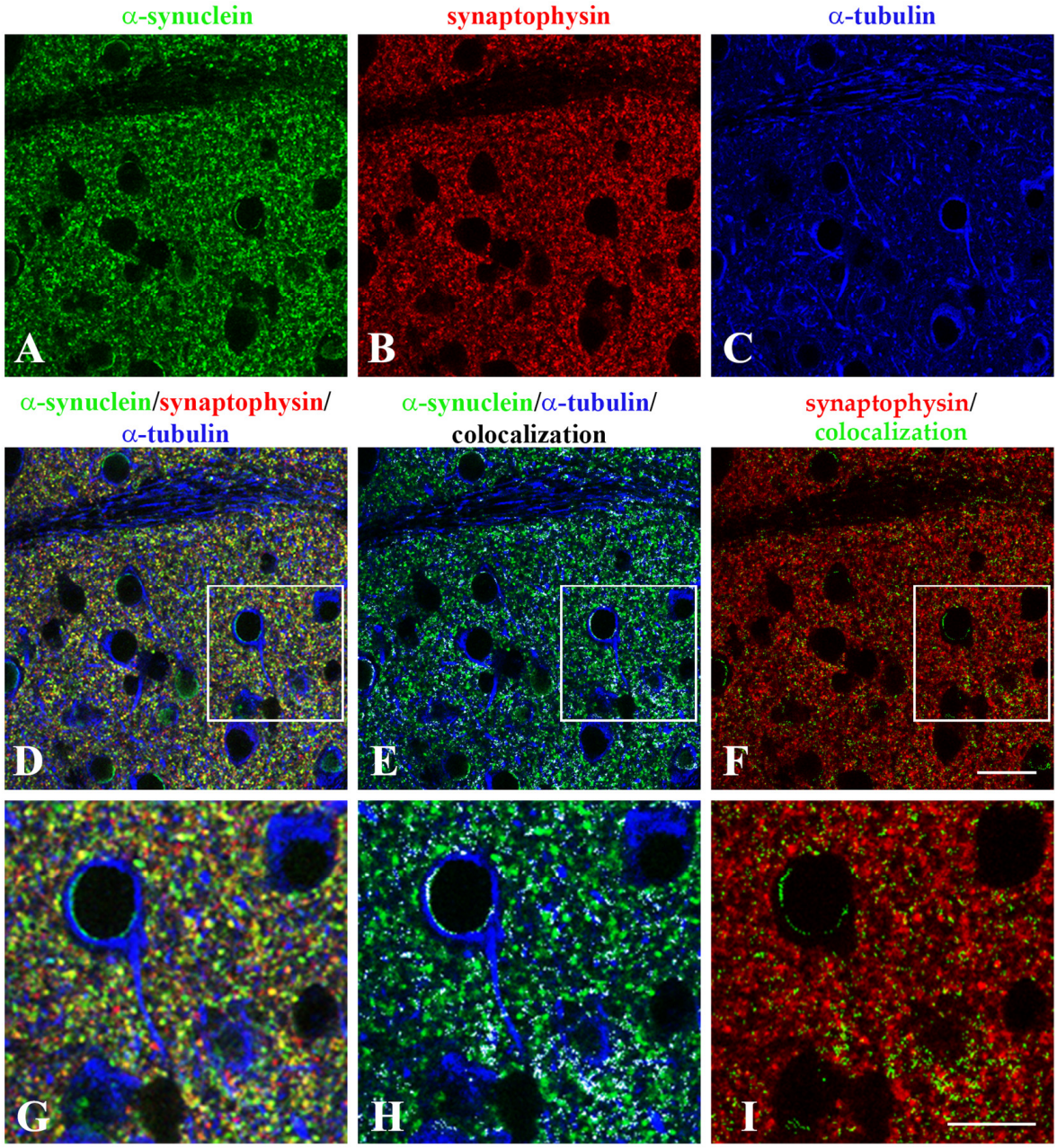

Figure 3. α-synuclein and α-tubulin colocalization in synaptic terminals in the *corpus striatum* of WT mice. Triple immunofluorescence for (**A**) α-synuclein (α-syn, green), (**B**) synaptophysin (syp, red), (**C**) α-tubulin (α-tub, blue) analyzed by confocal microscopy (n = 4 mice, at least 4 replicates). (**D**): Merge of the triple staining. The region marked by the white square in (**D**) is magnified in (**G**). (**E**): The colocalization between α-synuclein (green) and α-tubulin (blue) is shown as the white mask. The region marked by the white square in (**E**) is magnified in (**H**). (**F**): Merge of synaptophysin (red) with the α-synuclein/α-tubulin colocalization mask (green). The region marked by the white square in (**F**) is magnified in (**I**). Scale bars: 25 μm (**A**–**F**), 40 μm (**G**–**I**).

2.3. α-Synuclein and α-Tubulin Interact in Mouse Corpus Striatum and in Human Brain

Once the colocalization of α-synuclein and α-tubulin was revealed in the synaptic compartment, we decided to investigate whether the two proteins interact by means of brightfield PLA in both murine (Figure 4A,A') and human (Figure 5A,A') *corpus striatum*. First, we observed the association of the two proteins in murine brain (Figure 4A,A'). The specificity of the PLA technique was assessed by negative controls using two approaches: (i) omitting anti-α-synuclein primary antibody from PLA, and (ii) testing α-synuclein/α-tubulin PLA on sections of C57BL/6J OlaHsd mice carrying spontaneous deletion of the *SNCA* gene [53]. As expected, no staining was present with the omission of the primary antibody (Figure S4) and no specific signal was detected in the C57BL/6J OlaHsd mouse sections where α-synuclein is not expressed (Figure 4B,B'). Second, we found that α-synuclein also interacts with α-tubulin in the human brain (Figure 5A,A'). Here, we observed a diffused signal mostly in the grey matter in the *corpus striatum* (Figure 5A,A') and also in the cerebral cortex, especially in layer V (Figure 5A,A''), where a high synaptic density is observable. As a positive PLA control, we assessed the α-tubulin/βIII-tubulin interaction in the human brain sample and reported an expected pattern of staining that was more intense in the white matter (Figure 5B,B'), showing its specificity for regions that were rich in microtubules in the bundles of fibers of the *corpus striatum* (Figure 5B') or in the microtubules present in the apical dendrites of the pyramidal neurons (layer V, Figure 5B'').

Based on the evidence that α-synuclein and α-tubulin directly interact in mouse and human brain, we analyzed whether this interaction occurs in the synaptic compartment. We performed fluorescent PLA experiments in combination with synaptophysin immunofluorescence in order to detect the interaction between the two proteins in striatal synaptic terminals (Figure 6). The PLA signal (Figure S5A and Figure 6A) colocalized with synaptophysin labelling (Figure S5 and Figure 6A') in the merge images (Figure S5 and Figure 6A''). In detail, the fraction of PLA signal colocalizing with synaptophysin was more than half (obtained by Manders' coefficients, M1 = 0.65). Furthermore, only a small percentage (5%) of striatal synaptic terminals (M2 = 0.05) contained the association between the two proteins (Figure 6B). Then, analysing the intensity profiles (Figure 6C), we confirmed a complete superimposition of the α-synuclein and α-tubulin staining spectrum and the synaptophysin one, indicating that this interaction really occurred in some synapses.

Finally, we investigated the interaction of α-synuclein with α-tubulin inside the synapse at the ultrastructural level by carrying out a double immunogold pre-embedding localization. This technique enabled us to observe the presence of both antigens in the neuropilar structures of *corpus striatum* (Figure 7). The discrimination of intensified signals of α-synuclein and α-tubulin immunogold silver was possible through the double silver intensification of α-synuclein-related gold particles, which made them larger than the α-tubulin-related ones. First of all, the single immunolocalization of α-synuclein in some synaptic boutons (Figure 7A,F), and of α-tubulin in partially myelinated fibers (Figure 7D) and in some synaptic terminals (Figure 7B,E,F), reasserted our previous single ultrastructural immunolabelling (Figures S2 and S3) and gave us a validation of the double immunogold method. Moreover, our analysis confirmed the colocalization of the two proteins in dendritic shafts (Figure 7A,B,E,G) as well as in small (Figure 7A) and large (Figure 7C) axons. Finally, in the striatal neuropil, we identified a few small synaptic terminals, which contacted dendrites or dendritic spines and clearly displayed both α-synuclein- and α-tubulin-related gold particles, among or adjacent to small clear vesicles (Figure 7F,G).

In conclusion, our data demonstrated a strict relationship between α-synuclein and α-tubulin not only in different neuronal compartments such as dendrites and axons, but also in synapses where their mutual interaction could be crucial for neurotransmission.

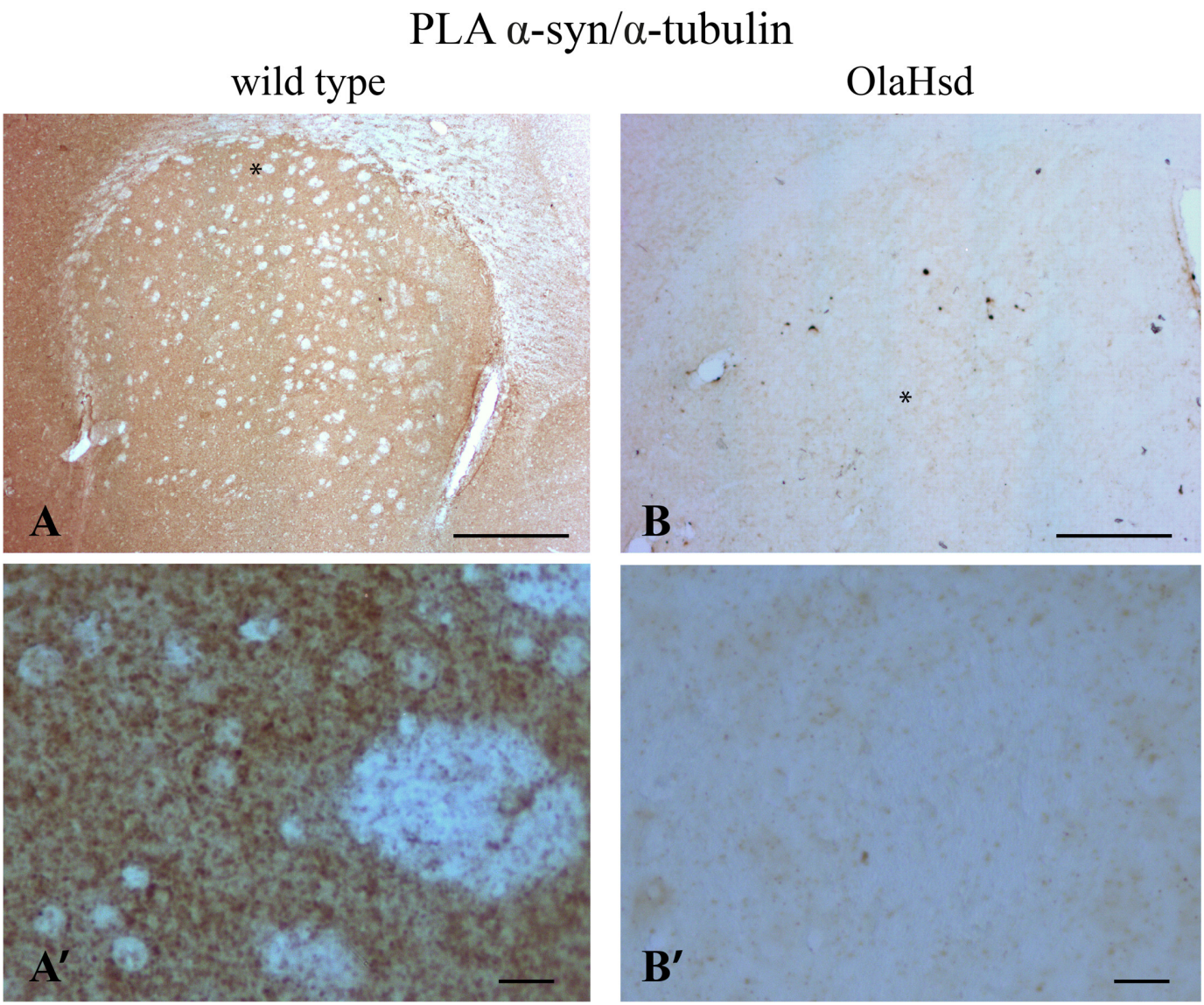

Figure 4. Brightfield PLA procedure for α-synuclein/α-tubulin PLA in *corpus striatum* of wild type and OlaHsd mice. (**A**,**B**): Low magnification image of PLA staining (brown) for α-synuclein/α-tubulin PLA in wild type mouse brain *corpus striatum* ((**A**), *n* = 4 mice) compared to the almost negative signal in OlaHsd mouse ((**B**), *n* = 4 mice). In (**A**), white dots are the typical bundles of fibers in the *striatum* that are negative for the PLA staining. (**A′**,**B′**): High magnification photomicrographs of the *striatum*. Asterisks in (**A**,**B**) indicate the regions that are magnified in (**A′**,**B′**), respectively. Scale bars: 500 µm (**A**,**B**), 10 µm (**A′**,**B′**).

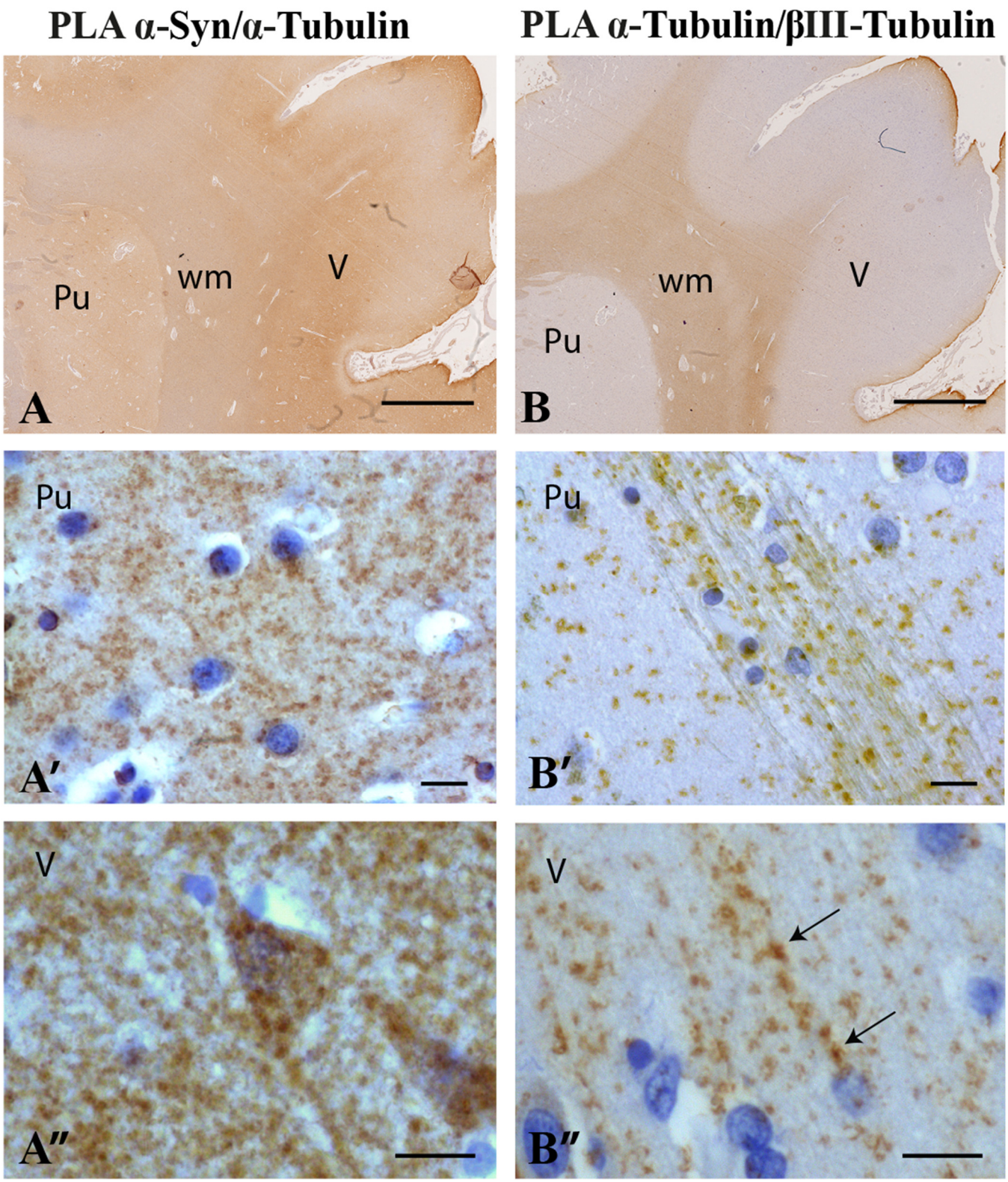

Figure 5. Brightfield PLA procedure for α-synuclein/α-tubulin PLA in human *corpus striatum* and cerebral cortex. (**A**): Low magnification image of PLA staining (brown) for α-synuclein/α-tubulin PLA in human brain *corpus striatum* and cerebral cortex (n = 4 subjects). (**A′**,**A″**): High magnification photomicrographs of putamen (Pu, (**A′**)) and cerebral cortex layer V (V, (**A″**)). (**B**): Low magnification image of PLA staining (brown) for α-tubulin/βIII-tubulin PLA in human *corpus striatum* and cerebral cortex used as a positive control. (**B′**,**B″**): High magnification photomicrographs of putamen (**B′**) and cerebral cortex layer V (**B″**). Arrows indicate signal in the apical dendrites of pyramidal neurons. Nuclei are counterstained with hematoxylin (violet). wm: white matter. Scale bars: 2.5 mm (**A**,**B**), 20 μm (**A′**–**B″**).

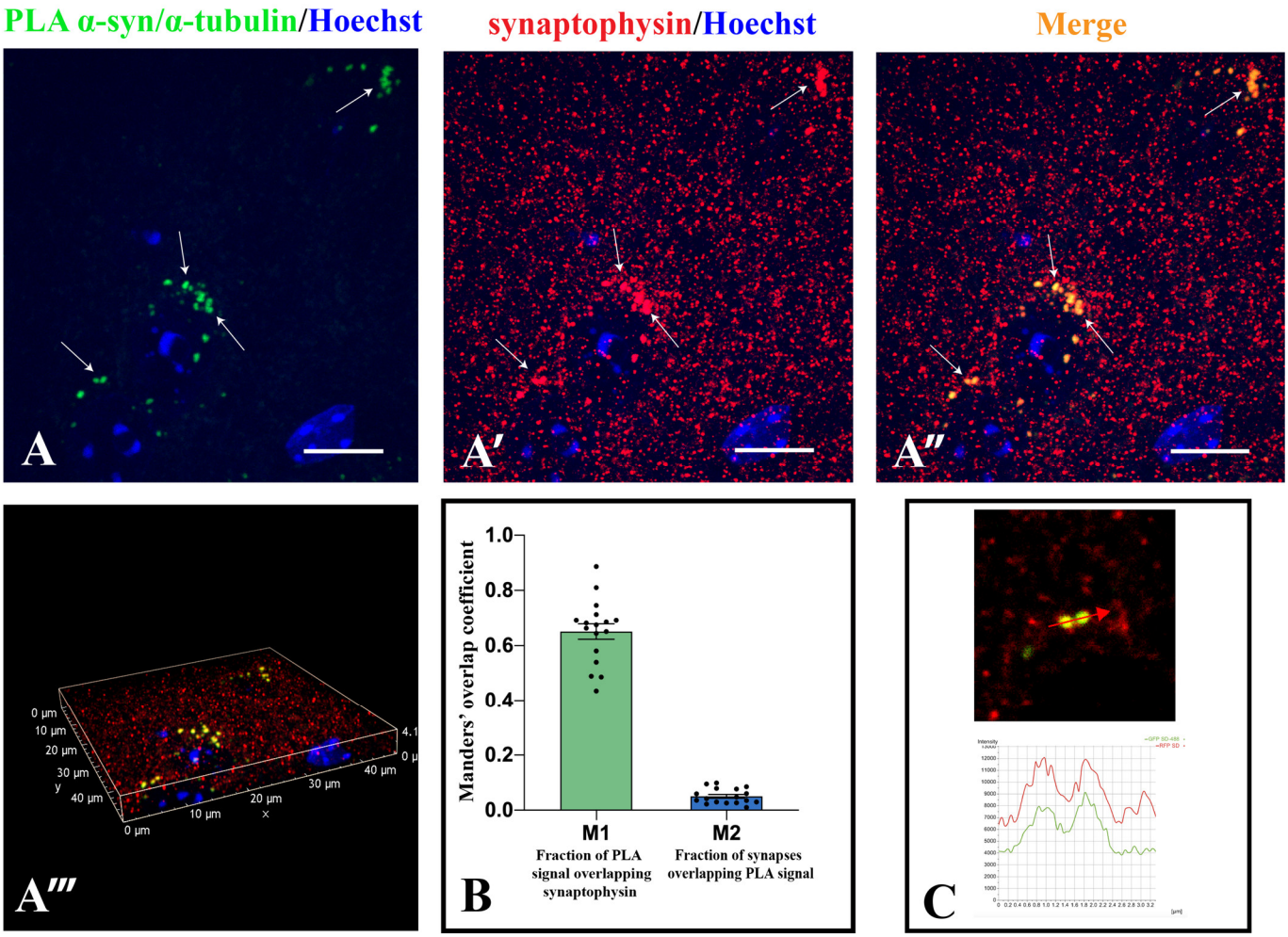

Figure 6. Spinning disk analysis of α-synuclein/α-tubulin interaction in the synapses of murine *corpus striatum*. (**A**): Representative maximum projection of α-synuclein/α-tubulin PLA staining (green, (**A**,**A″**)) and synaptophysin staining (red, (**A′**,**A″**)) in C57BL/6J mouse *corpus striatum*. White arrows point to some of the α-synuclein/α-tubulin PLA signals that colocalize with synaptophysin (yellow dots also in **A‴**,**C**). Nuclei are counterstained with Hoechst (blue). Scale bars: 5 µm (**A**–**A″**). (**A‴**): 3D reconstruction of all the immunofluorescence signals contained in the tissue slice is showed in the maximum projection. (**B**): M1 and M2 Manders' overlapping coefficient obtained from 16 images acquired from 4 different mice. (**C**): Intensity profile plot representing the values indicated by the red arrow in the single stack image of the merge channels stained for synaptophysin (red) and α-synuclein/α-tubulin PLA (green).

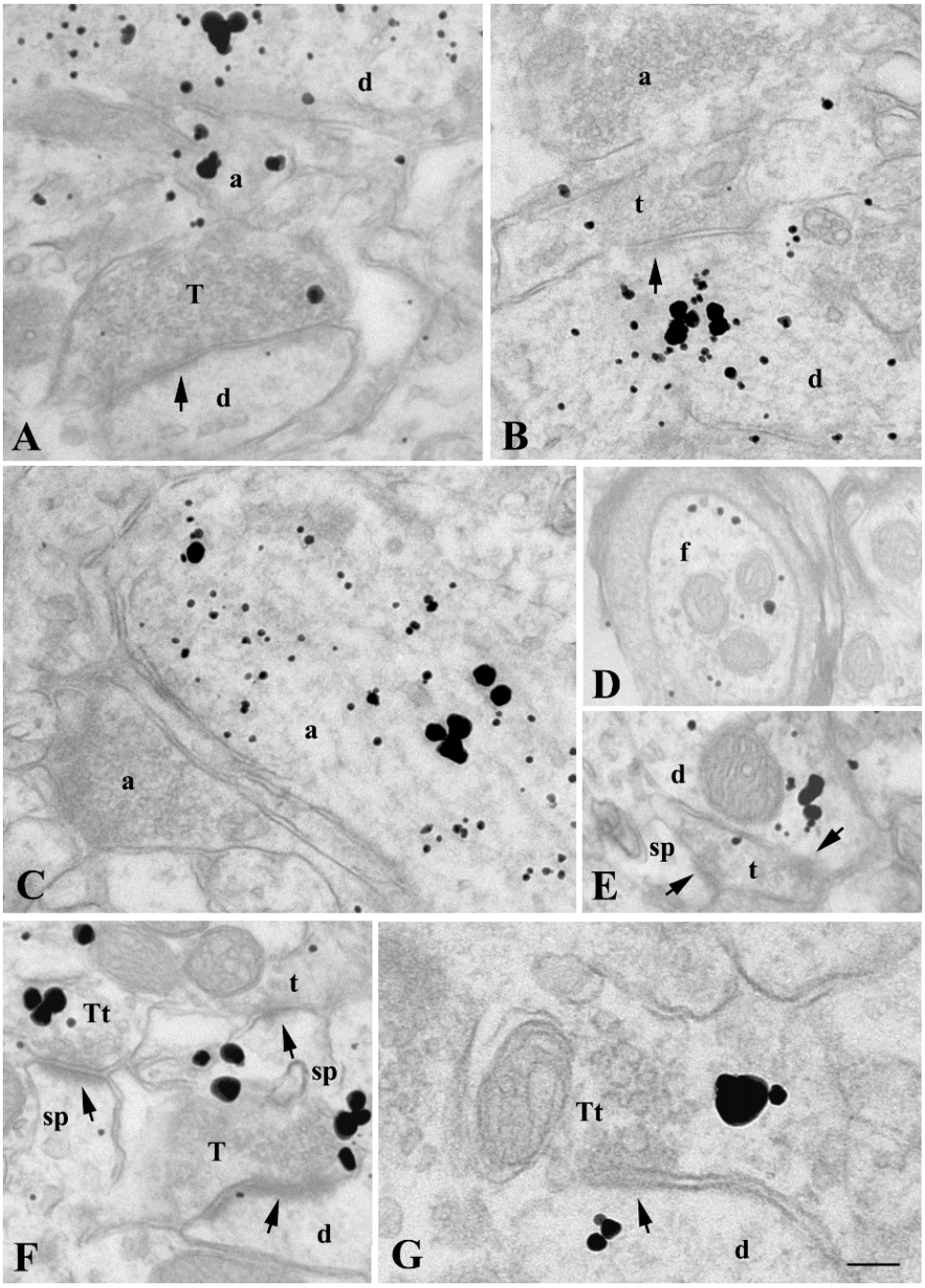

Figure 7. Immunoelectron microscopical localization of α-synuclein and α-tubulin in murine *corpus striatum*. Representative electron micrographs of α-synuclein (large silver-intensified gold particles) and α-tubulin (small silver-intensified gold particles) pre-embedding immunogold colocalization performed in C57BL/6J mice (n = 3). The colocalization appeared mainly in proximal dendrites (d), as shown in (**A**,**B**,**E**), as well as in some, but not all, small and large vesicle-containing axonal (a) profiles (**A**,**C**). Some poorly myelinated fibers (f in (**D**)) and small axonal terminals forming synaptic contacts (arrows) with proximal dendrites (d) and dendritic spines (sp) displayed single α-tubulin immunolabelling (t in (**B**,**E**,**F**)), whereas single α-synuclein immunolabelled synaptic boutons (T) contacting (arrows) distal dendrites (d) were shown in (**A**,**F**). Finally, double α-synuclein and α-tubulin immunogold staining was displayed by some small synaptic terminals (Tt) forming either asymmetric postsynaptic specializations (arrows in (**F**)) on dendritic spines (sp) or symmetric synapses (arrow in (**G**)) with α-tubulin-positive dendrites (d in (**G**)). Scale bar: 200 nm (**A**–**F**), 100 nm (**G**).

3. Discussion

Since α-synuclein was indicated as the major component of histopathological lesions in a spectrum of neurodegenerative diseases including PD, many studies were centered on its propensity to aggregate and form pathological inclusions [54], thus leaving its physiological role mainly elusive. Here, we addressed the emerging issue of the interplay of α-synuclein with microtubules. Beyond the differential expression of α-synuclein and its colocalization with α-tubulin in some areas of the murine brain, our data unravel the previously unexplored association of the two proteins that may be relevant for synaptic function in vivo. Given the impact of α-synuclein on the dynamic instability of microtubules in cell-free systems and neuronal cultured cells [38], the present work paves the way for investigating a novel physiological role of α-synuclein in the regulation of microtubule behavior at the synapse that, in turn, could help in the full comprehension of synaptic dysfunction occurring in both neurodegenerative and neurodevelopmental disorders.

The interaction of α-synuclein with tubulin and microtubules has been a matter of debate for a long time, mainly due to its potential implication in the pathogenesis of synucleinopathies [31,55,56]. However, the majority of data come from in vitro and cultured cell studies that were focused on understanding the consequences of their interplay on the biochemical properties of the two partners [30], microtubule dynamics [38] and axonal transport [37]. Nevertheless, few data are available regarding α-synuclein colocalization with tubulin in vivo. The presence of α-tubulin in Lewy bodies, pale bodies and Lewy neurites was highlighted by Alim et al. [33] in human brain affected by PD and other synucleinopathies, whereas a proximity of α-synuclein with axonal microtubules was observed exclusively by immunogold localization in cortical rat brain tissue [6]. Here, we reveal, for the first time, the colocalization of α-synuclein with microtubules in different areas of the murine brain, offering an overview on the regional differences. The highest levels of colocalization were found in the analyzed cortical areas, whereas both the *substantia nigra pars compacta* and the *corpus striatum* displayed low colocalization values in the total α-synuclein expression. In the *corpus striatum,* which is a focal structure that is rich in synaptic contacts originating from different brain areas (i.e., the cerebral cortex, brainstem and thalamus), we demonstrated the interaction of α-synuclein with α-tubulin using a PLA approach. Finally, we validated this interaction in human brain sections, not only in *corpus striatum*, which is clearly involved in PD and multiple system atrophy, but also in the cerebral cortex, a region that is relevant for dementia with Lewy bodies. Thus, our results point out α-tubulin as a novel interactor of α-synuclein, suggest that the colocalization of the two proteins is region-specific, and pose the question as to whether this could reflect a functional peculiarity.

The regional specificity in the colocalization could go together with the differential expression of α-synuclein in the brain as this work and previous papers demonstrate. Although no region-dependent changes were revealed by immunoblotting assays on brain lysates, the detailed analyses of brain areas by immunohistochemistry revealed significant alterations in the distribution and expression levels of α-synuclein in the forebrain (especially in distinct cortical areas) and ventral mesencephalon. Previous data based on semiquantitative analysis in the rodent brain suggest that many differences occur in the olfactory bulb, brainstem, thalamus, cerebellum, hippocampus, amygdala, *striatum* and neocortex [4,7,8,11]. Here, we moved to the first quantitative rating on α-synuclein distribution and revealed significant differences between entorhinal cortex/CA3 and other cortical and striatal areas. In particular, *substantia nigra pars compacta* appeared to be the least labelled area, showing a significant difference to all the other examined regions. Focusing on the midbrain region, an interesting scenario emerged from the analysis of α-synuclein distribution together with its colocalization with α-tubulin, which was completely unexplored until now. Beyond the differences observed in data coming from different laboratories that could depend on differences among antibodies, which is an important issue, as previously discussed [12], a common finding between ours and other papers [4,8] is the detection of somatic α-synuclein almost exclusively in the *substantia nigra pars compacta*. Collectively,

these results suggest that the differences in α-synuclein distribution inside neuronal compartments and protein interaction could mark this brain area and impose peculiar behavior in physiology, and perhaps in pathology.

The finding that α-synuclein interacts with tubulin at the synapse is groundbreaking as it sheds light on the emerging role of microtubules in this neuronal compartment. The presence of tubulin and microtubules in the synapse is a long-lasting controversial issue [57]. Even if, on the one hand, microtubules are easily visualized in axons and dendrites through multiple classical techniques, on the other hand, their synaptic localization has always been difficult due to their instability and scarcity in this neuronal compartment. Indeed, a long time ago, Gordon-Weeks et al. [42] demonstrated the presence of presynaptic microtubules in central synapses, autonomic varicosities, and also rat brain synaptosomes, at the ultrastructural level, by using specific fixatives enriched in EGTA, which avoided their disassembly by calcium influx. However, it was more than three decades later when evidence from live cell imaging experiments in *Drosophila* models definitively concluded that microtubules are present inside synaptic boutons [45]. More interestingly, recent studies in mammalian neurons demonstrated the role of microtubules in the release of synaptic vesicles [58]. Microtubule dynamics is crucial in excitatory en passant varicosities where they are able to correctly target synaptic vesicles to active zones, thus controlling neurotransmission [47]. In this scenario, the results we obtained through multiple approaches, from PLA and confocal spinning disk microscopy to double immunogold pre-embedding and electron microscopy, supply a novel insight into presynaptic functions. So, we speculate that the presynapse could be the privileged compartment where α-synuclein modulates synaptic release and activity via the regulation of microtubules due to its dynamase activity [38].

Our finding showing a direct interaction of α-synuclein and α-tubulin in a subset of striatal murine synapses strongly suggests that this could mark specific synapses. Interestingly, α-synuclein is mainly expressed in asymmetric synapses, generally excitatory, in the *corpus striatum* [5]. In parallel, a high grade of colocalization between α-synuclein and the vesicular glutamate transporter-1 (VGLUT1, a marker of most glutamatergic terminals) and the low colocalization with GAD67 (a GABAergic marker) were detected in the cerebral cortex [8]. Now, the challenge is to better understand if and how the expression of α-synuclein modulates the activity of specific synaptic terminals with different neurochemical properties and whether its interaction with α-tubulin plays a role. Indeed, based on the most recent studies on synaptic microtubules [49,57] and on our results, en passant synapses could represent the best example of sites where α-synuclein and α-tubulin might interact and cooperatively impact on synaptic activity. In this context, old data reporting ultrastructural characterization of dopaminergic striatal innervation revealed its en passant distribution in rat *striatum* [59]. On the other hand, we observed a high level of the two proteins colocalization in cortical areas that are notably rich in modulatory cholinergic, dopaminergic and serotoninergic en passant varicosities [60–62]. Lastly, studies regarding the modulatory role and localization of α-synuclein in brain synapses were mainly focused on excitatory glutamatergic synaptic contacts [8,9,16,63], where they could interact to orchestrate a fine tuning of glutamate release by specialized hotspots, as recently stated by Qu et al. [47] in hippocampal cell cultures. As a whole, these studies lay the foundation for further analyses designed to better understand the role of the α-synuclein/α-tubulin interplay in synaptic terminals that possess different neurochemical properties, where they could interact to orchestrate the fine tuning of neurotransmitter release.

Our data, which point to a novel aspect of α-synuclein biology (specifically, its interaction with tubulin at the synapse), open an interesting scenario to explain disease mechanisms in synucleinopathies and other disorders. One initial consideration is based on the "hub protein" role of α-synuclein that refers to its propensity to interact with multiple partners at the synapse and, in turn, to regulate neurotransmission [64]. Disruption of this complex network, even when just one partner is altered, triggers synaptic dysfunction. Notably, we know that α-synuclein mutants linked to familial PD impact the tubulin

system at different levels including impairment of tubulin polymerization [31,34,38], and that overexpression of α-synuclein disrupts the microtubule network [65], thus suggesting that defects in the interplay between α-synuclein and tubulin could be detrimental for neurons and lead to neurodegeneration. Furthermore, in a murine model of multiple system atrophy, α-synuclein/β-III tubulin protein complex was involved in synaptic vesicle release and reduces GABAergic inhibitory neurotransmission [15]. A second line of evidence comes from studies on the aggregation of α-synuclein. Oligomeric α-synuclein inhibits tubulin polymerization [35], whereas the modulation of tubulin system is effective in blocking the formation of pathological α-synuclein inclusions [66]. On these bases, our findings could suggest new investigations on the α-synuclein/α-tubulin interplay, not only in classical synucleinopathies but also in other neuropathologies such as Alzheimer's disease and epilepsy where synaptic dysfunction frequently underlies the respective clinical pictures [57]. Interestingly, studies on neurodevelopmental diseases point out the role of both deletion or partial duplication of the α-synuclein gene [67] and genetic mutations in microtubule-associated genes or defective regulation of microtubules in the pathophysiology of autism spectrum disorder [68]. This could permit speculation on the existence of a converging mechanism that involves both α-synuclein and tubulin cytoskeleton in neurodegenerative as well as neurodevelopmental disorders.

To conclude, our demonstration of the association between α-synuclein and α-tubulin in murine and human brain tissues proposes a new angle for looking at synaptic compartment and machinery in neuronal health, but also in disease.

4. Materials and Methods

Twelve male WT C57BL/6J mice at P60 were purchased from Charles River (Calco, Italy) and used for all experiments. The mice were maintained in pathogen-free conditions and bred with free access to water and standard pelleted diet. The mice were killed by decapitation or by intracardiac perfusion, to perform biochemical or immunohistochemical analysis, respectively. All procedures were compliant to Italian law (D. Lgs 2014/26, implementation of the Directive 2010/63/UE of the European Parliament and of the Council on the protection of animals used for scientific purposes) and approved by the University of Milan Animal Welfare Body and by the Italian Ministry of Health (project authorization number: 901/2015-PR).

Four C57BL/6J OlaHsd mice, a substrain of C57BL/6J mice carrying a spontaneous deletion of the SNCA gene, were stored at the University of Brescia and all procedures were approved by the Italian Ministry of Health (project authorization number: 719/2015-PR).

4.1. Human Samples

Formalin fixed paraffin embedded striatal sections were obtained from post-mortem human brains of four control subjects, including three females (64, 82, 93 years old) and one male (71 years old), in whom the absence of neurodegenerative pathologies was assessed (see [69] for details). Written informed consent was obtained from all subjects in compliance with relevant laws and institutional guidelines and approved by the appropriate committees.

4.2. Primary Antibodies

All primary antibodies, their epitope, host species, application dilution, source and catalogue number are summarized in Table 1.

Table 1. List of primary antibodies.

Primary Antibody	Epitope	Host Species	Application	Dilution	Source	Catalog Number
Actin	C-term fragment	Rabbit	WB	1:2000	Sigma-Aldrich St. Louis, MO, USA	A2066
α-synuclein	C-term human α-syn (aa 111–132)	Rabbit	WB	1:2000	Sigma-Aldrich St. Louis, MO, USA	S3062
			IF/EM	1:500		
			IHC	1:1500		
			PLA	1:100		
α-synuclein (clone Syn211)	C-term human α-syn (aa 121–125)	Mouse	IF	1:1500	Sigma-Aldrich St. Louis, MO, USA	S5566
			PLA	1:100		
α-tubulin (clone B-5-1-2)	C-term	Mouse	IF/EM	1:500	Sigma-Aldrich St. Louis, MO, USA	T6074
			PLA	1:50		
α-tubulin	N-term (aa 1–100)	Rabbit	PLA	1:50	Abcam, Cambridge, UK	ab4074
βIII-tubulin	C-term	Mouse	PLA	1:300	Sigma-Aldrich St. Louis, MO, USA	T8660
βIII-tubulin (clone EP1331Y)	C-term (within aa400)	Rabbit	PLA	1:250	Abcam, Cambridge, UK	ab52901
Synaptophysin1	Human synaptophysin1 (aa 301–313)	Guinea pig	IF	1:400	Synaptic Systems, Goettingen, Germany	101 004

4.3. Western Blot Analysis

Western blot analysis was performed on protein extracts obtained from brain regions of five mice. To obtain total proteins, *corpus striatum*, ventral midbrain, hippocampus, entorhinal, prefrontal and parietal cortices were immediately dissected on ice and mechanically homogenized and sonicated in sample buffer (SB1x: 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue and 62.5 mmol/L Tris, pH 6.8), containing protease and phosphatase inhibitors (Protease Inhibitor Cocktail, P8340 Sigma-Aldrich, St. Louis, MO, USA; Phosphatase Inhibitor Cocktail Set V, 524629, Millipore, Burlington, MA, USA). After centrifugation, soluble fractions were collected and protein concentration was measured with Pierce BCA protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of each sample were separated by SDS-PAGE and blotted onto PVDF membranes (Immobilon^{TM}-P, Millipore). Membranes were incubated for 30 min (min) at room temperature with 0.4% paraformaldehyde in PBS and then blocked with 3% bovine serum albumin (BSA) in 0.05% Tween20 Tris buffered saline (TBS-T) for 1 h (h) at room temperature and probed with the following antibodies: anti actin IgG (A2066) and anti α-synuclein (S3062). Membranes were washed for 30 min with TBS and incubated for 1 h at room temperature with HRP goat anti-rabbit IgG (1:4000; Cell Signaling Technology, Danvers, MA, USA). Chemiluminescent signals were detected using the Supersignal West Pico Chemiluminescent Substract kit (Pierce, Appleton, WI, USA). Acquisition and quantification were performed by ChemiDoc and Image Lab software (Bio-Rad, Hercules, CA, USA).

4.4. Immunohistochemistry

Mice were anesthetized with isoflurane and intraperitoneal 4% chloral hydrate (2 mL/100 g) and sacrificed by intracardiac perfusion as previously described [70]. Brains were immersed in 4% paraformaldehyde in phosphate buffer 0.1 M (PB), for 24 h at 4 °C. Next, brains were stored in PB, since they were cut in serial coronal brain sections (50 µm thick) with a VT1000S vibratome (Leica Microsystems). After intracardiac perfusion C57BL/6J OlaHsd mice brains were post-fixed for 2 h in 4% paraformaldehyde and conserved in 18% sucrose solution in phosphate buffered saline 0.01 M (PBS). The brains were then cut in 25 µm coronal sections with a cryostat and conserved in 60% glycerol.

According to Franklin and Paxinos [71], we chose the following sections for immunohistochemistry and densitometric analyses: (i) prefrontal cortex between +2.58 and

+1.14 mm from bregma (containing secondary motor region, also known as M2, or Fr2); (ii) somatosensory cortex and *corpus striatum* in proximity to −0.34 mm from bregma; (iii) *substantia nigra pars compacta* and *substantia nigra pars reticulata* at −3.64 mm from bregma; (iv) the entorhinal cortex and the hippocampus at −3.16 mm from bregma.

4.4.1. Immunoperoxidase Procedure

After aldehyde quenching with NH_4Cl (0.05 M in PBS) for 30 min and inactivation of endogenous peroxidases with 1% H_2O_2 (in PBS) for 30 min, sections were permeabilized with a mild pretreatment by ethanol (10%, 25%, 10% in PBS, 5 min each) to increase the immunoreagent penetration. The blocking solution constituted by 0.1% Triton X-100 in 1% BSA in PBS was then applied for 30 min. Next, slices were incubated overnight with anti-α-synuclein antibody (S3062) diluted in 0.1% BSA, at room temperature. This procedure was followed by incubation with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA), for 75 min. After washing, sections were treated with the avidin-biotin complex (ABC elite kit; Vector Laboratories; diluted 1:100) and then with a freshly prepared solution (0.075%) of 3-3′-diaminobenzidine tetrahydrochloride (Carl Roth GmbH & Co., Karlsruhe, Germany) and 0.002% H_2O_2. Finally, sections were mounted, dehydrated with ethanol (75%, 96% and 100% for 5 min each), immersed in xylene for 10 min and laid on coverslips with Eukitt® (O. Kindler GmbH, Freiburg, Germany). The specificity of primary antibodies was assessed by negative controls, e.g., omission of primary antiserum. In these cases, no specific staining was ever observed.

4.4.2. Densitometric Immunoperoxidase Analysis

To perform a densitometric analysis of α-synuclein on immunoperoxidase-stained sections, whole slices were acquired with the Nanozoomer S60 slide scanner (Hamamatsu, Tokyo, Japan). The images were then magnified and saved at 20× with the NDPview2 software (Hamamatsu) to show only the considered area. Different regions of interest (ROIs) were drawing for each brain areas using Fiji. The following areas were analyzed: prefrontal cortex (II/III layer and V layer), somatosensory cortex (II/III layer and V layer), *corpus striatum* (dorsal-lateral, dorsal-medial, and ventral), dentate gyrus, *Cornu Ammonis* region 3, entorhinal cortex, *substantia nigra pars compacta* and *reticulata*. Four images, at 20× for each area (one for each hemisphere at least in two different sections), were selected from the whole acquired sections (4 for each animal, two rostral sections for cortical and striatal areas, two caudal sections for nigral, hippocampal and entorhinal ones). The images were then deconvoluted and spatially calibrated using Fiji. For each image, a mean pixel signal intensity (in optical density; OD) inside the ROI was obtained and compared between the different areas. We analyzed at least 3–4 images per each area in each animal. We statistically tested whether subregional differences in α-synuclein intensity exist in the prefrontal cortex, somatosensory cortex, and *corpus striatum*. No subregional differences were detected; therefore, we decided to combine the data within each area.

4.4.3. Immunofluorescence Procedure

Sections were permeabilized and blocked as described for immunoperoxidase histochemistry. They were next incubated for two nights in a mixture of primary antibodies—anti-α-synuclein S3062 and anti-α-tubulin T6074—with or without anti-synaptophysin 1. After rinsing with PBS, an incubation with the following secondary antibodies was performed for 75 min: donkey anti-rabbit Alexa Fluor® 488 (Invitrogen, Waltham, MA, USA), donkey anti-mouse CF® 568 (Biotium, San Francisco, CA, USA), both diluted 1:200 in BSA 0.1%. For the triple immunostaining, the slices were incubated with a mixture of donkey anti-rabbit Alexa Fluor® 488 (1:200; Invitrogen), donkey anti-guinea pig CF® 568 (1:200; Biotium) and biotinylated horse anti-mouse secondary antibody (Vector Laboratories) in BSA 0.1% for 75 min at room temperature, followed by an incubation with Alexa Fluor® 647-conjugated streptavidin (1:200; Invitrogen) in PBS at room temperature for 2 h. Nuclei were stained using Hoechst 33342 (1:1000 in PBS; Sigma-Aldrich) for 15 min. Samples were

mounted on coverslips using Mowiol®-DABCO (Sigma-Aldrich). Double immunostaining images (4 for each area) were acquired with a Nikon A1 laser scanning confocal microscope with a 40× magnification objective, while triple fluorescent labelling images were acquired by a Leica TCS SP8 scanning confocal laser at 40× magnification with an optical zoom 2. The specificity of primary antibodies was assessed by negative controls, e.g., omission of primary antiserum. In these cases, no specific staining was ever observed.

4.5. Proximity Ligation Assay (PLA)

The in situ PLA enables the detection of protein-protein interactions in intact tissues [72,73]. For the PLA procedure, we analyzed murine wild type and OlaHsd brain sections, and human paraffin embedded brain sections, containing *corpus striatum* using the Duolink assay kit (Sigma-Aldrich), according to the manufacturer's instructions.

For the brightfield PLA procedure, after rehydration of the human brain slices, murine (WT and C57BL/6J OlaHsd) and human samples were then incubated with H_2O_2 for 20 min at room temperature and in 0.1% Triton X-100 in 1% BSA for 30 min at room temperature, sequentially followed by the mixture of: (i) the primary antibodies (for human sections: anti-α-synuclein antibody Syn211, S5566, anti-α-tubulin antibody, ab4074; anti-βIII-tubulin T8660; for murine sections: anti-α-synuclein S3062 and anti-α-tubulin T6074) incubated with 1% BSA for 1 h at 37 °C, then overnight at room temperature; (ii) the secondary antibodies donkey anti-mouse IgG conjugated with Duolink PLA MINUS oligonucleotides and anti-rabbit IgG secondary antibodies conjugated with Duolink PLA PLUS oligonucleotides diluted 1:5 in Duolink Antibody Diluent for 2 h at 37 °C; (iii) Duolink ligation solution (1:5) and ligase (1:40) for 1 h at 37 °C; (iv) Duolink amplification reagents (1:5) and polymerase (1:80) for 2 h at 37 °C; (v) Duolink detection solution (1:5) for 1 h at room temperature followed by the incubation of 3,3′ Diaminobenzidine as chromogen (DAB, Dako kit). The sections were then counterstained with hematoxylin and rapidly dehydrated before mounting with Eukitt® (O. Kindler GmbH).

For fluorescent PLA experiments, vibratome brain sections from WT mice (n = 4), after incubation in 0.1% Triton X-100 in 1% BSA for 30 min at room temperature, were treated with the mixture of primary antibodies (anti-α-synuclein S3062, anti-α-tubulin T6074 and anti-synaptophysin) diluted in Duolink PLA diluent for 2 h at 37 °C, then overnight at room temperature. In order to verify PLA signal specificity, vibratome brain sections from WT mice were incubated with anti-βIII tubulin ab52901, anti-α-tubulin T6074 and anti-synaptophysin 1as positive control. All primary antibodies were incubated with 0.1% Triton X-100 and 1% BSA for 1 h at 37 °C, then overnight at room temperature. After washing, all samples were incubated with the mix of secondary antibodies conjugated with Duolink PLA oligonucleotides and ligase, as described for the brightfield PLA procedure. After that, sections were incubated with a solution of Duolink amplification reagent green (1:5), Duolink polymerase (1:80) and donkey anti-guinea pig CF® 568 (1:200) at 37 °C for 2 h. Finally, Hoechst 33342 dye (1:5000) was used for nuclei counterstaining. The samples were mounted using Mowiol®-DABCO (Sigma). PLA labelled samples were examined both with a Nikon A1 laser scanning confocal microscope at 40× magnification (optical zoom 2) and with a Nikon microscope, equipped with CSI-W1 confocal scanner unit using a silicon-immersion 100× objective. Intensity profiles were obtained by the high-resolution images obtained with the spinning disk analyzed with the NIS elements AR software.

4.6. Colocalization Analysis

Confocal micrographs were analyzed with Fiji software. Identical parameters were used to acquire images for the same antigen, as previously described [74]. In brief, 4 nonoverlapping pictures were acquired in at least two different sections, so that double or triple immunolabelling and immunofluorescence PLA procedure were analyzed at least 4 fields per region in each animal. The degree of colocalization of different antigens was calculated by Manders' coefficients, computed with the ImageJ JACoP plug-in [75]. For PLA-synaptophysin colocalization, a fixed ROI around each neuron was analyzed

(at least 60 neurons for each mouse, n = 4 mice). In triple immunolabelling, the LASX software (Leica, Wetzlar, Germany) made it possible to obtain a colocalization mask for α-synuclein/α-tubulin signals. This mask was overlapped with the third signal, i.e., synaptophysin, and the degree of colocalization was calculated as the Manders' coefficient. In this way, we evaluated the presence of α-synuclein/α-tubulin double signal in synaptic terminals identified by synaptophysin.

4.7. Electron Microscopy

To preserve the ultrastructure of the tissues, 3 C57BL/6J WT mice at P60 were perfused with 4 % paraformaldehyde and 0.2% glutaraldehyde in PB. Sections with prefrontal cortex, *corpus striatum* and *substantia nigra pars compacta* were selected for immunoperoxidase or immunogold pre-embedding immunohistochemistry reaction. For immunoenzymatic α-synuclein localization, sections were processed as described in the immunoperoxidase procedure paragraph, only avoiding Triton X-100 treatment (see [76] for details). For immunogold single and double labelling after aldehyde quenching with 0.2% $NaBH_4$ in PBS for 30 min, sections were permeabilized with a mild treatment with ethanol (10%, 25%, 10% in PBS), rinsed with PBS and treated to block the unspecific interaction sites for 30 min with 1% BSA in PBS. Next, they were incubated overnight with one or the mixture of the primary antibodies anti-α-synuclein S3062 and anti-α-tubulin T6074 in 1% BSA at room temperature. This procedure was followed by rinsing with PBS and, after that, incubation with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories; diluted 1:100 in 0.1% BSA in PBS) for 4 h. After washing, sections were treated overnight with streptavidin-gold 6 nm (Aurion; diluted 1:10 in 0.1% BSA in PBS). The next day, slices were washed three times with PBS and three times with PB 0.1 M and then transferred in new glass boxes and rinsed with double-distilled water. Immunogold reaction was intensified with silver enhancement, using R-GENT SE-EM kit (Aurion, Wageningen, The Netherlands) for 90 min. Following washes with double-distilled water and with PB, the sections were incubated with a goat anti-mouse secondary antibody conjugated with Ultra-Small gold particles (Aurion) diluted 1:30 in BSA 0.1%. Following rinsing in PB, the sections were post-fixed with 1% glutaraldehyde in PB for 1 h, then rinsed with PB and double-distilled water in new glass boxes. The silver enhancement process was then repeated, using R-GENT SE-EM kit (Aurion) for 90 min. In this way, double immunolabelling gold particles that stain α-synuclein would be intensified two times and have a larger size compared to gold particles staining α-tubulin. Finally, both sections, processed according to the immunoperoxidase and immunogold procedures, were osmicated, dehydrated and epoxy embedded as previously described [70]. After polymerization, small areas from prefrontal cortex, *corpus striatum* and *substantia nigra pars compacta* sections were cut with a razor blade and glued to blank resin blocks for sectioning with a Reichert-Jung ultramicrotome (Leica). Ultrathin sections (50–70 nm) collected on Cu/Rh grids were counterstained with lead citrate, or left unstained, and examined with a Zeiss LEO912AB electron microscope (Zeiss).

4.8. Statistical Analysis

Data are given as mean values ± standard error of the mean (SEM) and represent data from a minimum of three independent experiments. Mean values of each experiment were subjected to logarithmic transformation and analyzed with one-way Analysis of Variance (ANOVA), using the Bonferroni *post-hoc* test. Statistical analysis was run with the GraphPad Prism 9.0.0 software. Unless otherwise indicated, detailed statistics are given in the figure legends.

Supplementary Materials: Supplementary materials can be found at https://www.mdpi.com/article/10.3390/ijms22179153/s1.

Author Contributions: Conceptualization, G.C.; methodology, A.A., A.M.C. and S.M.; investigation, A.A., S.P., D.M., A.C., A.M.C., L.M., G.F. and S.M.; project administration A.A., S.M. and G.C.; data

curation and formal analysis S.P., C.R., and A.C.; resources, A.A., S.M., G.F. and A.B.; writing—original draft preparation, A.A., S.P., A.C., D.M. and S.M.; writing—review and editing, G.C.; validation A.B.; visualization, A.A., S.P., A.C., C.R. and G.P.; supervision, G.C.; funding acquisition, G.P. and G.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Italian "5 × 1000" funding to Fondazione Grigioni per il Morbo di Parkinson (Milan, Italy), by University of Milano, Linea 2 2016 and 2017, to G.C. and A.A., and by Innovation Programme H2020-MSCA-ITN-2019-EJD-Grant agreement n. 860070 (TubInTrain, accessed date 1 October 2019, www.tubintrain.eu).

Institutional review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University of Milan (protocol code 66/21, 15 June 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets used and analyzed during the current study are available from the corresponding authors upon reasonable request.

Acknowledgments: The authors thank "Fondazione Grigioni per il Morbo di Parkinson" (Milan-Italy) for the salary provided to S.M. and for the long-lasting support given to G.C. We also thank Miriam Ascagni and Nadia Santo for technical support in confocal and electron microscopy analysis, respectively, carried out at NOLIMITS, an advanced imaging facility established by the Università degli Studi di Milano.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Burré, J. The synaptic function of α-synuclein. *J. Parkinsons. Dis.* **2015**, *5*, 699–713. [CrossRef]
2. Spillantini, M.G.; Schmidt, M.L.; Lee, V.M.Y.; Trojanowski, J.Q.; Jakes, R.; Goedert, M. α-synuclein in Lewy bodies. *Nature* **1997**, *388*, 839–840. [CrossRef]
3. Polymeropoulos, M.H.; Lavedan, C.; Leroy, E.; Ide, S.E.; Dehejia, A.; Dutra, A.; Pike, B.; Root, H.; Rubenstein, J.; Boyer, R.; et al. Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science* **1997**, *276*, 2045–2047. [CrossRef] [PubMed]
4. Andringa, G.; Du, F.; Chase, T.N.; Bennett, M.C. Mapping of rat brain using the Synuclein-1 monoclonal antibody reveals somatodendritic expression of α-synuclein in populations of neurons homologous to those vulnerable to Lewy body formation in human synucleopathies. *J. Neuropathol. Exp. Neurol.* **2003**, *62*, 1060–1075. [CrossRef] [PubMed]
5. Totterdell, S.; Hanger, D.; Meredith, G.E. The ultrastructural distribution of alpha-synuclein-like protein in normal mouse brain. *Brain Res.* **2004**, *1004*, 61–72. [CrossRef]
6. Zhang, L.; Zhang, C.; Zhu, Y.; Cai, Q.; Chan, P.; Uéda, K.; Yu, S.; Yang, H. Semi-quantitative analysis of α-synuclein in subcellular pools of rat brain neurons: An immunogold electron microscopic study using a C-terminal specific monoclonal antibody. *Brain Res.* **2008**, *1244*, 40–52. [CrossRef] [PubMed]
7. Vivacqua, G.; Casini, A.; Vaccaro, R.; Fornai, F.; Yu, S.; D'Este, L. Different sub-cellular localization of alpha-synuclein in the C57BL\6J mouse's central nervous system by two novel monoclonal antibodies. *J. Chem. Neuroanat.* **2011**, *41*, 97–110. [CrossRef]
8. Taguchi, K.; Watanabe, Y.; Tsujimura, A.; Tanaka, M. Brain region-dependent differential expression of alpha-synuclein. *J. Comp. Neurol.* **2016**, *524*, 1236–1258. [CrossRef] [PubMed]
9. Taguchi, K.; Watanabe, Y.; Tsujimura, A.; Tanaka, M. Expression of α-synuclein is regulated in a neuronal cell type-dependent manner. *Anat. Sci. Int.* **2019**, *94*, 11–22. [CrossRef]
10. Erskine, D.; Patterson, L.; Alexandris, A.; Hanson, P.S.; McKeith, I.G.; Attems, J.; Morris, C.M. Regional levels of physiological α-synuclein are directly associated with Lewy body pathology. *Acta Neuropathol.* **2018**, *135*, 153–154. [CrossRef]
11. Yu, S.; Li, X.; Liu, G.; Han, J.; Zhang, C.; Li, Y.; Xu, S.; Liu, C.; Gao, Y.; Yang, H.; et al. Extensive nuclear localization of α-synuclein in normal rat brain neurons revealed by a novel monoclonal antibody. *Neuroscience* **2007**, *145*, 539–555. [CrossRef]
12. Huang, Z.; Xu, Z.; Wu, Y.; Zhou, Y. Determining nuclear localization of alpha-synuclein in mouse brains. *Neuroscience* **2011**, *199*, 318–332. [CrossRef]
13. Kramer, M.L.; Schulz-Schaeffer, W.J. Presynaptic α-synuclein aggregates, not Lewy bodies, cause neurodegeneration in dementia with lewy bodies. *J. Neurosci.* **2007**, *27*, 1405–1410. [CrossRef]
14. Garcia-Reitböck, P.; Anichtchik, O.; Bellucci, A.; Iovino, M.; Ballini, C.; Fineberg, E.; Ghetti, B.; Della Corte, L.; Spano, P.; Tofaris, G.K.; et al. SNARE protein redistribution and synaptic failure in a transgenic mouse model of Parkinson's disease. *Brain* **2010**, *133*, 2032–2044. [CrossRef]
15. Ito, H.; Nakayama, K.; Jin, C.; Suzuki, Y.; Yazawa, I. α-Synuclein accumulation reduces GABAergic inhibitory transmission in a model of multiple system atrophy. *Biochem. Biophys. Res. Commun.* **2012**, *428*, 348–353. [CrossRef] [PubMed]

16. Eguchi, K.; Taoufiq, Z.; Thorn-Seshold, O.; Trauner, D.; Hasegawa, M.; Takahashi, T. Wild-type monomeric α-synuclein can impair vesicle endocytosis and synaptic fidelity via tubulin polymerization at the calyx of held. *J. Neurosci.* **2017**, *37*, 6043–6052. [CrossRef] [PubMed]
17. Bridi, J.C.; Hirth, F. Mechanisms of α-Synuclein induced synaptopathy in parkinson's disease. *Front. Neurosci.* **2018**, *12*, 1–18. [CrossRef] [PubMed]
18. Burré, J.; Sharma, M.; Südhof, T.C. Cell biology and pathophysiology of α-synuclein. *Cold Spring Harb. Perspect. Med.* **2018**, *8*, a024091. [CrossRef]
19. Larsen, K.E.; Schmitz, Y.; Troyer, M.D.; Mosharov, E.; Dietrich, P.; Quazi, A.Z.; Savalle, M.; Nemani, V.; Chaudhry, F.A.; Edwards, R.H.; et al. α-Synuclein overexpression in PC12 and chromaffin cells impairs catecholamine release by interfering with a late step in exocytosis. *J. Neurosci.* **2006**, *26*, 11915–11922. [CrossRef]
20. Burré, J.; Sharma, M.; Tsetsenis, T.; Buchman, V.; Etherton, M.R.; Südhof, T.C. α-Synuclein promotes SNARE-complex assembly in vivo and in vitro. *Science* **2010**, *329*, 1663–1667. [CrossRef]
21. Diao, J.; Burré, J.; Vivona, S.; Cipriano, D.J.; Sharma, M.; Kyoung, M.; Südhof, T.C.; Brunger, A.T. Native α-synuclein induces clustering of synaptic-vesicle mimics via binding to phospholipids and synaptobrevin-2/VAMP2. *Elife* **2013**, *2013*, 1–17. [CrossRef]
22. Wang, L.; Das, U.; Scott, D.A.; Tang, Y.; McLean, P.J.; Roy, S. α-Synuclein multimers cluster synaptic vesicles and attenuate recycling. *Curr. Biol.* **2014**, *24*, 2319–2326. [CrossRef] [PubMed]
23. Lai, Y.; Kim, S.; Varkey, J.; Lou, X.; Song, J.K.; Diao, J.; Langen, R.; Shin, Y.K. Nonaggregated α-synuclein influences snare-dependent vesicle docking via membrane binding. *Biochemistry* **2014**, *53*, 3889–3896. [CrossRef] [PubMed]
24. Butler, B.; Saha, K.; Rana, T.; Becker, J.P.; Sambo, D.; Davari, P.; Goodwin, J.S.; Khoshbouei, H. Dopamine transporter activity is modulated by α-synuclein. *J. Biol. Chem.* **2015**, *290*, 29542–29554. [CrossRef] [PubMed]
25. Guo, J.T.; Chen, A.Q.; Kong, Q.; Zhu, H.; Ma, C.M.; Qin, C. Inhibition of vesicular monoamine transporter-2 activity in α-synuclein stably transfected SH-SY5Y cells. *Cell. Mol. Neurobiol.* **2008**, *28*, 35–47. [CrossRef] [PubMed]
26. Zaltieri, M.; Grigoletto, J.; Longhena, F.; Navarria, L.; Favero, G.; Castrezzati, S.; Colivicchi, M.A.; Della Corte, L.; Rezzani, R.; Pizzi, M.; et al. α-synuclein and synapsin III cooperatively regulate synaptic function in dopamine neurons. *J. Cell Sci.* **2015**, *128*, 2231–2243. [CrossRef]
27. Somayaji, M.; Cataldi, S.; Choi, S.J.; Edwards, R.H.; Mosharov, E.V.; Sulzer, D. A dual role for α-synuclein in facilitation and depression of dopamine release from substantia nigra neurons in vivo. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 32701–32710. [CrossRef] [PubMed]
28. Sousa, V.L.; Bellani, S.; Giannandrea, M.; Yousuf, M.; Valtorta, F.; Meldolesi, J.; Chieregatti, E. α-Synuclein and its A30P mutant affect actin cytoskeletal structure and dynamics. *Mol. Biol. Cell* **2009**, *20*, 3725–3739. [CrossRef]
29. Bellani, S.; Sousa, V.L.; Ronzitti, G.; Valtorta, F.; Meldolesi, J.; Chieregatti, E. The regulation of synaptic function by α-synuclein. *Commun. Integr. Biol.* **2010**, *3*, 106–109. [CrossRef]
30. Carnwath, T.; Mohammed, R.; Tsiang, D. The direct and indirect effects of α-synuclein on microtubule stability in the pathogenesis of parkinson's disease. *Neuropsychiatr. Dis. Treat.* **2018**, *14*, 1685–1695. [CrossRef]
31. Calogero, A.M.; Mazzetti, S.; Pezzoli, G.; Cappelletti, G. Neuronal microtubules and proteins linked to Parkinson's disease: A relevant interaction? *Biol. Chem.* **2019**, *400*, 1099–1112. [CrossRef]
32. Payton, J.E.; Perrin, R.J.; Clayton, D.F.; George, J.M. Protein-protein interactions of alpha-synuclein in brain homogenates and transfected cells. *Mol. Brain Res.* **2001**, *95*, 138–145. [CrossRef]
33. Alim, M.A.; Hossain, M.S.; Arima, K.; Takeda, K.; Izumiyama, Y.; Nakamura, M.; Kaji, H.; Shinoda, T.; Hisanaga, S.; Uéda, K. Tubulin seeds α-synuclein fibril formation. *J. Biol. Chem.* **2002**, *277*, 2112–2117. [CrossRef]
34. Alim, M.A.; Ma, Q.L.; Takeda, K.; Aizawa, T.; Matsubara, M.; Nakamura, M.; Asada, A.; Saito, T.; Kaji, H.; Yoshii, M.; et al. Demonstration of a role for α-synuclein as a functional microtubule-associated protein. *J. Alzheimer's Dis.* **2004**, *6*, 435–442. [CrossRef]
35. Chen, L.; Jin, J.; Davis, J.; Zhou, Y.; Wang, Y.; Liu, J.; Lockhart, P.J.; Zhang, J. Oligomeric α-synuclein inhibits tubulin polymerization. *Biochem. Biophys. Res. Commun.* **2007**, *356*, 548–553. [CrossRef] [PubMed]
36. Zhou, R.M.; Huang, Y.X.; Li, X.L.; Chen, C.; Shi, Q.; Wang, G.R.; Tian, C.; Wang, Z.Y.; Jing, Y.Y.; Gao, C.; et al. Molecular interaction of α-synuclein with tubulin influences on the polymerization of microtubule in vitro and structure of microtubule in cells. *Mol. Biol. Rep.* **2010**, *37*, 3183–3192. [CrossRef]
37. Toba, S.; Jin, M.; Yamada, M.; Kumamoto, K.; Matsumoto, S.; Yasunaga, T.; Fukunaga, Y.; Miyazawa, A.; Fujita, S.; Itoh, K.; et al. Alpha-synuclein facilitates to form short unconventional microtubules that have a unique function in the axonal transport. *Sci. Rep.* **2017**, *7*, 1–19. [CrossRef] [PubMed]
38. Cartelli, D.; Aliverti, A.; Barbiroli, A.; Santambrogio, C.; Ragg, E.M.; Casagrande, F.V.M.; Cantele, F.; Beltramone, S.; Marangon, J.; De Gregorio, C.; et al. α-Synuclein is a Novel Microtubule Dynamase. *Sci. Rep.* **2016**, *6*, 1–13. [CrossRef]
39. Kelly, P.T.; Cotman, C.W. Synaptic Proteins. Characterization of Tubulin and Actin and Identification of a Distinct Postsynaptic Density Polypeptide The major proteins in isolated synaptic junctions (SJs) and postsynaptic densities (PSDs) have been compared to actin, tubulin. *J. Cell Biol.* **1978**, *79*, 173–183. [CrossRef] [PubMed]
40. Zisapel, N.; Levi, M.; Gozes, I. Tubulin: An Integral Protein of Mammalian Synaptic Vesicle Membranes. *J. Neurochem.* **1980**, *34*, 26–32. [CrossRef]

41. Gonzalez-lozano, M.A.; Klemmer, P.; Gebuis, T.; Hassan, C.; Van Nierop, P. Dynamics of the mouse brain cortical synaptic proteome during postnatal brain development. *Nat. Publ. Gr.* **2016**, *6*, 1–15. [CrossRef]
42. Gordon-Weeks, P.R.; Burgoyne, R.D.; Gray, E.G. Presynaptic microtubules: Organisation and assembly/disassembly. *Neuroscience* **1982**, *7*, 739–749. [CrossRef]
43. Westrum, L.E.; Gray, E.G.; Burgoyne, R.D.; Barron, J. Synaptic development and microtubule organization. *Cell Tissue Res.* **1983**, *231*, 93–102. [CrossRef]
44. Goellner, B.; Aberle, H. The synaptic cytoskeleton in development and disease. *Dev. Neurobiol.* **2012**, *72*, 111–125. [CrossRef] [PubMed]
45. Bodaleo, F.J.; Gonzalez-billault, C. The Presynaptic Microtubule Cytoskeleton in Physiological and Pathological Conditions: Lessons from Drosophila Fragile X Syndrome and Hereditary Spastic Paraplegias. *Front. Mol. Neurosci.* **2016**, *9*, 1–16. [CrossRef] [PubMed]
46. Borgen, M.A.; Giles, A.C.; Wang, D.; Grill, B. Synapse maintenance is impacted by ATAT-2 tubulin acetyltransferase activity and the RPM-1 signaling hub. *Elife* **2019**, *8*, 1–18. [CrossRef] [PubMed]
47. Qu, X.; Kumar, A.; Blockus, H.; Waites, C.; Bartolini, F. Activity-dependent nucleation of dynamic microtubules at presynaptic boutons is required for neurotransmission. *bioRxiv* **2019**, 4231–4240. [CrossRef]
48. Piriya, L.; Babu, A.; Wang, X.; Eguchi, X.; Guillaud, X.; Takahashi, X. Microtubule and Actin Differentially Regulate Synaptic Vesicle Cycling to Maintain High-Frequency Neurotransmission. *J. Neurosci.* **2020**, *40*, 131–142. [CrossRef] [PubMed]
49. Waites, C.; Qu, X.; Bartolini, F. The synaptic life of microtubules. *Curr. Opin. Neurobiol.* **2021**, *69*, 113–123. [CrossRef]
50. Braak, H.; Ghebremedhin, E.; Rüb, U.; Bratzke, H.; Del Tredici, K. Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Res.* **2004**, *318*, 121–134. [CrossRef]
51. Durante, V.; De Iure, A.; Loffredo, V.; Vaikath, N.; De Risi, M.; Paciotti, S.; Quiroga-Varela, A.; Chiasserini, D.; Mellone, M.; Mazzocchetti, P.; et al. Alpha-synuclein targets GluN2A NMDA receptor subunit causing striatal synaptic dysfunction and visuospatial memory alteration. *Brain* **2019**, *142*, 1365–1385. [CrossRef] [PubMed]
52. Klaus, A.; Da Silva, J.A.; Costa, R.M. What, If, and When to Move: Basal Ganglia Circuits and Self-Paced Action Initiation. *Annu. Rev. Neurosci.* **2019**, *42*, 459–483. [CrossRef]
53. Specht, C.G.; Schoepfer, R. Deletion of the alpha-synuclein locus in a subpopulation of C57BL/6J inbred mice. *BMC Neurosci.* **2001**, *2*, 11. [CrossRef]
54. Goedert, M.; Jakes, R.; Spillantini, M.G. The Synucleinopathies: Twenty Years on. *J. Park. Dis.* **2017**, *7*, S53–S71. [CrossRef]
55. Cartelli, D.; Cappelletti, G. Microtubule Destabilization Paves the Way to Parkinson's Disease. *Mol. Neurobiol.* **2017**, *54*, 6762–6774. [CrossRef]
56. Pellegrini, L.; Wetzel, A.; Grannó, S.; Heaton, G.; Harvey, K. Back to the tubule: Microtubule dynamics in Parkinson's disease. *Cell. Mol. Life Sci.* **2017**, *74*, 409–434. [CrossRef]
57. Dent, E.W. Dynamic microtubules at the synapse. *Curr. Opin. Neurobiol.* **2020**, *63*, 9–14. [CrossRef]
58. Guedes-Dias, P.; Nirschl, J.J.; Abreu, N.; Tokito, M.K.; Janke, C.; Magiera, M.M.; Holzbaur, E.L.F. Kinesin-3 Responds to Local Microtubule Dynamics to Target Synaptic Cargo Delivery to the Presynapse. *Curr. Biol.* **2019**, *29*, 268–282.e8. [CrossRef]
59. Hattori, T.; Takada, M.; Moriizumi, T.; Van Der Kooy, D. Single dopaminergic nigrostriatal neurons form two chemically distinct synaptic types: Possible transmitter segregation within neurons. *J. Comp. Neurol.* **1991**, *309*, 391–401. [CrossRef]
60. Sesack, S.R.; Hawrylak, V.A.; Matus, C.; Guido, M.A.; Levey, A.I. Dopamine axon varicosities in the prelimbic division of the rat prefrontal cortex exhibit sparse immunoreactivity for the dopamine transporter. *J. Neurosci.* **1998**, *18*, 2697–2708. [CrossRef]
61. Charnay, Y. Brain serotonergic circuitries. *Dialogues Clin. Neurosci.* **2010**, *12*, 471–487. [CrossRef]
62. Zhang, Z.W.; Burke, M.W.; Calakos, N.; Beaulieu, J.M.; Vaucher, E. Confocal analysis of cholinergic and dopaminergic inputs onto pyramidal cells in the prefrontal cortex of rodents. *Front. Neuroanat.* **2010**, *4*, 21. [CrossRef] [PubMed]
63. Taguchi, K.; Watanabe, Y.; Tsujimura, A.; Tatebe, H.; Miyata, S.; Tokuda, T.; Mizuno, T.; Tanaka, M. Differential expression of alpha-synuclein in hippocampal neurons. *PLoS ONE* **2014**, *9*, e89327. [CrossRef]
64. Longhena, F.; Faustini, G.; Spillantini, M.G.; Bellucci, A. Living in promiscuity: The multiple partners of alpha-synuclein at the synapse in physiology and pathology. *Int. J. Mol. Sci.* **2019**, *20*, 141. [CrossRef]
65. Lee, H.-G.; Zhu, X.; Takeda, A.; Perry, G.; Smith, M.A. Emerging evidence for the neuroprotective role of α-synuclein. *Exp. Neurol.* **2006**, *200*, 1–7. [CrossRef]
66. Outeiro, T.F.; McLean, P.J.; Hyman, B.T. Protein aggregation disorders. In *Neurobiology of Disease*; Gilman, S., Ed.; Elsevier-Academic Press: Amsterdam, The Netherlands, 2007.
67. Morato Torres, C.A.; Wassouf, Z.; Zafar, F.; Sastre, D.; Outeiro, T.F.; Schüle, B. The role of alpha-synuclein and other parkinson's genes in neurodevelopmental and neurodegenerative disorders. *Int. J. Mol. Sci.* **2020**, *21*, 5724. [CrossRef]
68. Cappelletti, G.; Cartelli, D. Acetylation of tubulin: A feasible protective target from neurodevelopment to neurodegeneration. In *Neuroprotection Autism, Schizophrenia and Alzheimer's Disease*; Gozes, I., Levine, J., Eds.; Academic Press: Cambridge, MA, USA, 2019; pp. 273–294. [CrossRef]
69. Mazzetti, S.; Basellini, M.J.; Ferri, V.; Cassani, E.; Cereda, E.; Paolini, M.; Calogero, A.M.; Bolliri, C.; De Leonardis, M.; Sacilotto, G.; et al. α-Synuclein oligomers in skin biopsy of idiopathic and monozygotic twin patients with Parkinson's disease. *Brain* **2020**, *143*, 920–931. [CrossRef]

70. Amadeo, A.; Coatti, A.; Aracri, P.; Ascagni, M.; Iannantuoni, D.; Modena, D.; Carraresi, L.; Brusco, S.; Meneghini, S.; Arcangeli, A.; et al. Postnatal Changes in K^+ /Cl^- Cotransporter-2 Expression in the Forebrain of Mice Bearing a Mutant Nicotinic Subunit Linked to Sleep-Related Epilepsy. *Neuroscience* **2018**, *386*, 91–107. [CrossRef]
71. Paxinos, G.; Franklin, K. *The Mouse Brain in Stereotaxic Coordinates, Compact*; Elsevier: Amsterdam, The Netherlands, 2008; ISBN 978-0-12-374244-5.
72. Longhena, F.; Faustini, G.; Missale, C.; Pizzi, M.; Bellucci, A. Dopamine transporter/α-synuclein complexes are altered in the post mortem caudate putamen of Parkinson's disease: An in situ proximity ligation assay study. *Int. J. Mol. Sci.* **2018**, *19*, 1611. [CrossRef]
73. Söderberg, O.; Gullberg, M.; Jarvius, M.; Ridderstråle, K.; Leuchowius, K.-J.; Jarvius, J.; Wester, K.; Hydbring, P.; Bahram, F.; Larsson, L.-G.; et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat. Methods* **2006**, *3*, 995–1000. [CrossRef]
74. Aracri, P.; Meneghini, S.; Coatti, A.; Amadeo, A.; Becchetti, A. α4β2* nicotinic receptors stimulate GABA release onto fast-spiking cells in layer V of mouse prefrontal (Fr2) cortex. *Neuroscience* **2017**, *340*, 48–61. [CrossRef] [PubMed]
75. Bolte, S.; Cordelières, F.P. A guided tour into subcellular colocalization analysis in light microscopy. *J. Microsc.* **2006**, *224*, 213–232. [CrossRef] [PubMed]
76. Paiardi, C.; Pasini, M.E.; Amadeo, A.; Gioria, M.; Berruti, G. The ESCRT-deubiquitinating enzyme USP8 in the cervical spinal cord of wild-type and Vps54-recessive (wobbler) mutant mice. *Histochem. Cell Biol.* **2014**, *141*, 57–73. [CrossRef] [PubMed]

Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2023**; 28(2): 33
https://doi.org/10.31083/j.fbl2802033

Review

Pathological Pathways and Alpha-Synuclein in Parkinson's Disease: A View from the Periphery

Milo Jarno Basellini[1,2,†], Josine Marie Kothuis[1,†], Alessandro Comincini[1,†], Gianni Pezzoli[3], Graziella Cappelletti[1,4,*,§], Samanta Mazzetti[1,3,*,§]

[1]Department of Biosciences, Università degli Studi di Milano, 20133 Milan, Italy
[2]Department of Biochemistry and Molecular Biology, Faculty of Biology, Universitat de Barcelona, 08028 Barcelona, Spain
[3]Fondazione Grigioni per il Morbo di Parkinson, 20125 Milan, Italy
[4]Center of Excellence of Neurodegenerative Diseases, Università degli Studi di Milano, 20133 Milan, Italy
*Correspondence: graziella.cappelletti@unimi.it (Graziella Cappelletti); samanta.mazzetti@gmail.com (Samanta Mazzetti)
[†]These authors contributed equally.
[§]These authors contributed equally.
Academic Editors: Xudong Huang, Fabio Moda and Giorgio Giaccone
Submitted: 20 December 2022 Revised: 3 February 2023 Accepted: 15 February 2023 Published: 23 February 2023

Abstract

Alpha-synuclein inclusions are the distinctive trait of brain areas affected by neurodegeneration in Parkinson's disease (PD). Nevertheless, PD is now considered as a multisystemic disorder, since alpha-synuclein pathology has been described also outside the central nervous system. In this regard, the early, non-motor autonomic symptoms point out an important role for the peripheral nervous system during disease progression. On this basis, we propose a review of the alpha-synuclein-related pathological processes observed at peripheral level in PD, starting from molecular mechanisms, through cellular processes to systemic modifications. We discuss their relevance in the etiopathogenesis of the disease, suggesting they are concurrent players in the development of PD, and that the periphery is an easily-accessible window to look at what is occurring in the central nervous system.

Keywords: alpha-synuclein; microtubules; synapses; mitochondria; autophagy; inflammation; neurodegeneration

1. Introduction

Parkinson's disease (PD) is a common progressive neuropathological condition clinically characterized by resting tremors, bradykinesia, postural instability and general rigidity. These cardinal manifestations are due to a depletion in the dopaminergic neuronal subpopulation of the Sömmering's *substantia nigra*, a basal ganglia structure located in the midbrain that projects to the striatum, establishing the so-called *nigro*-striatal pathway, a trait crucially involved in the circuits that ensure control and modulation of movement [1]. At brain level, PD is characterized by the presence of proteinaceous inclusion bodies within neuronal soma and neuropil, named Lewy bodies and Lewy neurites, respectively, which constitute the hallmark of the pathology and whose main component was found to be alpha-synuclein [2]. The discovery of the A53T mutation in the *SNCA* gene encoding for alpha-synuclein as the cause of a familial form of the disease also supported the crucial involvement of this protein in PD pathogenesis [3]. Alpha-synuclein is a natively unfolded protein that owes its name to its pre-synaptic and nuclear localization [4]. Human alpha-synuclein belongs to the synuclein family, which also includes beta- and gamma-synuclein. It is constitutively expressed in the nervous system, accounting for 1% of total neuronal cytosolic proteins, and in the brain, where it is predominantly found in the neocortex, hippocampus, *substantia nigra*, and *corpus striatum* [5,6]. Although the physiological role of alpha-synuclein has not been completely discovered, the known list of its interacting partners is abundant and includes lipid membranes, synaptic vesicles, tubulin, SNARE complex protein and proteins involved in calcium regulation and dopamine homeostasis [7] thus suggesting it could be involved in the modulation of neurotransmission, working on vesicle release and trafficking.

Together with PD, further pathologies were found to feature alpha-synuclein-positive inclusions, namely multiple system atrophy and Lewy body dementia, and thus they were classified as synucleinopathies [8]. Nevertheless, it is still a matter of debate whether alpha-synuclein aggregates are toxic, protective, or are an epiphenomenon related to progressive failure of cellular clearance mechanisms for aggregated proteins [9,10].

The proposed molecular mechanisms involved in PD pathogenesis include alteration of the microtubular cytoskeleton, synaptic dysfunctions, mitochondrial impairment, defective autophagy, impaired protein turnover and clearance, oxidative stress, and inflammation [11–13]. Interestingly, alpha-synuclein pathology was reported in multiple body regions and organs of patients affected by synucleinopathy, both in autoptic and living individuals [14,15], suggesting that it could also affect peripheral tissues. In addition, the early-onset non-motor symptoms that feature

the pathology include a wide range of autonomic manifestations such as hyposmia, hypotension, sleep disorder and gastrointestinal symptoms [16]. This paved the way to redefining PD as a multisystemic disorder and, in such a scenario, the study of the pathology in the periphery provides an easily-accessible window to the central nervous system, making it possible to evaluate pathological processes that may be mirrored in the brain, thus allowing to investigate the mechanisms underlying the disease's pathogenesis. Consequently, in this review we describe the pathological processes reported at the peripheral level in PD, microtubules, synapses, mitochondria, autophagy, protein clearance and inflammation, and we discuss their link to alpha-synuclein pathology and relevance to the etiopathogenesis of the disease.

2. Microtubule Dysfunction

Microtubules (MTs) are highly dynamic polymers of $\alpha\beta$ tubulin heterodimers. The specialization of MTs is regulated by the "tubulin code" which embodies the expression of different α- and β-tubulin isotypes and post-translational modification of tubulin (PTMs). In addition, different microtubule-associated proteins (MAPs) represent a further regulation mechanism. MTs are crucial not only during cell division, when they form the mitotic spindle, but also in post-mitotic cells like neurons, in which they ensure the correct specialization and polarization [17]. Both anterograde and retrograde axonal transport in neurons is ensured by the interaction between MTs and motor proteins, and also synaptic plasticity has been proven to depend on MT dynamics (Fig. 1A).

Given their importance in neuron physiology, numerous studies have demonstrated how microtubule impairment can lead to neurodegenerative disorders and *in vitro* and *in vivo* studies have indicated that microtubule dysfunction is linked to the pathogenesis of PD (Fig. 1B) [7,18]. First of all, Lewy bodies, the pathological hallmark of PD, have been found to contain a large number of cytoskeletal proteins, including tubulin, MAPs, and neurofilaments, suggesting cytoskeletal alterations in PD [19,20]. More recent studies have offered novel insights into the molecular composition of Lewy bodies. Using STED (Stimulated emission depletion microscopy)-based super resolution microscopy and correlative high-resolution imaging and biophysical approaches, the presence of crowded membranous materials including membrane fragments, dysmorphic mitochondria and structures resembling lysosomes, together with cytoskeletal elements were revealed in Lewy bodies [21]. Interestingly, the analysis of the subcellular rearrangement of the aggregated forms of alpha-synuclein unraveled the presence of cytoskeletal elements such as tubulin and neurofilaments at the periphery of Lewy bodies and suggested their role in the morphogenesis of Lewy bodies [22]. Further evidence as to the role of microtubules in PD pathogenesis come from genome-wide association studies in familial events of the pathology, that identified mutations in microtubule-related protein genes including *MAPT* [23], which is also independent of *SNCA* mutations. Looking at toxin-based models of PD, the parkinsonism-induced neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its toxic metabolite 1-methyl-4-phenylpyridinium (MPP^+) have been associated with changes in the tubulin network and the degeneration of dopaminergic neurons *in vitro* and *in vivo* [24–27]. Notably, it has been shown that exposure to MPP^+ in differentiated PC12 cells induces microtubule changes very early, long before mitochondrial impairment appears [28]. Taken together, these findings in a neurotoxin-based model suggest that microtubule alterations may play a crucial role during the early phases of PD pathogenesis. In this regard, several PD-linked mutations involved in familial forms of the pathology, including Parkin, LRRK2, and alpha-synuclein, affect some proteins that are known to interact, to different extents, with the microtubule system [29–32].

The interplay between microtubules and alpha-synuclein have gained increasing interest over time. In 2001, Payton and colleagues [33] first reported the co-immunoprecipitation of alpha-synuclein with both alpha- and beta-tubulin from zebra finch and mouse brain homogenates, which was later confirmed also in rat and hamster brain [34–36]. Besides, a study in HeLa cells reported that alpha-synuclein co-localizes with MTs [36]. More recently, Cartelli and colleagues demonstrated that alpha-synuclein forms a complex with an $\alpha_2\beta_2$-tubulin tetramer *in vitro* and folds upon this interaction [29]. Despite the direct evidence for the interaction of alpha-synuclein with tubulin and MTs, the exact binding region is still under debate and further research is needed [32]. Controversial results have also been published on the effect of alpha-synuclein on tubulin dynamics: one research group showed that monomeric alpha-synuclein does not interfere with the tubulin network *in vitro* [36], whereas other studies demonstrated that alpha-synuclein inhibits [37] or promotes [34,35] tubulin polymerization. Interestingly, Cartelli *et al.* [29] proposed that alpha-synuclein is able to function as a so-called "dynamase", by inducing both MT nucleation and catastrophe, and that it modulates the partitioning between tubulin dimers and MTs at the neuronal growth cone [38].

Few data are actually reported on microtubule dysfunction in the periphery with respect to PD. Toba and coworkers [39] demonstrated that bi-directional axonal transport is severely affected in alpha-synuclein depleted rat dorsal root ganglion neurons, and that, alpha-synuclein interaction with βIII-tubulin occurs in rat femoral nerve, where, together with dynein, allow the transport of short microtubules. As regards studies on humans, skin fibroblasts exhibit reduced microtubule mass and impaired microtubule stability in patients affected with both idiopathic and genetic PD and, interestingly, pharmacological and genetic

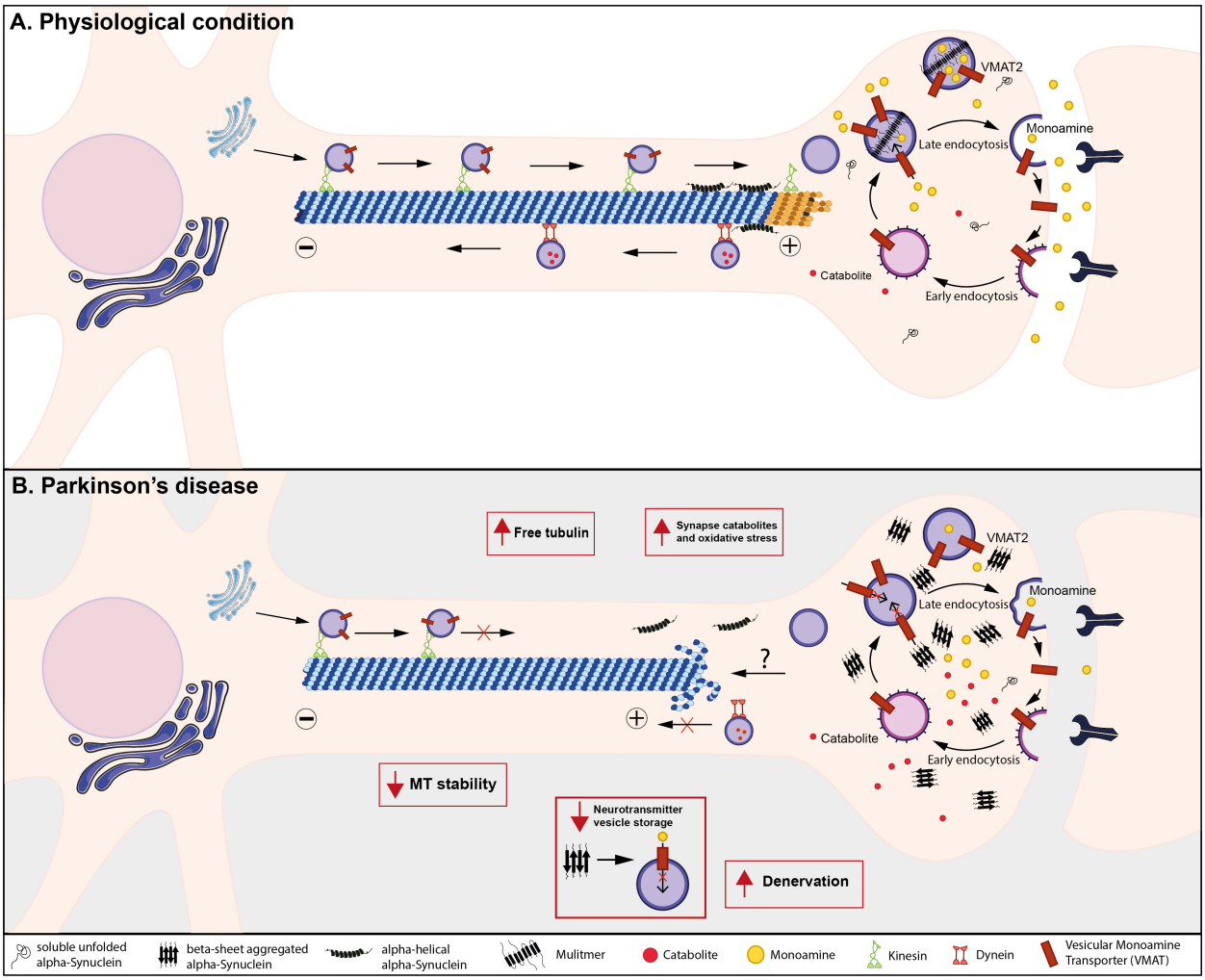

Fig. 1. Suggested model for alpha-synuclein impact on microtubule homeostasis and synaptic functioning in PD. (A) Under physiological conditions, MTs are conventional and well-organized; vesicles use MTs as tracks to reach the synaptic boutons from the cell body, moving toward the distal tip of the axon in an anterograde movement. Alpha-helical alpha-synuclein interacts with MTs at the neuronal growth cone. In the synaptic compartment, vesicular monoamine transporter 2 (VMAT2) loads presynaptic vesicles with monoamines, maintaining the releasable pool as well as the recycling pool. The retrograde movement sustains catabolite clearance, avoiding harmful interactions with the synaptic components. (B) In Parkinson's disease, alpha-helical alpha-synuclein may lose its interaction with MTs and alpha-synuclein aggregation leads to MT destabilization, thus impairing transport towards and away from the synapses. This could trigger the accumulation of reactive catabolites and/or aberrant proteins, likely increasing oxidative stress damage at the synaptic level. Additionally, the alteration of VMAT2 functioning by a possible direct interaction with alpha-synuclein aggregates could reduce the extent of the neurotransmitter vesicular storage and releasable pool which lead to synaptic transmission defects and, eventually, to neuronal death.

approaches resulted in the rescue of microtubule defects [40]. This study showed, for the first time, microtubule dysfunction in PD patients. More recently, elevated levels of autoantibodies against tubulin have been measured in sera of patients with PD, indicating that they may function as a peripheral biomarker for PD diagnosis [41]. Furthermore, Esteves and colleagues [42] investigated microtubule changes in a PD cytoplasmic hybrid (cybrid) cell line in which platelet mitochondria from a PD subject were transferred to NT2 neuronal cells previously depleted of endogenous mitochondrial DNA. These NT2 cells are known to show reduced complex I activity and decreased levels of ATP in comparison to control cybrid cell lines [43]. Interestingly, they demonstrated an increased free/polymerized tubulin ratio and oligomeric alpha-synuclein levels in the PD cybrid cell line compared to the control cell line. Moreover, the addition of the microtubule stabilizer 'taxol' to the PD cybrid cell line normalizes the free/polymerized ratio and reduces the levels of oligomeric alpha-synuclein. A follow up study demonstrated improved microtubule-dependent trafficking after NAP (Davunetide) administration in PD cybrid cell lines compared to control cell lines

[44]. Together, this research group showed microtubule alterations and increased oligomeric alpha-synuclein in cybrid cells obtained from PD patients, suggesting that mitochondrial dysfunction may destabilize the microtubule network and increases the levels of oligomeric alpha-synuclein in PD. Overall, these data suggest that microtubule dysfunctions play an important role in PD onset and progression. In addition, while wild-type alpha-synuclein may have a favorable impact on microtubule functioning (Fig. 1A), misfolded and oligomeric variants seem to be responsible for microtubule destabilization (Fig. 1B).

3. Synaptic Dysfunction

Synaptic homeostasis is crucial to maintain correct neuronal functioning as well as to guarantee the proper transmission of information within the nervous system. Even though the exact physiological role of alpha-synuclein in neuronal cells has not yet been elucidated, its interaction with different proteins in the synaptic compartment and its prevalence in the pre-synapse point to a possible biological role as a wide regulator of synaptic activity in the central nervous system (CNS) [45,46]. Among the physiological interactions proposed for alpha-synuclein, its activity as a mediator of the neuronal trafficking in the synapse stands out [47]. A series of discoveries have indeed outlined a role of alpha-synuclein as a Soluble N-ethylmaleimide Sensitive Factor Attachment Protein (SNAP) receptor (SNARE)-chaperone that helps SNARE-mediated vesicle-membrane fusion, thus promoting exocytosis in a physiological context. Further evidence found in the CNS indicates that other alpha-synuclein putative interactions with synaptic proteins are certainly worth noting, including the reuptake neurotransmitter proteins such as dopamine transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2) [48,49]. It has also been demonstrated that alpha-synuclein interacts with the family of the synapsins, a group of proteins largely implicated in synaptic plasticity, exocytosis and in the regulation of the vesicle releasable pool [50] (Fig. 1A). Experimental results were obtained by analyzing these pathways in a pathological background of alpha-synuclein mutation/overexpression or in the presence of misfolded aggregates, namely oligomers and fibrils, to elucidate how this could induce synaptic dysfunction throughout the CNS.

In a PD-related experimental setup, Garcia-Reitböck and colleagues [51] carried out a double staining immunohistochemistry between alpha-synuclein and the t-SNARE proteins SNAP-25 or syntaxin-1, and demonstrated their co-localization in the cerebral cortex of a PD-mouse model expressing full-length human mutant A30P alpha-synuclein. They found an accumulation and redistribution of SNARE proteins in the same transgenic mice compared to controls. In addition, to correlate this finding with the disease in humans, SNARE proteins repositioning was unraveled in the *striatum* of patients affected by early onset PD [47], hinting that alpha-synuclein mutations may have a harmful effect on synaptic vesicle exocytosis. Evidence in primary mouse cortical neurons and adult mouse hippocampal neurons revealed a dose-dependent reduction of pre-synaptic markers levels, and synaptic vesicle recycling upon recombinant alpha-synuclein administration [52]. These results are in line with the loss of synaptic terminals reported in the brain [53]. As mentioned, multiple interactions with synaptic transporters of monoamine have also been proposed as physiological functions of alpha-synuclein. Alterations in these putative modulation roles were suggested to be concurrent enhancers in the onset and progression of synaptic dysfunction. Indeed, the reduced total amount and the simultaneous clustered pattern of DAT, which co-localized in the caudate-putamen with alpha-synuclein and phospho-alpha-synuclein, was demonstrated by immunofluorescence labeling [54]. DAT/alpha-synuclein complexes were also detected by means of "*in situ*" proximity ligation assay (PLA), which showed a marked DAT redistribution in the caudate-putamen of PD patients [53]. Likewise, interactions with dopamine membrane transporters were reported after adeno-associated virus type 6 (AAV6) injection in the caudate-putamen of adult rats overexpressing human alpha-synuclein. Immunohistochemical analysis revealed axonal and synaptic swellings after 8–16 weeks, as well as inclusions positive for both alpha-synuclein and VMAT2 [55], suggesting an impairment in dopamine vesicle uptake and a cytosolic accumulation of this neurotransmitter, which may produce DA-derived, cytotoxic reactive species (Fig. 1B). Accordingly, in a similar model alpha-synuclein preformed fibrils (PFFs) were injected in the *substantia nigra*, and VMAT2 expression resulted significantly lower in those neurons where the phospho-alpha-synuclein pathology was more severe compared to controls who had not received PFFs injection [56]. Since alpha-synuclein is regarded to interact directly with lipid membranes [57], the effect of alpha-synuclein aggregation on membrane damage was also investigated. A covalent modification of alpha-synuclein by a dopamine catabolite (DOPAL) which provokes its oligomerization was noticed *in vitro* as well as in cellular models. Such aggregates were shown to induce dopamine leak through synaptic vesicle disruption or permeabilization and their effect was correlated with increased cell death [58]. All these effects, reported to happen when alpha-synuclein switches to create pathological small inclusions, further foster the idea of a putative detrimental role of oligomers and/or amyloid fibrils in membrane homeostasis that may ultimately contribute to neurodegeneration by causing increased oxidative stress, altered neurotransmitter storage and vesicle integrity (Fig. 1B).

Alpha-synuclein pathology seems to progressively move from the periphery to the spinal cord and brainstem in a centripetal movement [59,60] by exploiting a prion-like diffusion through the neuronal network [61,62].

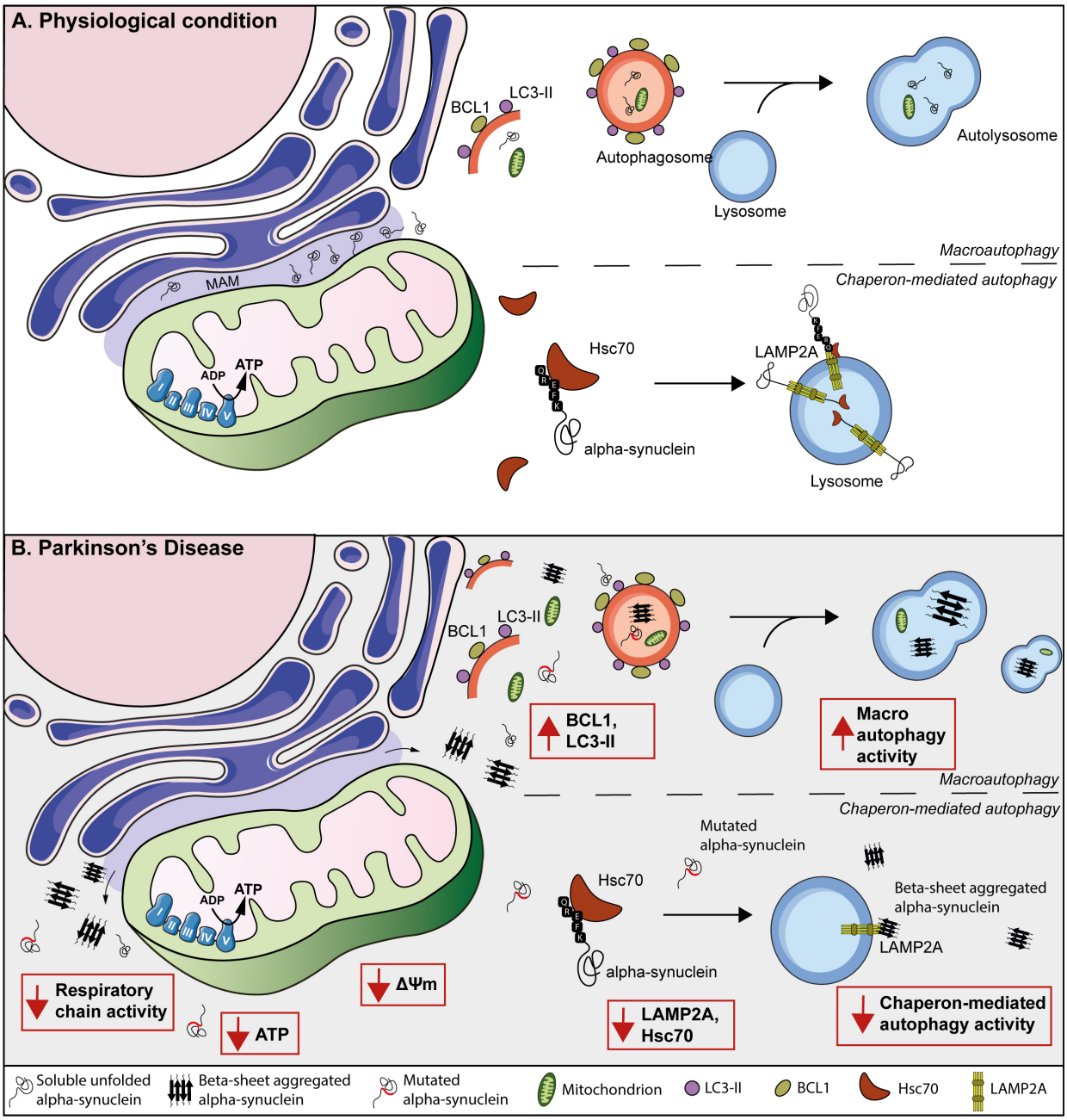

Fig. 2. Suggested model for mitochondria and autophagy contribution to PD evolution and progression in peripheral tissues. (A) Representation of the endoplasmic reticulum, the mitochondrial associated endoplasmic reticulum membrane (MAM), and a mitochondrion containing the mitochondrial respiratory chain. Physiologically, alpha-synuclein (black tangles) is localized at the MAM. At the same time, the macroautophagy machinery forms double-membrane organelles, called autophagosomes, mediated by LC3-II and BCL1. Then, autophagosomes incorporate cytosolic cargo, (including i.e., alpha-synuclein, mitochondria), fuse with lysosomes, and form autolysosomes to achieve cargo degradation. Besides, during chaperon-mediated autophagy, the KFERQ domain of the alpha-synuclein protein is recognized by Hsc70, which interacts with LAMP2A, and promotes its degradation via lysosomes. (B) In PD patients, mutated and/or oligomeric variants of alpha-synuclein affect its localization in MAM and move towards the cytosol. Moreover, these alpha-synuclein species causes mitochondrial dysfunction, including reduced mitochondrial complex I activity, decreased ATP production and, finally, a reduction in the mitochondrial membrane potential. PD peripheral tissue demonstrated an increase in autophagic structures (i.e., autophagosomes, lysosomes, and autolysosomes), suggesting that peripheral tissue of PD patients relies on an auto-activated compensatory mechanism of degradation. However, it might eventually be difficult to sustain this compensatory increase of autophagy, causing an elevation of alpha-synuclein aggregates, toxicity, and neuronal death. Moreover, reduced chaperone-mediated autophagy, including LAMP2A and Hsc70, has been observed in peripheral tissue derived from PD patients.

The hypothesis was supported by analyzing patients' peripheral tissues clearly reporting denervation [63–65] and alpha-synuclein or phospho-alpha-synuclein inclusions in autonomic neurons [15,66–69]. This peripheral degeneration may reflect the autonomic symptoms starting in the early stages of synucleinopathies. Although most synucleinopathies ultimately affect the CNS, little is known regarding the role that alpha-synuclein aggregation might play in the PNS. Recently, Challis and colleagues [70] showed gut dysfunction and motor symptoms in aged mice after PFFs duodenal seeding, showing a significant increase in brainstem phospho-alpha-synuclein and a reduced striatal dopamine in aged mice inoculated with PFFs, compared to PFF-inoculated younger WT mice or monomer-inoculated aged mice. Axonal phospho-alpha-synuclein aggregates and degeneration of the cardiac sympathetic nerve were already known to anticipate neuronal cell loss in the paravertebral sympathetic ganglia of patients affected by incidental Lewy body dementia [71], thus supporting the idea of a centripetal distal-driven degeneration in the peripheral nervous system in PD. This fits in the dying-back mechanism of cell death suggested for synucleinopathies, in which cell body damage occurs following synaptic dysfunction in a retrograde axonal degeneration [72,73]. Interestingly, the impairment of the synaptic compartment in peripheral tissues from PD patients was linked to synuclein oligomers in skin biopsies by Mazzetti and colleagues [74]. The authors managed to evaluate the synaptic density, described as the ratio between the area of synaptic terminals and the total area of the targeted structure, recalling the procedure used for the assessment of denervation in PD samples [75]. By highlighting the synaptic compartment with synaptophysin, a reduced synaptic density was reported in 26% of PD patients, whereas only 3% of consecutive controls presented this feature, thus indicating an increased degeneration of peripheral synapses.

Additionally, *in vivo* non-invasive imaging techniques, such as ^{123}I-meta-iodobenzylguanidine (MIBG) scintigraphy, and ^{18}F-dopamine positron emission tomography (PET), allowed to visualize and quantify the extent of denervation and the loss of nerve terminals in patients affected by synucleinopathies [76]. The use of different radiotracers analogous to the endogenous norepinephrine and dopamine respectively, reveals the *in vivo* extent and distribution of these neurotransmitters by substituting them at the post-ganglionic synapses of the sympathetic nervous system [63,71]. Denervation and significant reduction of heart-to-mediastinum ratios were observed in PD patients with orthostatic hypotension compared to healthy controls [77,78]. Reduced levels of MIBG uptake were seen in PD patients with REM sleep behavior disorder [79] and in most patients with idiopathic REM sleep behavior disorder, which very frequently degenerates in PD or Lewy body dementia [80], showed a significant lower cardiac MIBG uptake, similar to the levels found patients [81].

Since synaptic dysfunction was reported to precede neuronal death in patients, the impairment caused by alpha-synuclein aggregation in the synaptic compartment may assume a pivotal role in determining the onset of PD in the peripheral nervous system (PNS) and ultimately in the CNS. Together with the emerging evidence of synaptic dysfunction in human tissues, peripheral synapses might be considered as putative initiators or supporters of alpha-synuclein aggregation in the early stages of the pathology.

4. Mitochondrial Dysfunction

Impaired mitochondrial functioning has been associated with several disorders such as cancer and diabetes type-II, but also ageing and neurodegenerative diseases, including PD [82]. Multiple groups have reported the presence of alpha-synuclein in mitochondria [83–85], and, more specifically, localized at the mitochondria-associated endoplasmic reticulum membranes (MAM), a distinct domain of the endoplasmic reticulum [86] (Fig. 2A).

The first evidence of a link between mitochondrial dysfunction and PD came from an observation in young drug addicts who developed parkinsonism after accidentally injecting themselves with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [87]. Its toxic metabolite MPP^+ is shown to inhibit complex-I of the mitochondrial respiratory chain. The reduction of complex-I activity leads to decreased energy production and increased oxidative stress, which eventually causes cell death. Several years later, mitochondrial complex-I deficiency was reported in the *substantia nigra pars compacta* (SNpc) of *post-mortem* brains from PD patients [88]. More recently, it has been shown that decreased complex-I activity is not limited to the SNpc but occurs throughout the brain of individuals with PD [89]. Increased oxidative stress, as a result of increased reactive oxygen species (ROS), is thought to play a critical role in the dopaminergic neuronal loss of patients with PD. *Post-mortem* studies reported elevated levels of oxidized lipids, proteins, and DNA oxidation products in SN neurons from PD patients in comparison with controls [90–93]. Further evidence for mitochondria-driven PD pathogenesis comes from genome-wide association studies in familial events of the pathology, that identified mutations in mitochondria-related genes including parkin, DJ-1, and PINK-1 [94,95]. Focusing on alpha-synuclein, Guardia-Laguarta and colleagues [86] demonstrated that alpha-synuclein point mutations A30P and A53T affect their localization with mitochondria-associated endoplasmic reticulum membranes (MAM), reduce MAM functioning, decrease ER-mitochondrial connectivity and increase mitochondrial fragmentation in HeLa cells and in human and mouse brain samples (Fig. 2B). Furthermore, the induced expression of the familial alpha-synuclein mutant A53T in human fetal dopaminergic primary neurons causes a significant reduction of mitochondrial complex I activity and increased production of ROS [96]. Interestingly,

accumulation of wild-type alpha-synuclein in mitochondria also resulted in a significantly, albeit at a slower rate than that observed for mutant A53T, reduced complex I activity and an increased ROS production. Alpha-synuclein did not induce any significant disruption of other complexes, suggesting its preferential interaction with mitochondrial complex I. Moreover, increased levels of cholesterol metabolites derived from ROS have been reported in cortices from patients with Lewy Body dementia, and importantly, they demonstrated that this increase in metabolites accelerates alpha-synuclein aggregation [90].

Mitochondrial dysfunction in PD is not only reported in the CNS but has also been shown and widely investigated in the periphery (Fig. 2B). A reduced activity of the respiratory chain's complexes has been unraveled in multiple cell types from PD patients, including platelets, fibroblasts, lymphocytes and myocytes, suggesting a widespread reduction of mitochondrial activity in PD [97–99]. Chen and colleagues [100] revealed increased oxidative stress and elevated levels of leukocyte 8-hydroxydeoxyguanosine (8-OHdG) and malondialdehyde in blood plasma derived from PD patients. Likewise, Baumuratov and colleagues [101] reported aberrant mitochondrial morphology in colon biopsies from PD patients, reporting that colon ganglia from patients contained significantly more mitochondria per ganglion and exhibited a higher total mitochondrial mass compared to controls. Interestingly, expression of recombinant wild-type alpha-synuclein has also been shown to cause impaired mitochondrial complex I activity in human foetal enteric neurons [102].

Recently, morphometric mitochondrial changes have been demonstrated also in skin fibroblasts from individuals with idiopathic PD [103] together with an impaired mitochondrial membrane potential. Finally, the effect of alpha-synuclein mutations on mitochondrial dysfunction has also been investigated in human fibroblasts derived from a patient with parkinsonism carrying a triplication in the alpha-synuclein gene, which revealed a reduced complex I activity, decreased ATP production and a reduction in the mitochondrial membrane potential compared to controls [104].

In conclusion, these studies provide substantial evidence that mitochondrial dysfunctions are features of PD both in the CNS and in a broad range of different peripheral samples, including skeletal muscle, lymphocytes, platelets, blood plasma, skin fibroblasts and colon ganglia. This makes peripheral samples, naturally easily-accessible and less-invasive, a reliable option for the investigation of mitochondria- related dysfunctions in PD, considering that further research needs to be conducted into the role of alpha-synuclein in the physiology and pathology of mitochondria.

5. Autophagy and Protein Clearance

The main cellular mechanisms responsible for the degradation of large, long-lived proteins and cytoplasmic organelles, and, particularly, those that are toxic or damaged, are the ubiquitin-proteasome pathway (UPP) and autophagy [105,106].

The impairment of both UPP and the autophagy machinery has been associated with the pathogenesis of neurodegenerative diseases, including PD [107]. Under physiological conditions, alpha-synuclein can be degraded via UPP in which it is tagged with ubiquitin molecules and transferred to the proteosome complex for ATP dependent degradation [106] as well as via the autophagic pathways (Fig. 2A) [108]. UPP failure has been reported in PD patients. In fact, in post-mortem human brains significantly decreased chymotrypsin-like and trypsin-like proteasome activity in the substantia nigra of PD patients compared to controls has been reported, together with a reduction in proteasome subunit levels. The observation that alpha-synuclein staining was more sensitive and specific than ubiquitin, indicates that alpha-synuclein aggregation precedes ubiquitination, that in turn promotes degradation by proteasome [8]. Proteasome dysfunction together with alpha-synuclein aggregates induce the involvement of both macroautophagy (Fig. 2A) and chaperone-mediated autophagy [109]. Previous studies have demonstrated that intracellular degradation of alpha-synuclein via the macroautophagic pathway is modulated, at least partly, by the autophagic regulator protein Beclin-1 [110,111]. For chaperone-mediated autophagy, the alpha-synuclein KFERQ domain is recognized by hsp70, interacts with LAMP2A, and is subsequently degraded in the lysosome by proteases [111]. In 1997, Anglade and colleagues [112] already described autophagic alterations in PD, reporting an accumulation of autophagic vacuoles in nigral dopaminergic cells from *post-mortem* PD patients. Since then, growing evidence from pathological studies suggests that autophagy is dysregulated in PD. Key proteins, Beclin-1 and LC3II, involved in the formation and recognition of autophagosomes, respectively, are found to be increased in the *substantia nigra* of PD patients [113] and in brain samples from patients with Lewy body dementia [114]. Additionally, disrupted autophagosomes and endolysosomes have recently been found within Lewy bodies, thus suggesting the involvement of autophagic mechanisms in the formation of intracellular inclusions [115]. In contrast, proteins involved in chaperone-mediated autophagy, including chaperone Hsc70 and LAMP2A, are reduced in several brain areas of individuals diagnosed with PD [116,117], suggesting that autophagy pathways are defective in PD brains. Substantial evidence has shown a link between autophagy and alpha-synuclein toxicity in synucleinopathies, including PD, Lewy bodies dementia, and multiple system atrophy [108]. First of all, LC3 is shown to co-localize with alpha-synuclein in Lewy Bodies and Lewy neurites in the *substantia nigra* of PD patients [116], and with Lewy bodies in the hippocampus [118] and temporal cortex [119] of people with Lewy body dementia. Co-localization of LC3 with phosphorylated variants of alpha-synuclein has

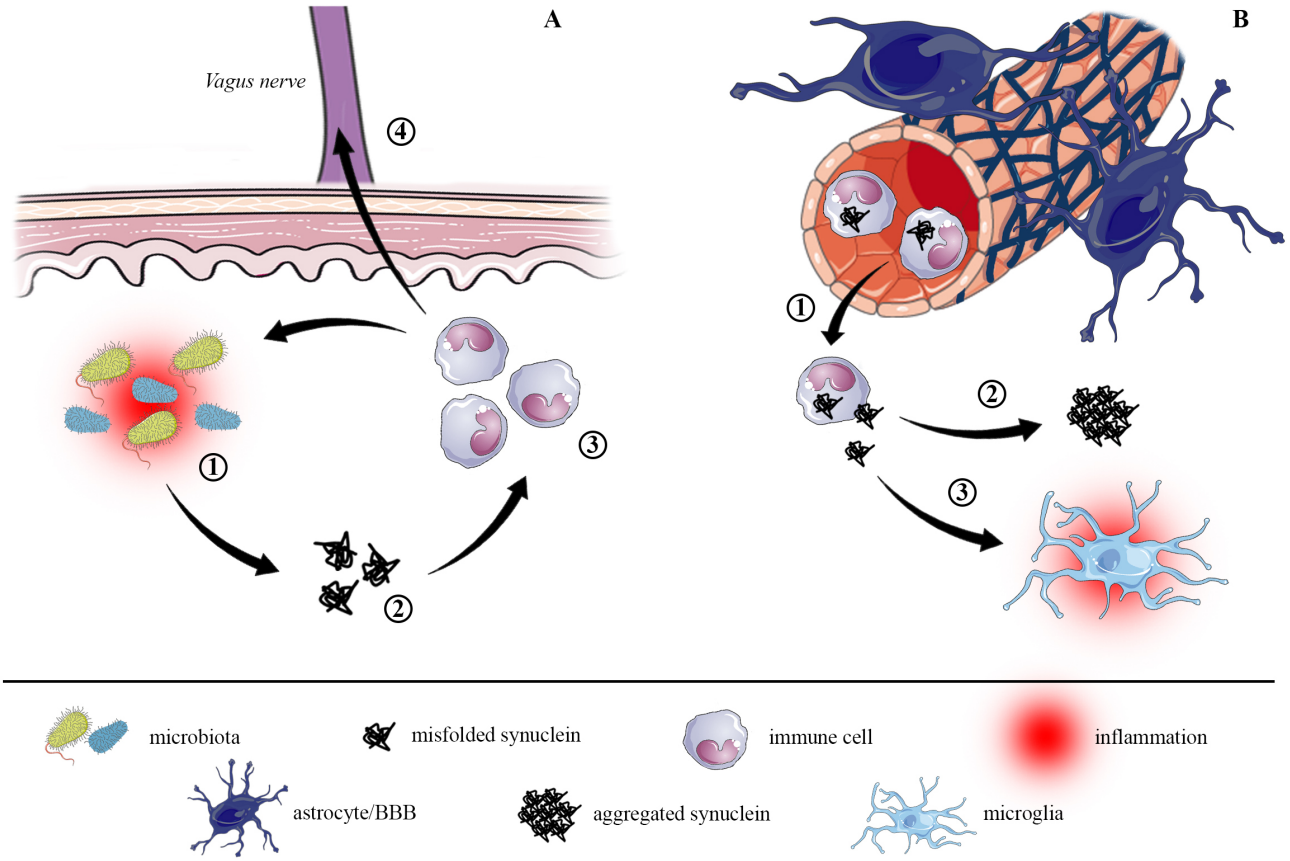

Fig. 3. Proposed pathways for alpha-synuclein dissemination from gut to brain. (A) The inflammatory environment in gastrointestinal regions, probably triggered by external stimuli and sustained by dysbiosis (1), increases alpha-synuclein expression and misfolding, leading to the formation of small alpha-synuclein aggregates (oligomers, 2). Misfolded and oligomeric synuclein species activate the peripheral immune response by recruiting immune cells (mostly monocytes and neutrophils, 3), which are primed by the inflammatory environment. The immune cells uptake alpha-synuclein and, in turn, further promote inflammation and protein misfolding and aggregation. The increased intestinal permeability allows immune cells to migrate through the vagus nerve (4) directly towards the dorsal motor nucleus of vagus in the brainstem, from where it starts to spread in the brain. (B) At the same time, chronic local inflammation promotes a systemic inflammatory status that can affect permeability of the blood brain barrier, allowing alpha-synuclein to be transported to the brain (1). There, alpha-synuclein fosters the formation of bigger aggregates (2), resulting in the neuropathological lesions hallmark of synucleinopathies, and it triggers microglial activation (3) that perpetuates neuroinflammation.

been observed in glial cytoplasmic inclusions in patients affected by multiple system atrophy [120]. Moreover, histone deacetylase-6 (HDAC6), involved in the transport of misfolded proteins to the aggresome, also localizes with alpha-synuclein in Lewy bodies from PD and Lewy body dementia patients and in Papp Lantos bodies in multiple system atrophy [121,122]. Together, these findings suggest an impaired clearance of autophagosomes in synucleinopathies. Notably, in neuronal cultures, wild-type alpha-synuclein can be degraded by autophagy [123–125], whereas mutated or structurally-changed variants impact on the degradation pathways, blocking the chaperone-mediated autophagy pathway, thereby, affecting protein degradation [123]. Interestingly, the blockage of chaperone-mediated autophagy by mutant A53T increases the activation of macroautophagy, although this compensatory result appears to have a negative effect causing neuronal cell death [124]. Furthermore, the inhibition of chaperone-mediated autophagy by 3-Methyladenine induces the formation of high molecule weight or detergent-insoluble oligomeric species of alpha-synuclein [125], thus suggesting that proper chaperone-mediated autophagy is important for the prevention and degradation of alpha-synuclein variants. Likewise, the failure of the autophagic machinery in clearing misfolded and/or aggregated alpha-synuclein has been proposed as a key mechanism in the formation of Lewy bodies [126]. More controversial results have been published concerning the pharmacological inhibition of macroautophagy. A study in PC12 cells demonstrated an expected increase in wild-type alpha-synuclein after inhibiting macroautophagy with 3-Methyladenine, whereas others reported that 3-Methyladenine macroautophagy inhibition results in a sig-

nificant increase of mutant A53T but not wild-type alpha-synuclein [127]. Furthermore, pharmacological stimulation of macroautophagy, using rapamycin, significantly increases the clearance of wild-type, A30P, and A53T alpha-synuclein. Together, these somewhat controversial results suggest that macroautophagy is involved in the degradation of both wild-type and mutated variants of alpha-synuclein. A more recent study proved that the accumulation of alpha-synuclein also led to increased damage to the integrity of lysosomal membranes, and that a mutation of the pore-forming lysosomal protein TMEM175 is associated with an increased loss of dopaminergic neurons and an increased formation of alpha-synuclein pathological variants [128]. As regards studies on human-derived cells, few data are available regarding peripheral aspects of UPP impairment. In blood mononuclear cells (PBMCs) and blood lymphocytes collected from PD patients a reduced activity of the 20S proteasome subunit was observed, with an inverse correlation with disease duration and severity, with no differences in ubiquitin levels [129].

Prigione and colleagues [130] identified the induction of the macroautophagy response in PBMCs of PD patients compared to controls. Moreover, in line with results from the CNS, studies reported elevated levels of LC3II, and reduced levels of Hsc70 and LAMP2A in PBMCs obtained from individuals with PD [131–134], suggesting a potential dysregulation of autophagy also in the periphery of PD patients (Fig. 2B). Furthermore, a whole-transcriptome assay revealed mRNA downregulation of 6 regulators involved in the formation of the autophagosome (i.e., ULK3, ATG2A, ATG4B, ATG5, ATG16L1, HDAC6), whereas protein levels of upstream autophagy markers (i.e., ULK1, Beclin-1, autophagy/beclin-1 regulator 1) were elevated in PD-derived PBMCs [135]. The levels of some autophagy-related proteins were significantly correlated with mutant alpha-synuclein, but, even more importantly, all autophagy-related proteins demonstrated a strong correlation with oligomeric alpha-synuclein. This suggests that alpha-synuclein, and, in particular, the oligomeric variants, may influence the macroautophagic pathway. Besides PBMCs, one study investigated the role of macroautophagy on PD in skin fibroblasts from patients with late-onset PD [136]. They displayed a striking increase of autophagosomes and autolysosomes in PD fibroblasts compared to healthy controls. Interestingly, pharmacological blockage of macroautophagy resulted in an even greater autophagic flux, with a widespread collection of macroautophagic structures.

Supporting the hypothesis that clearance mechanisms are key players in the etiopathogenesis of the disease, some familial forms of PD can be ascribed to mutations in genes involved in the autophagic machinery. Mutations in the *LRKK2* gene are known to be the most common cause of dominant familial PD (2% of sporadic PD events) [137], affecting the autophagic pathway [138] and increasing the propensity to accumulate alpha-synuclein [139]. Ho and colleagues [140] demonstrated that the LRRK2 R1441G mutation in mouse embryonic fibroblasts induces more perinuclear clustering of lysosomes, whose redistribution indicates impaired degradation of aberrant proteins. Similarly, the same redistribution was also observed in aged LRRK2 R1441G knock-in mouse brain. Furthermore, the rate of cellular clearance of alpha-synuclein using the photoactivatable fluorescence-based cell assay in knock-in mouse embryonic fibroblasts was significantly lower than the wild type one [140].

According to genome wide association studies, mutations in the *GBA1* gene, encoding for glucocerebrosidase, are the most common genetic risk factor for PD. This mutation is causative for Gaucher disease, a lysosomal storage disorder [141], and a decreased enzyme activity has been reported in PD patients with or without *GBA1* mutations [142]. Emerging experimental evidence both in the central and peripheral nervous systems suggests a correlation between this decreased activity and alpha-synuclein accumulation [143–145]. In particular, the L444P mutation resulted in the exacerbation of both motor and gastrointestinal deficits found in the A53T mouse model of Parkinson's disease [145].

In conclusion, these studies suggest that the impairment of the autophagy machinery, characterising PD affected brains is also present at the peripheral level.

6. Inflammatory Processes

A neuroinflammatory environment is a common feature shared by neurodegenerative diseases, including PD. Indeed, mutations in immune system-related genes, namely *LRRK2*, *DJ-1* and *HLA-DR*, are known to be responsible for familial forms of PD [146]. Initial signs of neuroinflammation found in brains affected by synuclein-related pathologies include an extensive activation of microglial cells and a high density of astrocytes, as well as the infiltration of blood-derived mononuclear phagocytes and T lymphocytes. In addition, a significant increase in levels of pro-inflammatory cytokines (e.g., IL-1, IL-6 and TNF) has been described in the brain and CSF of PD patients [147,148]. In support of this, the use of non-steroidal anti-inflammatory drugs (NSAIDs) has been associated with a decreased risk of developing PD, despite the fact they are inefficient in the treatment of the late stages of the disease, thus suggesting an early involvement of neuroinflammatory processes in its pathogenesis [149]. However, the topic is controversial and neither epidemiological studies nor meta-analyses have demonstrated that NSAIDs decrease the risk of PD or modify disease progression [150].

Universal consensus as to the actual contribution of inflammation to neurodegeneration is still lacking, and it remains unclear whether alpha-synuclein pathology is a cause or consequence of a neuroinflammatory environment. Indeed, microglia activation is reported to be triggered by

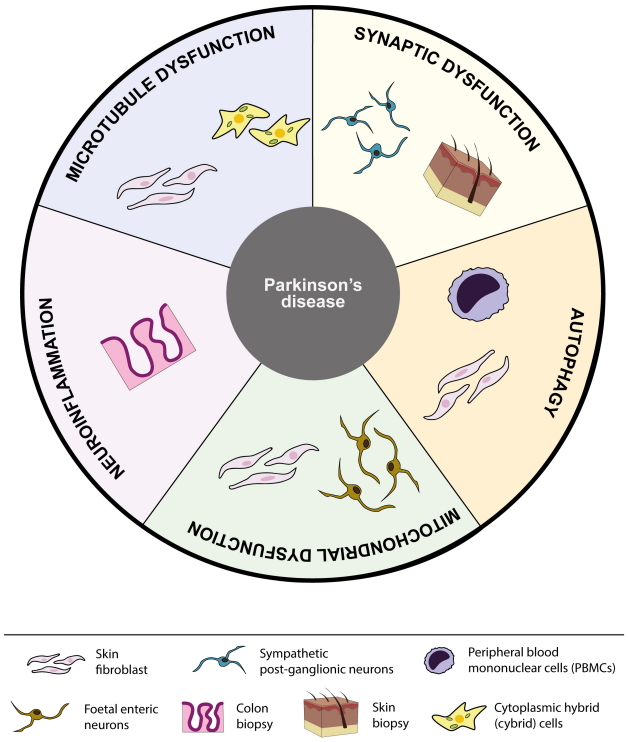

Fig. 4. Schematic summary of peripheral tissues linked to each pathological pathway involved in PD. Overview of the five different pathological pathways discussed in this review, including microtubule dysfunction, synaptic dysfunction, autophagy, mitochondrial dysfunction and neuroinflammation. Each pathological pathway shows a schematic representation of all peripheral tissues investigated for that particular pathway to date.

misfolded, nitrated and aggregated alpha-synuclein, while inflammatory processes are known to elicit an oxidative environment that can, in turn, enhance protein misfolding, aggregation and cell death [151]. Studies *in vitro* and in animal models suggest that the extracellular aggregated and misfolded alpha-synuclein is recognized by microglial Toll-Like receptors, triggering protein uptake, microglial activation and inflammatory response prior to neuronal loss [152].

Intriguingly, an unexpected relationship seems to exist between peripheric inflammation and neurodegenerative diseases. In particular, there is a noteworthy overlap between genetic variants linked to PD and gut disorders and, likewise, inflammatory processes of the gastrointestinal tract have been reported to increase the risk of PD [153]. In addition, dysbiosis of gastrointestinal microbiota has been described in synucleinopathies as well as in several CNS disorders, and linked to the inflammatory environment observed in disease [154–156]. Similarly, population studies have shown that PD patients exhibit an increased prevalence of dermatological inflammatory disorders associated with bacterial colonization [157,158]. However, no studies have questioned the correlation between skin microbiota and neurodegenerative diseases, and the few investigations into other vulnerable sites like oral and nasal mucosa showed no significant association [159]. On the contrary, a study on wild type mice reports that intraperitoneal injection of bacterial lipopolysaccharides (LPS) together with alpha-synuclein strains results in leukocytes infiltration and increased microglial population at the central level [160]. In the model proposed by the authors, LPS-activated inflammatory monocytes could internalize alpha-synuclein and move to the brain promoting its propagation at the central level. Similar indications arose from studies in synuclein-overexpressing mouse models. Sampson and colleagues [161] described an enhanced neuroinflammation as well as worsened motor deficits and increased synuclein pathology in mice colonized with microbiota derived from PD patients. In addition, in a study by Kishimoto and colleagues [162], mild gut inflammation is reported to reduce the age of PD onset and increase motor deficits and inflammation markers in mouse brain. At the same time, both synuclein and phospho-synuclein immunoreactivity are increased in the brain and gut, while a selective decrease in the neuronal dopaminergic population has been found in the *substantia nigra*. Consistently, alpha-synuclein pathology has been proven to spread from the gut to brain through the vagal nerve and reach the dorsal motor nucleus of the *vagus* [163] and, at the same time, LPS-mediated inflammation seems to enhance blood-brain barrier permeability, facilitating alpha-synuclein transit towards the brain [164]. The putative involvement of peripheral inflammation in neurodegenerative disease has been investigated also in living patients. Devos and colleagues [165] collected ascending colon biopsies from PD patients and age/sex matched healthy controls, who were tested for inflammatory status and alpha-synuclein deposition. The authors reported a significant increase in mRNA levels of pro-inflammatory cytokines (TNF-alpha, IL-6 and IL-1-beta) and a greater extent of glial markers, glial fibrillar acid protein and sox-10 in PD samples. Furthermore, two-thirds of the biopsies from PD patients resulted positive for phospho-alpha-synuclein immunostaining. Interestingly, levels of mRNAs displayed a negative correlation with disease duration and no correlation with disease severity (evaluated using the UPDRS scale) or enteric synuclein pathology, thus suggesting that inflammatory processes are involved predominantly in the early phases of PD and that the gastrointestinal tract might be the priming site of disease onset. An interesting study by Stolzenberg and colleagues [166] investigated the relationship between alpha-synuclein and gut inflammation in a pediatric cohort of 42 children with upper gastrointestinal pathologies (i.e., duodenitis, gastritis, *Helicobacter pylori*, reactive gastropathy) with no CNS disease. They evaluated by immunohistochemistry the extent of synuclein pathology and inflammatory status in endoscopic biopsies. Interestingly, the severity of inflammation, rated as neutrophil and mononuclear leukocyte density, was found to correlate with the enteric alpha-synuclein

burden and, in addition, both monomeric and aggregated species of alpha-synuclein were proven to be chemotactic towards neutrophils and monocytes and to promote dendritic cell maturation. In support of their data, the authors reported that biopsies collected before the onset of infections were devoid of synuclein deposits and that follow-up in patients with acute and non-chronic infection showed a decreased gastrointestinal alpha-synuclein. The association between peripheral inflammation and neurodegeneration was directly tested in a noteworthy study by Moghaddam and colleagues [167], who investigated a cohort 388 drug-naïve PD patients and 148 controls. The authors assessed nigrostriatal dysfunction by DAT Single Photon Emission Computed Tomography (SPECT) imaging and evaluated the inflammatory status, rated as neutrophil to lymphocyte ratio, in patients' blood samples. As expected, the inflammatory status was significantly higher in PD subjects compared to controls and, intriguingly, it was negatively correlated with the severity of nigrostriatal dysfunction. This is coherent with the observation that PD progression is reported to not follow a linear model, and striatal neurons depletion is faster in the early stages of the disease, while a way slower progression is observed in advanced stages [168]. Taken together these results endorse the hypothesis that an inflammatory environment is required in the initial phase of neurodegenerative diseases. Alpha-synuclein, in turn, could act as a mediator for immune cell recruitment and trigger an enhanced inflammatory response, both in the periphery and in the CNS, through microglial activation. In addition, the finding of blood-derived leukocytes in affected brain suggests an increased blood brain barrier permeability which increases the transport of misfolded and aggregated alpha-synuclein towards the brain. These pathways, summarized in Fig. 3, can potentially act together in the neurodegenerative process in a non-exclusive fashion, resulting in a systemic inflammatory state and alpha-synuclein deposition both in the periphery and in the CNS.

7. Discussion and Conclusions

Perceiving PD as a pathology confined to the CNS is now an outdated picture. The non-motor symptoms include several autonomic, non-dopaminergic manifestation affecting multiple body districts and, interestingly, these symptoms characterize the prodromal phase of the pathology and can be discrete predictors for the development of clinical PD. This evidence endorses the hypothesis that the pathology can even start at the periphery, likely through susceptible sites and with a multifactorial etiology that embodies genetic predisposition and environmental factors [169]. The neuropathological staging proposed by Braak and colleagues seems to confirm this hypothesis, on the grounds that the first brain areas affected by Lewy pathology are the dorsal motor nucleus of *vagus* and olfactory bulb, both receiving afferences from the peripheral nervous system and associated with body regions acting as an interface with the outside and so more susceptible to environmental triggers (namely the gastrointestinal tract and olfactory mucosa). According to this hypothesis, alpha-synuclein aggregates enter the brain simultaneously via two routes: nasal, with a progression to the amydgala, and gastric, with retrograde diffusion to the dorsal motor nucleus. Recently, it has been suggested that rather than this "dual-hit" scenario, the "single-hit olfactory bulb (OB)/brain or autonomic" hypothesis is taking place [170]. The dual-hit hypothesis is supported by the fact that constipation, REM sleep behavior disorder (RBD), and hyposmia prior to diagnosis are present in the prodromal phase. However, clinical observation of this phase could be more complicated, as constipation is manifested in fewer than half of PD patients in the early stage, RBD affects approximately only a third, and hyposmia around 70% before diagnosis. These data suggest that not all patients fit into the dual-hit hypothesis, including a clinical "OB/brain-first" or "body first" subtype. This is also supported by a very recent analysis of two datasets (Vanta and Tokyo) in which it is emerged that alpha-synuclein pathology in the enteric nervous system or lower brainstem very rarely present OB pathology, while patients with mild amygdala-prevalence nearly always present OB pathology.

On this basis, looking at the processes that occur at the periphery may represent an easily-accessible window to look at what is occurring in CNS. In this review, we have provided a comprehensive report of the pathological mechanisms described in extra-brain districts, ranging from molecular to systemic levels (Fig. 4).

Many of the studies performed on peripheral samples collected from patients involved blood mononuclear cells and skin fibroblasts that have proven to be easily-accessible models. They revealed impaired microtubular and mitochondrial physiology, as well as a defective autophagic machinery at the periphery in PD. However, the relevance of these data is limited, as the models lack the organizational complexity of a tissue and, in addition, the intracellular high specialization of a neuron, where the microtubule-mediated connection between synapses and soma is complex and highly regulated. Despite the lack of molecular insights, the collection of body fluids and tissue biopsies from skin and gut could be useful and provide an overview of the systemic modifications occurring in the periphery in the pathological environment of PD, as demonstrated for the increased inflammatory state and a progressive loss of synaptic terminals. Therefore, this paper wants to draw attention to the importance of the periphery as a field of investigation into CNS diseases, aiming to study the mechanistic features of the pathology. Each of the reported processes could potentially trigger PD, as suggested by familial forms of the pathology, and it may impact reciprocally on the others due to the close interconnection between these processes. To conclude, we emphasize how the peripheral aspects of PD warrant further studies as they may become

a reliable predictive element as well as an accessible target for disease modifying agents, especially in light of a possible peripheral onset.

The pathological mechanisms we have discussed in this review are potentially linked to each other. The dying back neurodegeneration that starts from the synapses could be fostered by microtubule dysfunction, inducing mitochondrial impairment and autophagic failure as a consequence of the defective transport towards the cell body. This degeneration then elicits inflammation, especially through microglia activation. Additionally, all these pathological processes may have a mutual impact on the others, since synaptic functioning could be impaired firstly by autophagy or mitochondrial mutations, such as *Parkin*, *LRRK2*, *GBA* and *TMEM175* mutations, known to be factors for PD, which increase during aging. Lastly, the pivotal emerging role of the microtubule system, as suggested by *MAPT* mutations, definitely warrants more in depth investigation, since it connects the synapses and the cell bodies and could play an additional pivotal role both in axonal transport and synaptic activity. Notably, the peripheral aspects of PD are undoubtedly worthy of further studies as they may represent the onset of CNS pathology and, if this were to be proven, they could become reliable predictors PD development as well as an accessible target for disease modifying agents.

Author Contributions

MJB, JMK, and AC wrote the manuscript and designed the figures. GP, GC and SM critically reviewed it. All the authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

The authors thank "Fondazione Grigioni per il Morbo di Parkinson" (Milan-Italy) for long-lasting support to GC. We are grateful to all the members of our team for stimulating discussion and to Karen Doyle for reading and editing the paper.

Funding

This research was funded by Italian "5×1000" funding to Fondazione Grigioni per il Morbo di Parkinson (Milan, Italy) and by Innovation Programme H2020-MSCA-ITN-2019-EJD-Grant agreement n. 860070 (TubInTrain, accessed date 1 October 2019, https://www.tubintrain.eu/).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Dickson DW. Parkinson's disease and parkinsonism: neuropathology. Cold Spring Harbor Perspectives in Medicine. 2012; 2: a009258.

[2] Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature. 1997; 388: 839–840.

[3] Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, *et al*. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science. 1997; 276: 2045–2047.

[4] Maroteaux L, Campanelli JT, Scheller RH. Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 1988; 8: 2804–2815.

[5] Amadeo A, Pizzi S, Comincini A, Modena D, Calogero AM, Madaschi L, *et al*. The Association between α-Synuclein and α-Tubulin in Brain Synapses. International Journal of Molecular Sciences. 2021; 22: 9153.

[6] Stefanis L. α-Synuclein in Parkinson's disease. Cold Spring Harbor Perspectives in Medicine. 2012; 2: a009399.

[7] Pellegrini L, Wetzel A, Grannó S, Heaton G, Harvey K. Back to the tubule: microtubule dynamics in Parkinson's disease. Cellular and Molecular Life Sciences. 2017; 74: 409–434.

[8] Goedert M, Jakes R, Spillantini MG. The Synucleinopathies: Twenty Years On. Journal of Parkinson's Disease. 2017; 7: S51–S69.

[9] Riederer P, Berg D, Casadei N, Cheng F, Classen J, Dresel C, *et al*. α-Synuclein in Parkinson's disease: causal or bystander? Journal of Neural Transmission. 2019; 126: 815–840.

[10] Espay AJ. Movement disorders research in 2021: cracking the paradigm. The Lancet. Neurology. 2022; 21: 10–11.

[11] Burré J, Sharma M, Südhof TC. Cell Biology and Pathophysiology of α-Synuclein. Cold Spring Harbor Perspectives in Medicine. 2018; 8: a024091.

[12] Hirsch EC, Jenner P, Przedborski S. Pathogenesis of Parkinson's disease. Movement Disorders: Official Journal of the Movement Disorder Society. 2013; 28: 24–30.

[13] Mazzetti S, Calogero AM, Pezzoli G, Cappelletti G. Cross-talk between α-synuclein and the microtubule cytoskeleton in neurodegeneration. Experimental Neurology. 2023; 359: 114251.

[14] Schneider SA, Boettner M, Alexoudi A, Zorenkov D, Deuschl G, Wedel T. Can we use peripheral tissue biopsies to diagnose Parkinson's disease? A review of the literature. European Journal of Neurology. 2016; 23: 247–261.

[15] Beach TG, Adler CH, Sue LI, Vedders L, Lue L, White Iii CL, *et al*. Multi-organ distribution of phosphorylated alpha-synuclein histopathology in subjects with Lewy body disorders. Acta Neuropathologica. 2010; 119: 689–702.

[16] Stern MB, Lang A, Poewe W. Toward a redefinition of Parkinson's disease. Movement Disorders: Official Journal of the Movement Disorder Society. 2012; 27: 54–60.

[17] Janke C, Magiera MM. The tubulin code and its role in controlling microtubule properties and functions. Nature Reviews. Molecular Cell Biology. 2020; 21: 307–326.

[18] Cartelli D, Cappelletti G. Microtubule Destabilization Paves the Way to Parkinson's Disease. Molecular Neurobiology. 2017; 54: 6762–6774.

[19] Forno LS, Sternberger LA, Sternberger NH, Strefling AM, Swanson K, Eng LF. Reaction of Lewy bodies with antibodies to phosphorylated and non-phosphorylated neurofilaments. Neuroscience Letters. 1986; 64: 253–258.

[20] Galloway PG, Grundke-Iqbal I, Iqbal K, Perry G. Lewy bodies contain epitopes both shared and distinct from Alzheimer neurofibrillary tangles. Journal of Neuropathology and Experimen-

tal Neurology. 1988; 47: 654–663.
[21] Shahmoradian SH, Lewis AJ, Genoud C, Hench J, Moors TE, Navarro PP, *et al*. Lewy pathology in Parkinson's disease consists of crowded organelles and lipid membranes. Nature Neuroscience. 2019; 22: 1099–1109.
[22] Moors TE, Maat CA, Niedieker D, Mona D, Petersen D, Timmermans-Huisman E, *et al*. The subcellular arrangement of alpha-synuclein proteoforms in the Parkinson's disease brain as revealed by multicolor STED microscopy. Acta Neuropathologica. 2021; 142: 423–448.
[23] Elbaz A, Ross OA, Ioannidis JPA, Soto-Ortolaza AI, Moisan F, Aasly J, *et al*. Independent and joint effects of the MAPT and SNCA genes in Parkinson disease. Annals of Neurology. 2011; 69: 778–792.
[24] Cappelletti G, Pedrotti B, Maggioni MG, Maci R. Microtubule assembly is directly affected by MPP(+) *in vitro*. Cell Biology International. 2001; 25: 981–984.
[25] Cappelletti G, Surrey T, Maci R. The parkinsonism producing neurotoxin MPP+ affects microtubule dynamics by acting as a destabilising factor. FEBS Letters. 2005; 579: 4781–4786.
[26] Schmidt DE, Ebert MH, Lynn JC, Whetsell WO, Jr. Attenuation of 1-methyl-4-phenylpyridinium (MPP+) neurotoxicity by deprenyl in organotypic canine substantia nigra cultures. Journal of Neural Transmission. 1997; 104: 875–885.
[27] Cartelli D, Casagrande F, Busceti CL, Bucci D, Molinaro G, Traficante A, *et al*. Microtubule alterations occur early in experimental parkinsonism and the microtubule stabilizer epothilone D is neuroprotective. Scientific Reports. 2013; 3: 1837.
[28] Cartelli D, Ronchi C, Maggioni MG, Rodighiero S, Giavini E, Cappelletti G. Microtubule dysfunction precedes transport impairment and mitochondria damage in MPP+ -induced neurodegeneration. Journal of Neurochemistry. 2010; 115: 247–258.
[29] Cartelli D, Aliverti A, Barbiroli A, Santambrogio C, Ragg EM, Casagrande FVM, *et al*. α-Synuclein is a Novel Microtubule Dynamase. Scientific Reports. 2016; 6: 33289.
[30] Ren Y, Jiang H, Hu Z, Fan K, Wang J, Janoschka S, *et al*. Parkin mutations reduce the complexity of neuronal processes in iPSC-derived human neurons. Stem Cells (Dayton, Ohio). 2015; 33: 68–78.
[31] Law BMH, Spain VA, Leinster VHL, Chia R, Beilina A, Cho HJ, *et al*. A direct interaction between leucine-rich repeat kinase 2 and specific β-tubulin isoforms regulates tubulin acetylation. The Journal of Biological Chemistry. 2014; 289: 895–908.
[32] Calogero AM, Mazzetti S, Pezzoli G, Cappelletti G. Neuronal microtubules and proteins linked to Parkinson's disease: a relevant interaction? Biological Chemistry. 2019; 400: 1099–1112.
[33] Payton JE, Perrin RJ, Clayton DF, George JM. Protein-protein interactions of alpha-synuclein in brain homogenates and transfected cells. Brain Research. Molecular Brain Research. 2001; 95: 138–145.
[34] Alim MA, Hossain MS, Arima K, Takeda K, Izumiyama Y, Nakamura M, *et al*. Tubulin seeds alpha-synuclein fibril formation. The Journal of Biological Chemistry. 2002; 277: 2112–2117.
[35] Alim MA, Ma QL, Takeda K, Aizawa T, Matsubara M, Nakamura M, *et al*. Demonstration of a role for alpha-synuclein as a functional microtubule-associated protein. Journal of Alzheimer's Disease: JAD. 2004; 6: 435–435–42; discussion 443–9.
[36] Zhou RM, Huang YX, Li XL, Chen C, Shi Q, Wang GR, *et al*. Molecular interaction of α-synuclein with tubulin influences on the polymerization of microtubule in vitro and structure of microtubule in cells. Molecular Biology Reports. 2010; 37: 3183–3192.
[37] Chen L, Jin J, Davis J, Zhou Y, Wang Y, Liu J, *et al*. Oligomeric alpha-synuclein inhibits tubulin polymerization. Biochemical and Biophysical Research Communications. 2007; 356: 548–553.
[38] Cartelli D, Cappelletti G. α-Synuclein regulates the partitioning between tubulin dimers and microtubules at neuronal growth cone. Communicative & Integrative Biology. 2017; 10: e1267076.
[39] Toba S, Jin M, Yamada M, Kumamoto K, Matsumoto S, Yasunaga T, *et al*. Alpha-synuclein facilitates to form short unconventional microtubules that have a unique function in the axonal transport. Scientific Reports. 2017; 7: 16386.
[40] Cartelli D, Goldwurm S, Casagrande F, Pezzoli G, Cappelletti G. Microtubule destabilization is shared by genetic and idiopathic Parkinson's disease patient fibroblasts. PLoS ONE. 2012; 7: e37467.
[41] Salama M, Shalash A, Magdy A, Makar M, Roushdy T, Elbalkimy M, *et al*. Tubulin and Tau: Possible targets for diagnosis of Parkinson's and Alzheimer's diseases. PLoS ONE. 2018; 13: e0196436.
[42] Esteves AR, Arduíno DM, Swerdlow RH, Oliveira CR, Cardoso SM. Microtubule depolymerization potentiates alpha-synuclein oligomerization. Frontiers in Aging Neuroscience. 2010; 1: 5.
[43] Esteves ARF, Domingues AF, Ferreira IL, Januário C, Swerdlow RH, Oliveira CR, *et al*. Mitochondrial function in Parkinson's disease cybrids containing an nt2 neuron-like nuclear background. Mitochondrion. 2008; 8: 219–228.
[44] Esteves AR, Gozes I, Cardoso SM. The rescue of microtubule-dependent traffic recovers mitochondrial function in Parkinson's disease. Biochimica et Biophysica Acta. 2014; 1842: 7–21.
[45] Westphal CH, Chandra SS. Monomeric synucleins generate membrane curvature. The Journal of Biological Chemistry. 2013; 288: 1829–1840.
[46] Varkey J, Isas JM, Mizuno N, Jensen MB, Bhatia VK, Jao CC, *et al*. Membrane curvature induction and tubulation are common features of synucleins and apolipoproteins. The Journal of Biological Chemistry. 2010; 285: 32486–32493.
[47] Burré J, Sharma M, Tsetsenis T, Buchman V, Etherton MR, Südhof TC. Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. Science. 2010; 329: 1663–1667.
[48] Swant J, Goodwin JS, North A, Ali AA, Gamble-George J, Chirwa S, *et al*. α-Synuclein stimulates a dopamine transporter-dependent chloride current and modulates the activity of the transporter. The Journal of Biological Chemistry. 2011; 286: 43933–43943.
[49] Butler B, Saha K, Rana T, Becker JP, Sambo D, Davari P, *et al*. Dopamine Transporter Activity Is Modulated by α-Synuclein. The Journal of Biological Chemistry. 2015; 290: 29542–29554.
[50] Mirza FJ, Zahid S. The Role of Synapsins in Neurological Disorders. Neuroscience Bulletin. 2018; 34: 349–358.
[51] Garcia-Reitböck P, Anichtchik O, Bellucci A, Iovino M, Ballini C, Fineberg E, *et al*. SNARE protein redistribution and synaptic failure in a transgenic mouse model of Parkinson's disease. Brain: a Journal of Neurology. 2010; 133: 2032–2044.
[52] Bate C, Gentleman S, Williams A. α-synuclein induced synapse damage is enhanced by amyloid-β1-42. Molecular Neurodegeneration. 2010; 5: 55.
[53] Bellucci A, Navarria L, Falarti E, Zaltieri M, Bono F, Collo G, *et al*. Redistribution of DAT/α-synuclein complexes visualized by "*in situ*" proximity ligation assay in transgenic mice modelling early Parkinson's disease. PLoS ONE. 2011; 6: e27959.
[54] Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, *et al*. alpha-Synuclein is phosphorylated in synucleinopathy lesions. Nature Cell Biology. 2002; 4: 160–164.
[55] Lundblad M, Decressac M, Mattsson B, Björklund A. Impaired neurotransmission caused by overexpression of α-synuclein in nigral dopamine neurons. Proceedings of the National Academy

of Sciences of the United States of America. 2012; 109: 3213–3219.
[56] Thakur P, Breger LS, Lundblad M, Wan OW, Mattsson B, Luk KC, *et al*. Modeling Parkinson's disease pathology by combination of fibril seeds and α-synuclein overexpression in the rat brain. Proceedings of the National Academy of Sciences of the United States of America. 2017; 114: E8284–E8293.
[57] Wersinger C, Rusnak M, Sidhu A. Modulation of the trafficking of the human serotonin transporter by human alpha-synuclein. The European Journal of Neuroscience. 2006; 24: 55–64.
[58] Plotegher N, Berti G, Ferrari E, Tessari I, Zanetti M, Lunelli L, *et al*. DOPAL derived alpha-synuclein oligomers impair synaptic vesicles physiological function. Scientific Reports. 2017; 7: 40699.
[59] Braak H, Del Tredici K, Rüb U, de Vos RAI, Jansen Steur ENH, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiology of Aging. 2003; 24: 197–211.
[60] Hawkes CH, Del Tredici K, Braak H. Parkinson's disease: a dual-hit hypothesis. Neuropathology and Applied Neurobiology. 2007; 33: 599–614.
[61] Prusiner SB, Woerman AL, Mordes DA, Watts JC, Rampersaud R, Berry DB, *et al*. Evidence for α-synuclein prions causing multiple system atrophy in humans with parkinsonism. Proceedings of the National Academy of Sciences of the United States of America. 2015; 112: E5308–5317.
[62] Desplats P, Lee HJ, Bae EJ, Patrick C, Rockenstein E, Crews L, *et al*. Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106: 13010–13015.
[63] Goldstein DS, Holmes C, Li ST, Bruce S, Metman LV, Cannon RO, 3rd. Cardiac sympathetic denervation in Parkinson disease. Annals of Internal Medicine. 2000; 133: 338–347.
[64] Tipre DN, Goldstein DS. Cardiac and extracardiac sympathetic denervation in Parkinson's disease with orthostatic hypotension and in pure autonomic failure. Journal of Nuclear Medicine: Official Publication, Society of Nuclear Medicine. 2005; 46: 1775–1781.
[65] Melli G, Vacchi E, Biemmi V, Galati S, Staedler C, Ambrosini R, *et al*. Cervical skin denervation associates with alpha-synuclein aggregates in Parkinson disease. Annals of Clinical and Translational Neurology. 2018; 5: 1394–1407.
[66] Donadio V, Incensi A, Leta V, Giannoccaro MP, Scaglione C, Martinelli P, *et al*. Skin nerve α-synuclein deposits: a biomarker for idiopathic Parkinson disease. Neurology. 2014; 82: 1362–1369.
[67] Mu L, Sobotka S, Chen J, Su H, Sanders I, Adler CH, *et al*. Alpha-synuclein pathology and axonal degeneration of the peripheral motor nerves innervating pharyngeal muscles in Parkinson disease. Journal of Neuropathology and Experimental Neurology. 2013; 72: 119–129.
[68] Stokholm MG, Danielsen EH, Hamilton-Dutoit SJ, Borghammer P. Pathological α-synuclein in gastrointestinal tissues from prodromal Parkinson disease patients. Annals of Neurology. 2016; 79: 940–949.
[69] Hilton D, Stephens M, Kirk L, Edwards P, Potter R, Zajicek J, *et al*. Accumulation of α-synuclein in the bowel of patients in the pre-clinical phase of Parkinson's disease. Acta Neuropathologica. 2014; 127: 235–241.
[70] Challis C, Hori A, Sampson TR, Yoo BB, Challis RC, Hamilton AM, *et al*. Gut-seeded α-synuclein fibrils promote gut dysfunction and brain pathology specifically in aged mice. Nature Neuroscience. 2020; 23: 327–336.
[71] Orimo S, Uchihara T, Nakamura A, Mori F, Kakita A, Wakabayashi K, *et al*. Axonal alpha-synuclein aggregates herald centripetal degeneration of cardiac sympathetic nerve in Parkinson's disease. Brain: a Journal of Neurology. 2008; 131: 642–650.
[72] Roy S, Zhang B, Lee VMY, Trojanowski JQ. Axonal transport defects: a common theme in neurodegenerative diseases. Acta Neuropathologica. 2005; 109: 5–13.
[73] Sleigh JN, Rossor AM, Fellows AD, Tosolini AP, Schiavo G. Axonal transport and neurological disease. Nature Reviews. Neurology. 2019; 15: 691–703.
[74] Mazzetti S, Basellini MJ, Ferri V, Cassani E, Cereda E, Paolini M, *et al*. α-Synuclein oligomers in skin biopsy of idiopathic and monozygotic twin patients with Parkinson's disease. Brain: a Journal of Neurology. 2020; 143: 920–931.
[75] Navarro-Otano J, Casanova-Mollà J, Morales M, Valls-Solé J, Tolosa E. Cutaneous autonomic denervation in Parkinson's disease. Journal of Neural Transmission. 2015; 122: 1149–1155.
[76] Knudsen K, Borghammer P. Imaging the Autonomic Nervous System in Parkinson's Disease. Current Neurology and Neuroscience Reports. 2018; 18: 79.
[77] Goldstein DS, Holmes CS, Dendi R, Bruce SR, Li ST. Orthostatic hypotension from sympathetic denervation in Parkinson's disease. Neurology. 2002; 58: 1247–1255.
[78] Katagiri A, Asahina M, Araki N, Poudel A, Fujinuma Y, Yamanaka Y, *et al*. Myocardial (123)I-MIBG Uptake and Cardiovascular Autonomic Function in Parkinson's Disease. Parkinson's Disease. 2015; 2015: 805351.
[79] Nomura T, Inoue Y, Högl B, Uemura Y, Kitayama M, Abe T, *et al*. Relationship between (123)I-MIBG scintigrams and REM sleep behavior disorder in Parkinson's disease. Parkinsonism & Related Disorders. 2010; 16: 683–685.
[80] Iranzo A, Tolosa E, Gelpi E, Molinuevo JL, Valldeoriola F, Serradell M, *et al*. Neurodegenerative disease status and post-mortem pathology in idiopathic rapid-eye-movement sleep behaviour disorder: an observational cohort study. The Lancet. Neurology. 2013; 12: 443–453.
[81] Miyamoto T, Miyamoto M, Inoue Y, Usui Y, Suzuki K, Hirata K. Reduced cardiac 123I-MIBG scintigraphy in idiopathic REM sleep behavior disorder. Neurology. 2006; 67: 2236–2238.
[82] Nunnari J, Suomalainen A. Mitochondria: in sickness and in health. Cell. 2012; 148: 1145–1159.
[83] Parihar MS, Parihar A, Fujita M, Hashimoto M, Ghafourifar P. Mitochondrial association of alpha-synuclein causes oxidative stress. Cellular and Molecular Life Sciences. 2008; 65: 1272–1284.
[84] Shavali S, Brown-Borg HM, Ebadi M, Porter J. Mitochondrial localization of alpha-synuclein protein in alpha-synuclein overexpressing cells. Neuroscience Letters. 2008; 439: 125–128.
[85] Li WW, Yang R, Guo JC, Ren HM, Zha XL, Cheng JS, *et al*. Localization of alpha-synuclein to mitochondria within midbrain of mice. Neuroreport. 2007; 18: 1543–1546.
[86] Guardia-Laguarta C, Area-Gomez E, Rüb C, Liu Y, Magrané J, Becker D, *et al*. α-Synuclein is localized to mitochondria-associated ER membranes. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 2014; 34: 249–259.
[87] Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science. 1983; 219: 979–980.
[88] Schapira AH, Holt IJ, Sweeney M, Harding AE, Jenner P, Marsden CD. Mitochondrial DNA analysis in Parkinson's disease. Movement Disorders: Official Journal of the Movement Disorder Society. 1990; 5: 294–297.
[89] Fløtnes IH, Fernandez-Vizarra E, Lykouri M, Brakedal B, Skeie GO, Miletic H, *et al*. Neuronal complex I deficiency occurs throughout the Parkinson's disease brain, but is not associated with neurodegeneration or mitochondrial DNA damage. Acta Neuropathologica. 2018; 135: 409–425.
[90] Bosco DA, Fowler DM, Zhang Q, Nieva J, Powers ET, Went-

worth P, Jr, *et al*. Elevated levels of oxidized cholesterol metabolites in Lewy body disease brains accelerate alpha-synuclein fibrilization. Nature Chemical Biology. 2006; 2: 249–253.
[91] Floor E, Wetzel MG. Increased protein oxidation in human substantia nigra pars compacta in comparison with basal ganglia and prefrontal cortex measured with an improved dinitrophenylhydrazine assay. Journal of Neurochemistry. 1998; 70: 268–275.
[92] Zhang J, Perry G, Smith MA, Robertson D, Olson SJ, Graham DG, *et al*. Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons. The American Journal of Pathology. 1999; 154: 1423–1429.
[93] Alam ZI, Jenner A, Daniel SE, Lees AJ, Cairns N, Marsden CD, *et al*. Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. Journal of Neurochemistry. 1997; 69: 1196–1203.
[94] Norris KL, Hao R, Chen LF, Lai CH, Kapur M, Shaughnessy PJ, *et al*. Convergence of Parkin, PINK1, and α-Synuclein on Stress-induced Mitochondrial Morphological Remodeling. The Journal of Biological Chemistry. 2015; 290: 13862–13874.
[95] van der Merwe C, Jalali Sefid Dashti Z, Christoffels A, Loos B, Bardien S. Evidence for a common biological pathway linking three Parkinson's disease-causing genes: parkin, PINK1 and DJ-1. The European Journal of Neuroscience. 2015; 41: 1113–1125.
[96] Devi L, Raghavendran V, Prabhu BM, Avadhani NG, Anandatheerthavarada HK. Mitochondrial import and accumulation of alpha-synuclein impair complex I in human dopaminergic neuronal cultures and Parkinson disease brain. The Journal of Biological Chemistry. 2008; 283: 9089–9100.
[97] del Hoyo P, García-Redondo A, de Bustos F, Molina JA, Sayed Y, Alonso-Navarro H, *et al*. Oxidative stress in skin fibroblasts cultures from patients with Parkinson's disease. BMC Neurology. 2010; 10: 95.
[98] Yoshino H, Nakagawa-Hattori Y, Kondo T, Mizuno Y. Mitochondrial complex I and II activities of lymphocytes and platelets in Parkinson's disease. Journal of Neural Transmission. Parkinson's Disease and Dementia Section. 1992; 4: 27–34.
[99] Bindoff LA, Birch-Machin MA, Cartlidge NE, Parker WD, Jr, Turnbull DM. Respiratory chain abnormalities in skeletal muscle from patients with Parkinson's disease. Journal of the Neurological Sciences. 1991; 104: 203–208.
[100] Chen CM, Liu JL, Wu YR, Chen YC, Cheng HS, Cheng ML, *et al*. Increased oxidative damage in peripheral blood correlates with severity of Parkinson's disease. Neurobiology of Disease. 2009; 33: 429–435.
[101] Baumuratov AS, Antony PMA, Ostaszewski M, He F, Salamanca L, Antunes L, *et al*. Enteric neurons from Parkinson's disease patients display *ex vivo* aberrations in mitochondrial structure. Scientific Reports. 2016; 6: 33117.
[102] Braidy N, Gai WP, Xu YH, Sachdev P, Guillemin GJ, Jiang XM, *et al*. Alpha-synuclein transmission and mitochondrial toxicity in primary human foetal enteric neurons *in vitro*. Neurotoxicity Research. 2014; 25: 170–182.
[103] Antony PMA, Kondratyeva O, Mommaerts K, Ostaszewski M, Sokolowska K, Baumuratov AS, *et al*. Fibroblast mitochondria in idiopathic Parkinson's disease display morphological changes and enhanced resistance to depolarization. Scientific Reports. 2020; 10: 1569.
[104] Mak SK, Tewari D, Tetrud JW, Langston JW, Schüle B. Mitochondrial dysfunction in skin fibroblasts from a Parkinson's disease patient with an alpha-synuclein triplication. Journal of Parkinson's Disease. 2011; 1: 175–183.
[105] Nixon RA. The role of autophagy in neurodegenerative disease. Nature Medicine. 2013; 19: 983–997.
[106] Lehtonen Š, Sonninen TM, Wojciechowski S, Goldsteins G, Koistinaho J. Dysfunction of Cellular Proteostasis in Parkinson's Disease. Frontiers in Neuroscience. 2019; 13: 457.
[107] Guo F, Liu X, Cai H, Le W. Autophagy in neurodegenerative diseases: pathogenesis and therapy. Brain Pathology (Zurich, Switzerland). 2018; 28: 3–13.
[108] Arotcarena ML, Teil M, Dehay B. Autophagy in Synucleinopathy: The Overwhelmed and Defective Machinery. Cells. 2019; 8: 565.
[109] Bi M, Du X, Jiao Q, Chen X, Jiang H. Expanding the role of proteasome homeostasis in Parkinson's disease: beyond protein breakdown. Cell Death & Disease. 2021; 12: 154.
[110] Spencer B, Potkar R, Trejo M, Rockenstein E, Patrick C, Gindi R, *et al*. Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 2009; 29: 13578–13588.
[111] Lopes da Fonseca T, Villar-Piqué A, Outeiro TF. The Interplay between Alpha-Synuclein Clearance and Spreading. Biomolecules. 2015; 5: 435–471.
[112] Anglade P, Vyas S, Javoy-Agid F, Herrero MT, Michel PP, Marquez J, *et al*. Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. Histology and Histopathology. 1997; 12: 25–31.
[113] Dehay B, Bové J, Rodríguez-Muela N, Perier C, Recasens A, Boya P, *et al*. Pathogenic lysosomal depletion in Parkinson's disease. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 2010; 30: 12535–12544.
[114] Miki Y, Tanji K, Mori F, Utsumi J, Sasaki H, Kakita A, *et al*. Alteration of Upstream Autophagy-Related Proteins (ULK1, ULK2, Beclin1, VPS34 and AMBRA1) in Lewy Body Disease. Brain Pathology (Zurich, Switzerland). 2016; 26: 359–370.
[115] Mahul-Mellier AL, Burtscher J, Maharjan N, Weerens L, Croisier M, Kuttler F, *et al*. The process of Lewy body formation, rather than simply α-synuclein fibrillization, is one of the major drivers of neurodegeneration. Proceedings of the National Academy of Sciences of the United States of America. 2020; 117: 4971–4982.
[116] Alvarez-Erviti L, Rodriguez-Oroz MC, Cooper JM, Caballero C, Ferrer I, Obeso JA, *et al*. Chaperone-mediated autophagy markers in Parkinson disease brains. Archives of Neurology. 2010; 67: 1464–1472.
[117] Murphy KE, Gysbers AM, Abbott SK, Spiro AS, Furuta A, Cooper A, *et al*. Lysosomal-associated membrane protein 2 isoforms are differentially affected in early Parkinson's disease. Movement Disorders: Official Journal of the Movement Disorder Society. 2015; 30: 1639–1647.
[118] Higashi S, Moore DJ, Minegishi M, Kasanuki K, Fujishiro H, Kabuta T, *et al*. Localization of MAP1-LC3 in vulnerable neurons and Lewy bodies in brains of patients with dementia with Lewy bodies. Journal of Neuropathology and Experimental Neurology. 2011; 70: 264–280.
[119] Crews L, Spencer B, Desplats P, Patrick C, Paulino A, Rockenstein E, *et al*. Selective molecular alterations in the autophagy pathway in patients with Lewy body disease and in models of alpha-synucleinopathy. PLoS ONE. 2010; 5: e9313.
[120] Tanji K, Odagiri S, Maruyama A, Mori F, Kakita A, Takahashi H, *et al*. Alteration of autophagosomal proteins in the brain of multiple system atrophy. Neurobiology of Disease. 2013; 49: 190–198.
[121] Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell. 2003; 115: 727–738.
[122] Mazzetti S, De Leonardis M, Gagliardi G, Calogero AM, Basellini MJ, Madaschi L, *et al*. Phospho-HDAC6 Gathers Into Protein Aggregates in Parkinson's Disease and Atypical Parkinsonisms. Frontiers in Neuroscience. 2020; 14: 624.

[123] Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science. 2004; 305: 1292–1295.
[124] Xilouri M, Vogiatzi T, Vekrellis K, Park D, Stefanis L. Abberant alpha-synuclein confers toxicity to neurons in part through inhibition of chaperone-mediated autophagy. PLoS ONE. 2009; 4: e5515.
[125] Vogiatzi T, Xilouri M, Vekrellis K, Stefanis L. Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. The Journal of Biological Chemistry. 2008; 283: 23542–23556.
[126] Fares MB, Jagannath S, Lashuel HA. Reverse engineering Lewy bodies: how far have we come and how far can we go? Nature Reviews. Neuroscience. 2021; 22: 111–131.
[127] Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. Alpha-Synuclein is degraded by both autophagy and the proteasome. The Journal of Biological Chemistry. 2003; 278: 25009–25013.
[128] Wie J, Liu Z, Song H, Tropea TF, Yang L, Wang H, *et al*. A growth-factor-activated lysosomal K^+ channel regulates Parkinson's pathology. Nature. 2021; 591: 431–437.
[129] Blandini F, Sinforiani E, Pacchetti C, Samuele A, Bazzini E, Zangaglia R, *et al*. Peripheral proteasome and caspase activity in Parkinson disease and Alzheimer disease. Neurology. 2006; 66: 529–534.
[130] Prigione A, Piazza F, Brighina L, Begni B, Galbussera A, Difrancesco JC, *et al*. Alpha-synuclein nitration and autophagy response are induced in peripheral blood cells from patients with Parkinson disease. Neuroscience Letters. 2010; 477: 6–10.
[131] Wu G, Wang X, Feng X, Zhang A, Li J, Gu K, *et al*. Altered expression of autophagic genes in the peripheral leukocytes of patients with sporadic Parkinson's disease. Brain Research. 2011; 1394: 105–111.
[132] Sala G, Arosio A, Stefanoni G, Melchionda L, Riva C, Marinig D, *et al*. Rotenone upregulates alpha-synuclein and myocyte enhancer factor 2D independently from lysosomal degradation inhibition. BioMed Research International. 2013; 2013: 846725.
[133] Papagiannakis N, Xilouri M, Koros C, Stamelou M, Antonelou R, Maniati M, *et al*. Lysosomal alterations in peripheral blood mononuclear cells of Parkinson's disease patients. Movement Disorders: Official Journal of the Movement Disorder Society. 2015; 30: 1830–1834.
[134] Vavilova JD, Boyko AA, Troyanova NI, Ponomareva NV, Fokin VF, Fedotova EY, *et al*. Alterations in Proteostasis System Components in Peripheral Blood Mononuclear Cells in Parkinson Disease: Focusing on the HSP70 and p62 Levels. Biomolecules. 2022; 12: 493.
[135] Miki Y, Shimoyama S, Kon T, Ueno T, Hayakari R, Tanji K, *et al*. Alteration of autophagy-related proteins in peripheral blood mononuclear cells of patients with Parkinson's disease. Neurobiology of Aging. 2018; 63: 33–43.
[136] Teves JMY, Bhargava V, Kirwan KR, Corenblum MJ, Justiniano R, Wondrak GT, *et al*. Parkinson's Disease Skin Fibroblasts Display Signature Alterations in Growth, Redox Homeostasis, Mitochondrial Function, and Autophagy. Frontiers in Neuroscience. 2018; 11: 737.
[137] Cookson MR. The role of leucine-rich repeat kinase 2 (LRRK2) in Parkinson's disease. Nature Reviews. Neuroscience. 2010; 11: 791–797.
[138] Bravo-San Pedro JM, Niso-Santano M, Gómez-Sánchez R, Pizarro-Estrella E, Aiastui-Pujana A, Gorostidi A, *et al*. The LRRK2 G2019S mutant exacerbates basal autophagy through activation of the MEK/ERK pathway. Cellular and Molecular Life Sciences. 2013; 70: 121–136.
[139] Zhao Y, Keshiya S, Perera G, Schramko L, Halliday GM, Dzamko N. LRRK2 kinase inhibitors reduce alpha-synuclein in human neuronal cell lines with the G2019S mutation. Neurobiology of Disease. 2020; 144: 105049.
[140] Ho PWL, Leung CT, Liu H, Pang SYY, Lam CSC, Xian J, *et al*. Age-dependent accumulation of oligomeric SNCA/α-synuclein from impaired degradation in mutant LRRK2 knockin mouse model of Parkinson disease: role for therapeutic activation of chaperone-mediated autophagy (CMA). Autophagy. 2020; 16: 347–370.
[141] Sidransky E, Nalls MA, Aasly JO, Aharon-Peretz J, Annesi G, Barbosa ER, *et al*. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. The New England Journal of Medicine. 2009; 361: 1651–1661.
[142] Murphy KE, Gysbers AM, Abbott SK, Tayebi N, Kim WS, Sidransky E, *et al*. Reduced glucocerebrosidase is associated with increased α-synuclein in sporadic Parkinson's disease. Brain: a Journal of Neurology. 2014; 137: 834–848.
[143] Gegg ME, Burke D, Heales SJR, Cooper JM, Hardy J, Wood NW, *et al*. Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains. Annals of Neurology. 2012; 72: 455–463.
[144] Mazzulli JR, Xu YH, Sun Y, Knight AL, McLean PJ, Caldwell GA, *et al*. Gaucher disease glucocerebrosidase and α-synuclein form a bidirectional pathogenic loop in synucleinopathies. Cell. 2011; 146: 37–52.
[145] Fishbein I, Kuo YM, Giasson BI, Nussbaum RL. Augmentation of phenotype in a transgenic Parkinson mouse heterozygous for a Gaucher mutation. Brain: a Journal of Neurology. 2014; 137: 3235–3247.
[146] Lill CM. Genetics of Parkinson's disease. Molecular and Cellular Probes. 2016; 30: 386–396.
[147] Lim S, Chun Y, Lee JS, Lee SJ. Neuroinflammation in Synucleinopathies. Brain Pathology (Zurich, Switzerland). 2016; 26: 404–409.
[148] Williams GP, Marmion DJ, Schonhoff AM, Jurkuvenaite A, Won WJ, Standaert DG, *et al*. T cell infiltration in both human multiple system atrophy and a novel mouse model of the disease. Acta Neuropathologica. 2020; 139: 855–874.
[149] De Virgilio A, Greco A, Fabbrini G, Inghilleri M, Rizzo MI, Gallo A, *et al*. Parkinson's disease: Autoimmunity and neuroinflammation. Autoimmunity Reviews. 2016; 15: 1005–1011.
[150] Liu TW, Chen CM, Chang KH. Biomarker of Neuroinflammation in Parkinson's Disease. International Journal of Molecular Sciences. 2022; 23: 4148.
[151] Lema Tomé CM, Tyson T, Rey NL, Grathwohl S, Britschgi M, Brundin P. Inflammation and α-synuclein's prion-like behavior in Parkinson's disease–is there a link? Molecular Neurobiology. 2013; 47: 561–574.
[152] Caputi V, Giron MC. Microbiome-Gut-Brain Axis and Toll-Like Receptors in Parkinson's Disease. International Journal of Molecular Sciences. 2018; 19: 1689.
[153] Houser MC, Tansey MG. The gut-brain axis: is intestinal inflammation a silent driver of Parkinson's disease pathogenesis? NPJ Parkinson's Disease. 2017; 3: 3.
[154] Barichella M, Severgnini M, Cilia R, Cassani E, Bolliri C, Caronni S, *et al*. Unraveling gut microbiota in Parkinson's disease and atypical parkinsonism. Movement Disorders: Official Journal of the Movement Disorder Society. 2019; 34: 396–405.
[155] Sharon G, Sampson TR, Geschwind DH, Mazmanian SK. The Central Nervous System and the Gut Microbiome. Cell. 2016; 167: 915–932.
[156] Jiang C, Li G, Huang P, Liu Z, Zhao B. The Gut Microbiota and Alzheimer's Disease. Journal of Alzheimer's Disease: JAD. 2017; 58: 1–15.
[157] Skorvanek M, Bhatia KP. The Skin and Parkinson's Disease: Review of Clinical, Diagnostic, and Therapeutic Issues. Movement Disorders Clinical Practice. 2016; 4: 21–31.

[158] Belkaid Y, Segre JA. Dialogue between skin microbiota and immunity. Science. 2014; 346: 954–959.
[159] Shen L. Gut, oral and nasal microbiota and Parkinson's disease. Microbial Cell Factories. 2020; 19: 50.
[160] Peralta Ramos JM, Iribarren P, Bousset L, Melki R, Baekelandt V, Van der Perren A. Peripheral Inflammation Regulates CNS Immune Surveillance Through the Recruitment of Inflammatory Monocytes Upon Systemic α-Synuclein Administration. Frontiers in Immunology. 2019; 10: 80.
[161] Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, *et al*. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell. 2016; 167: 1469–1480.e12.
[162] Kishimoto Y, Zhu W, Hosoda W, Sen JM, Mattson MP. Chronic Mild Gut Inflammation Accelerates Brain Neuropathology and Motor Dysfunction in α-Synuclein Mutant Mice. Neuromolecular Medicine. 2019; 21: 239–249.
[163] Holmqvist S, Chutna O, Bousset L, Aldrin-Kirk P, Li W, Björklund T, *et al*. Direct evidence of Parkinson pathology spread from the gastrointestinal tract to the brain in rats. Acta Neuropathologica. 2014; 128: 805–820.
[164] Sui YT, Bullock KM, Erickson MA, Zhang J, Banks WA. Alpha synuclein is transported into and out of the brain by the blood-brain barrier. Peptides. 2014; 62: 197–202.
[165] Devos D, Lebouvier T, Lardeux B, Biraud M, Rouaud T, Pouclet H, *et al*. Colonic inflammation in Parkinson's disease. Neurobiology of Disease. 2013; 50: 42–48.
[166] Stolzenberg E, Berry D, Yang D, Lee EY, Kroemer A, Kaufman S, *et al*. A Role for Neuronal Alpha-Synuclein in Gastrointestinal Immunity. Journal of Innate Immunity. 2017; 9: 456–463.
[167] Sanjari Moghaddam H, Ghazi Sherbaf F, Mojtahed Zadeh M, Ashraf-Ganjouei A, Aarabi MH. Association Between Peripheral Inflammation and DATSCAN Data of the Striatal Nuclei in Different Motor Subtypes of Parkinson Disease. Frontiers in Neurology. 2018; 9: 234.
[168] Marek K, Innis R, Van Dyck C, Fussell B, Early M, Eberly S, *et al*. [123I] β-CIT SPECT imaging assessment of the rate of Parkinson's disease progression. Neurology. 2001; 57: 2089–2094.
[169] Obeso JA, Stamelou M, Goetz CG, Poewe W, Lang AE, Weintraub D, *et al*. Past, present, and future of Parkinson's disease: A special essay on the 200th Anniversary of the Shaking Palsy. Movement Disorders: Official Journal of the Movement Disorder Society. 2017; 32: 1264–1310.
[170] Borghammer P, Just MK, Horsager J, Skjærbæk C, Raunio A, Kok EH, *et al*. A postmortem study suggests a revision of the dual-hit hypothesis of Parkinson's disease. NPJ Parkinson's Disease. 2022; 8: 166.

PART III

Table of Contents

SIDE RESEARCHS:

Published paper 2

Samanta Mazzetti †, Michela Barichella, Federica Giampietro, Angelica Giana, Alessandra Maria Calogero, Alida Amadeo, Nicola Palazzi, **Alessandro Comincini**, Giorgio Giaccone, Manuela Bramerio, Serena Caronni, Viviana Cereda, Emanuele Cereda, Graziella Cappelletti, Chiara Rolando and Gianni Pezzoli.
"A protective astrocyte subtype expresses Vitamin D-activating enzyme in Parkinson's disease." *CNS Neuroscience & Therapeutics*. **2022**; 28:703-713.

Received: 17 September 2021 | Revised: 21 December 2021 | Accepted: 26 December 2021

DOI: 10.1111/cns.13801

CNS Neuroscience & Therapeutics Open Access WILEY

ORIGINAL ARTICLE

Astrocytes expressing Vitamin D-activating enzyme identify Parkinson's disease

Samanta Mazzetti[1,2] | Michela Barichella[2,3] | Federica Giampietro[1] | Angelica Giana[1,3] | Alessandra M Calogero[1] | Alida Amadeo[1,7] | Nicola Palazzi[1] | Alessandro Comincini[1] | Giorgio Giaccone[4] | Manuela Bramerio[5] | Serena Caronni[2,3] | Viviana Cereda[2,3] | Emanuele Cereda[6] | Graziella Cappelletti[1,7] | Chiara Rolando[1] | Gianni Pezzoli[2,3]

[1]Department of Biosciences, Università degli Studi di Milano, Milan, Italy

[2]Fondazione Grigioni per il Morbo di Parkinson, Milan, Italy

[3]Parkinson Institute, ASST "G.Pini-CTO," Milan, Milan, Italy

[4]Unit of Neuropathology and Neurology, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy

[5]S. C. Divisione Oncologia Falck and S. C. Divisione Anatomia Patologica, Ospedale Niguarda Ca' Granda, Milan, Italy

[6]Clinical Nutrition and Dietetics Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

[7]Center of Excellence on Neurodegenerative Diseases, Università degli Studi di Milano, Milan, Italy

Correspondence

Samanta Mazzetti, Department of Biosciences, Università degli Studi di Milano, Fondazione Grigioni per il Morbo di Parkinson, Milan, Italy.
Email: samanta.mazzetti@gmail.com

Graziella Cappelletti, Department of Biosciences, Università degli Studi di Milano, Center of Excellence on Neurodegenerative Diseases, Università degli Studi di Milano, Milan, Italy.
Email: graziella.cappelletti@unimi.it

Abstract

Introduction: Astrocytes are involved in Parkinson's disease (PD) where they could contribute to α-Synuclein pathology but also to neuroprotection via α-Synuclein clearance. The molecular signature underlying their dual role is still elusive. Given that vitamin D has been recently suggested to be protective in neurodegeneration, the aim of our study was to investigate astrocyte and neuron vitamin D pathway alterations and their correlation with α-Synuclein aggregates (ie, oligomers and fibrils) in human brain obtained from PD patients.

Methods: The expression of vitamin D pathway components CYP27B1, CYP24A1, and VDR was examined in brains obtained from PD patients (Braak stage 6; $n = 9$) and control subjects ($n = 4$). We also exploited proximity ligation assay to identified toxic α-Synuclein oligomers in human astrocytes.

Results: We found that vitamin D-activating enzyme CYP27B1 identified a subpopulation of astrocytes exclusively in PD patients. CYP27B1 positive astrocytes could display neuroprotective features as they sequester α-Synuclein oligomers and are associated with Lewy body negative neurons.

Conclusion: The presence of CYP27B1 astrocytes distinguishes PD patients and suggests their contribution to protect neurons and to ameliorate neuropathological traits.

KEYWORDS

astrocytes, CYP27B1, Parkinson's disease, Vitamin D, α-Synuclein oligomers

Samanta Mazzetti, Michela Barichella, Federica Giampietro, Graziella Cappelletti, Chiara Rolando and Gianni Pezzoli contributed equally to this work.

1 | INTRODUCTION

Astrocytes are key players in Parkinson's disease (PD).[1-3] α-Synuclein misfolding and aggregation in neurons is the key hallmark of PD that associates with neuropathological deterioration.[4] Astrocytes positively respond in PD as they can internalize and degrade α-Synuclein aggregates thus protecting neurons.[1,5]. On the contrary, the acquisition of inflammatory and altered metabolic properties in astrocytes results in detrimental effects and can potentially exacerbate the damage.[6,7] Therefore, identifying glial specific mechanisms that promote the acquisition of protective phenotype can be beneficial for treating PD.

The activation of vitamin D pathway has been linked to neuroprotection.[8] Vitamin D_3 (25-hydroxyvitamin D [25(OH)D]) is present in the serum, and it needs to be activated by 25-hydroxyvitamin D1-α-hydroxylase (CYP27B1) in order to exert its biological function.[9] CYP27B1 and 1,25-dihydroxyvitamin D receptor (VDR) are expressed by neurons and glial cells in *post-mortem* human brain indicating that the vitamin D can be locally activated.[10,11] PD patients are usually characterized by lower 25(OH)D levels than controls[12] and reduced serum 25(OH)D and polymorphisms in VDR have been associated with increased susceptibility to PD.[13] Furthermore, lower serum concentrations have been observed in patients with the more severe symptoms including impaired cognitive functions.[14-17] Interestingly, vitamin D supplementation can slow Hoehn-Yahr stage deterioration, particularly in patients carrying vitamin D receptor polymorphisms.[13] In addition, vitamin D supplementation in PD patients has been recently suggested to play a protective role also in COVID-19 symptoms.[18]

Despite the important role of vitamin D in PD, it is unknown whether the expression of the key signaling components is altered during the disease.[14] We therefore aimed to analyze the distribution and localization of vitamin D-activating and degrading enzymes CYP27B1 and CYP24A1 (25-Hydroxyvitamin D-24-hydroxylase) and VDR in *post-mortem* human brain (Braak stage 6) of PD patients. We observed that CYP27B1 was specifically increased in an astrocyte subpopulation of PD patients, exclusively in brain regions involved in the pathology. Furthermore, we investigated the involvement of CYP27B1 positive astrocytes in α-Synuclein pathology and we correlated their expression with α-Synuclein oligomers or Lewy bodies. These data all together could provide novel insights into vitamin D involvement in human astrocyte response during PD.

2 | MATERIALS AND METHODS

2.1 | Patients

The clinical diagnosis of PD was performed using the UK Brain Bank criteria[19,20] and confirmed by neuropathological analysis carried out by two experts (GG and MB) in agreement with the current BrainNet Europe Consortium guidelines.[21]

Post-mortem human brains obtained from PD patients ($n = 9$; eight Braak stage 6 of α-Synuclein pathology; one amigdala prevalence) and from control subjects ($n = 4$) clinically free from neurological diseases were used (Table 1; Nervous Tissues Bank of Milan). Written informed consent was obtained from all subjects in compliance with relevant

TABLE 1 Demographic and clinical characteristics of the subjects included in the present study. All the patients were enrolled and followed during their disease by neurologists experienced in movement disorders at the Parkinson's Institute ASST G. Pini-CTO of Milan

Group	Gender	Age at onset (years)	Age at death (years)	Disease duration (years)	Hoehn-Yahr stage[a]	Dementia[b]
CTRL	M	/	71	/	/	No
CTRL	F	/	93	/	/	No
CTRL	F	/	82	/	/	No
CTRL	F	/	64	/	/	No
PD	M	62	80	18	4	No
PD	M	62	73	11	3	No
PD	M	59	87	28	3	No
PD	M	57	71	14	5	Yes
PD	M	57	75	18	3	No
PD	F	53	91	38	5	Yes
PD	F	59	79	20	4	No
PD	F	55	79	24	4	No
PD	F	43	72	29	5	Yes

Note: Abbreviations: CTRL, Control; F, female; M, male; PD, Parkinson's disease.

[a]As assessed 1 year before death;

[b]As defined by UPDRS-Part I, item 1 score ≥ 2 and also mini-mental state examination (MMSE) if necessary.

laws and institutional guidelines and approved by the appropriate institutional committees.

Brains were fixed in 10% buffered formalin for at least 1 month at 20°C. After dehydration and clearing steps, selected areas were paraffin embedded and 5 μm thick frontal cortex, mesencephalic sections, containing *substantia nigra*, pons, and bulb were cut and processed for the following analysis.

2.2 | Reagents

Primary and secondary antibodies and kit are summarized in Table 2.

2.3 | Immunohistochemistry

Antigen retrieval (80% formic acid for 20 min at room temperature, RT) was necessary only for VDR staining. Samples were incubated for 20 min with 3% H_2O_2 followed by 1% BSA diluted in 0.01 M phosphate saline buffer (PBS) containing 0.1% Triton X-100 (PBS-T). Primary antibody (anti-VDR, anti-CYP27B1, anti-Glial fibrillary acidic protein, GFAP) in 1% BSA diluted in PBS-T was incubated overnight at RT. Antigen-antibody bound was visualized using goat anti-mouse (for VDR) or anti-rabbit (for CYP27B1 and GFAP) secondary antibody (1 h, RT) and with 3,3'-Diaminobenzidine as chromogen (DAB kit).

TABLE 2 Primary and secondary antibodies and kits used in this study

Antigen	Code	Host	Dilution
Primary antibodies			
25-hydroxyvitamin D1-α-hydroxylase (CYP27B1)	ABN182 Merck	Rabbit	1:50 (IHC) 1:1500 (IF)
25-Hydroxyvitamin D – 24-hydroxylase (CYP24A1)	HPA022261 Merck	Rabbit	1:100
Apolipoprotein E (ApoE)	AB947 Merck	Goat	1:300
C3D Complement (C3)	A0063 Dako	Rabbit	1:100
Glial Fibrillary Acidic Protein (GFAP)	Z0334 Dako	Rabbit	1:300
Lysosome-associate Membrane Protein 1 (LAMP1)	L1418 Sigma	Rabbit	1:100
S100β	Z0311 Dako	Rabbit	1:250
S100β	287006 Synaptic Systems	Chicken	1:1000
Tyrosine Hydroxylase (TH)	PA – 18372 Thermo Fisher	Goat	1:200
Vitamin D Receptor (VDR)	Sc – 13133 clone D – 6 Santa Cruz	Mouse	1:50
α-Synuclein	Ab27766 clone LB509 Abcam	Mouse	1:500
α-Synuclein	S3062 Merck	Rabbit	1:2000
Fluorochrome/Enzyme Antibody	**Code**	**Host**	**Dilution**
Secondary antibodies			
Alexa Fluor® 488 anti-rabbit	A21206 Thermo Fisher	Donkey	1:200
Alexa Fluor® 568 anti-goat	A11057 Thermo Fisher	Donkey	1:200
Alexa Fluor® 647 anti-rabbit	A32795 Thermo Fisher	Donkey	1:200
Alexa Fluor® 647 anti-goat	Jackson ImmunoResearch	Donkey	1:400
Cy3 anti-chicken	Jackson ImmunoResearch	Donkey	1:400
HRP anti-rabbit	Jackson ImmunoResearch	Donkey	1:5000
EnVision System-HRP Labeled polymer anti-mouse	K4001 Dako Omnis	Goat	1:1
EnVision System-HRP Labeled polymer anti-rabbit	K4003 Dako Omnis	Goat	1:1
ImmPRESS™-AP anti- rabbit	MP – 5401Vector	Horse	1:1
Commercial assay			
Duolink® in situ probe marker MINUS	DUO920101KT Merck	-	*
Duolink® in situ probe marker PLUS	DUO920091KT Merck	-	*
Duolink® In Situ Detection Reagents Red	DUO92008 Merck	-	*
EnVision FLEX DAB + SubstrateChromogen System	K3468 Dako	-	*
EnVision™ Flex Target retrieval solution high pH	K8004 Dako	-	*
Fast Blue B salt	F3378-1G Merck	-	1 mg/ml
Hoescht 33342	62249 Thermo Fisher	-	1:5000
TSA® Plus Fluoresceine	NEL741001KT Akoya Biosciences	-	1:50

*Used as indicated by the manufacture instruction.

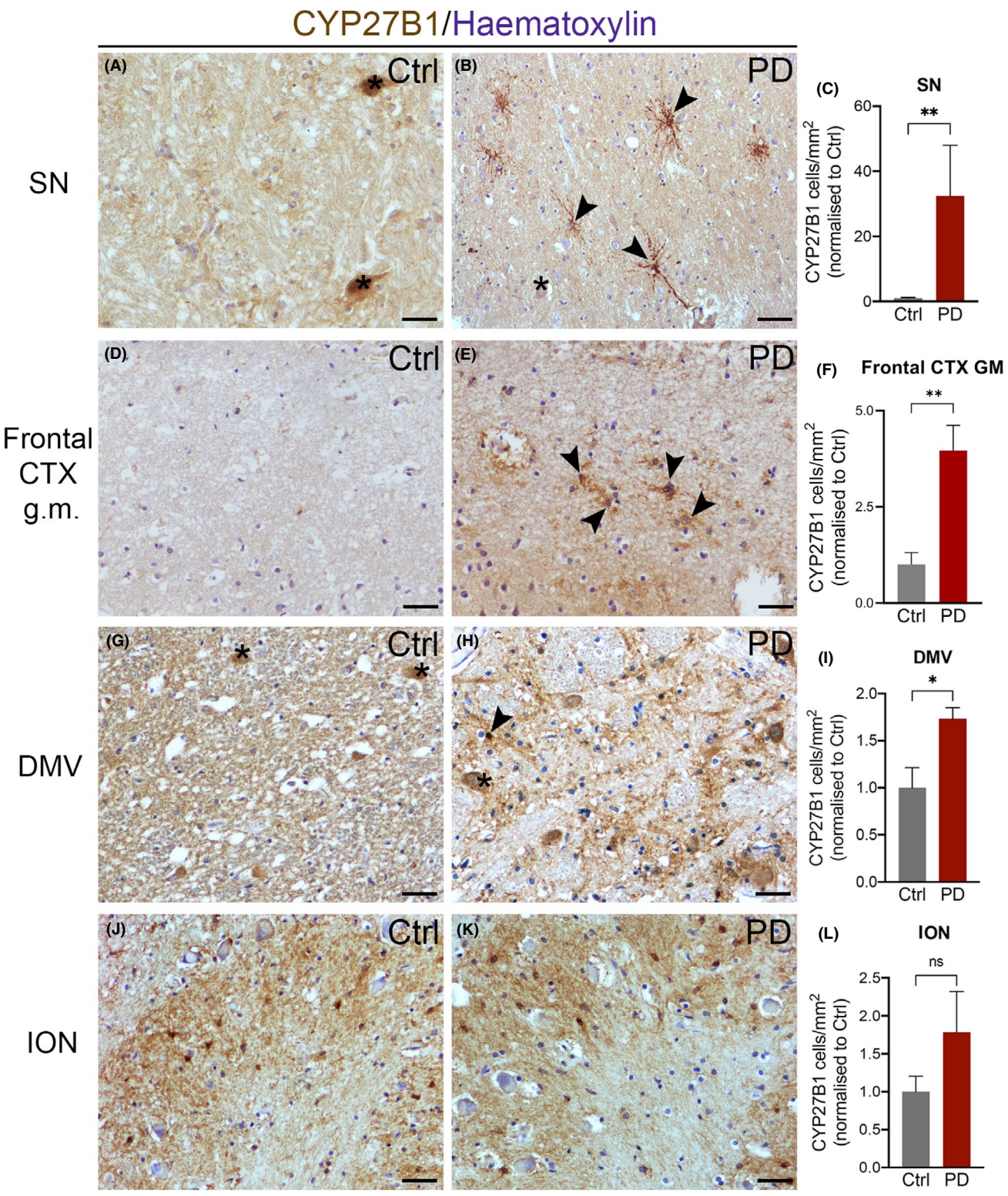

FIGURE 1 CYP27B1 distribution in *post-mortem* human brain. In *substantia nigra* (SN; A and B) and in dorsal motor nucleus of vagus (DMV; G and H), CYP27B1 staining is mainly localized in neuronal cell bodies and in neuropil of control subjects (Ctrl; A and G), while in PD patients, CYP27B1 intense staining is visible in astrocytes with a morphology resembling varicose projection astrocytes (PD; B and H; black arrowheads). In layer I of frontal cortex gray matter (Frontal CTX g.m., D and E), a low staining is detectable in controls (D), while in PD patients CYP27B1 astrocytes are well visible (E). In inferior olivary nucleus (ION; J and K), CYP27B1 staining is present in cell bodies of astrocytes in both control and PD samples. Nuclei are stained with hematoxylin. Black asterisks: dopaminergic neurons containing neuromelanin. Scale bar, 40 μm. Graphs show the density of CYP27B1 positive astrocytes in controls and PD patients in SN (C, 22 *vs* 694), Frontal Cortex GM (F, 245 *vs* 2307), DMV (I, 428 *vs* 793), and ION (L, 374 *vs* 461). Data in graphs are reported as mean ± standard error; Mann-Whitney test (* $p < 0.05$, ** $p < 0.01$)

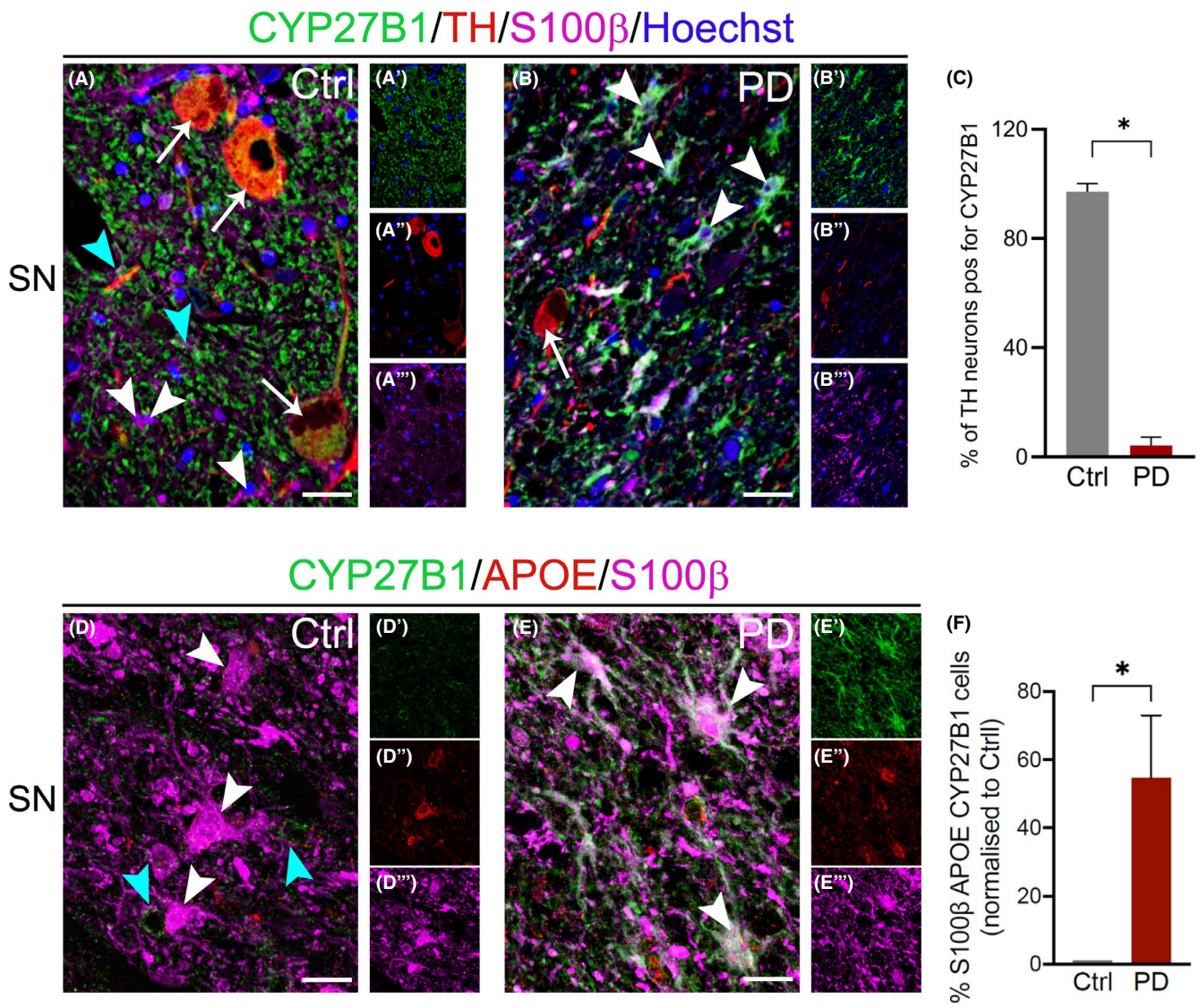

FIGURE 2 Confocal analysis of CYP27B1 positive cells in *substantia nigra* of control subjects and PD patients. CYP27B1 staining is intense in TH positive neurons (A, B; white arrows) in controls (A-A'') but not in PD (B-B''). On the contrary, in S100β positive astrocytes (A, B; white arrowheads), CYP27B1 staining is intense in PD (B, B', B'''), while it is restricted to astrocyte processes (A, A', A'''; cyan arrowheads) in controls. Nuclei are counterstained with Hoechst. Scale bar, 30 μm. Graph shows the percentage of TH neurons positive for CYP27B1 (113 neurons analyzed for controls, $n = 3$, and 214 for PD, $n = 6$). CYP27B1 staining is intense and diffuse in cell bodies and processes of ApoE and S100β positive astrocytes (white arrowheads) in PD samples (E-E'''), while it is visible in astrocytic end-feet of controls (D-D'''; cyan arrowheads). Scale bar, 15 μm. Graph (F) shows the percentage of astrocytes positive for S100β, APOE, and CYP27B1 (137 astrocytes analyzed for controls, $n = 3$, and 118 for PD, $n = 3$). Data in graphs (C, F) are reported as mean ± standard error; Mann-Whitney test (* $p < 0.05$)

In order to associate Lewy body pathology to CYP27B1 staining, we performed a double immunoenzymatic procedure using anti α-Synuclein (1:500, LB509). The samples were also pretreated with 20% acetic acid for 20 min to inactivate endogenous alkaline phosphatase. The secondary antibodies used were goat anti-mouse conjugated with HRP and rabbit conjugated with alkaline phosphatase. Then, a substrate solution of 0.1 M Tris-HCl (pH 9.2–9.4), 1-Naphthyl phosphate disodium salt (1mg/ml), and Fast Blue B salt (1mg/ml) was used to develop the CYP27B1 staining and DAB incubation to visualize α-Synuclein.

2.4 | Immunofluorescence and confocal analysis

To detect CYP27B1, we used tyramide signal amplification system. The sections were incubated with: (i) CYP27B1 (1:1500) in 1% BSA and 0.3% Triton X-100 diluted in TN (0.1 M Tris-HCl, 0.15 M NaCl) overnight at RT; (ii) HRP-conjugated secondary antibodies (donkey anti-rabbit, 1:5000) in TN for 2 h at RT; (iii) FITC-labeled tyramide (1:50) in Amplification Diluent for 2 min at RT. Classical immunofluorescence was performed with different primary antibodies (anti-GFAP, 1:300 or S100β 1:250, and Apolipoprotein E 1:300; or C3D Complement 1:100;

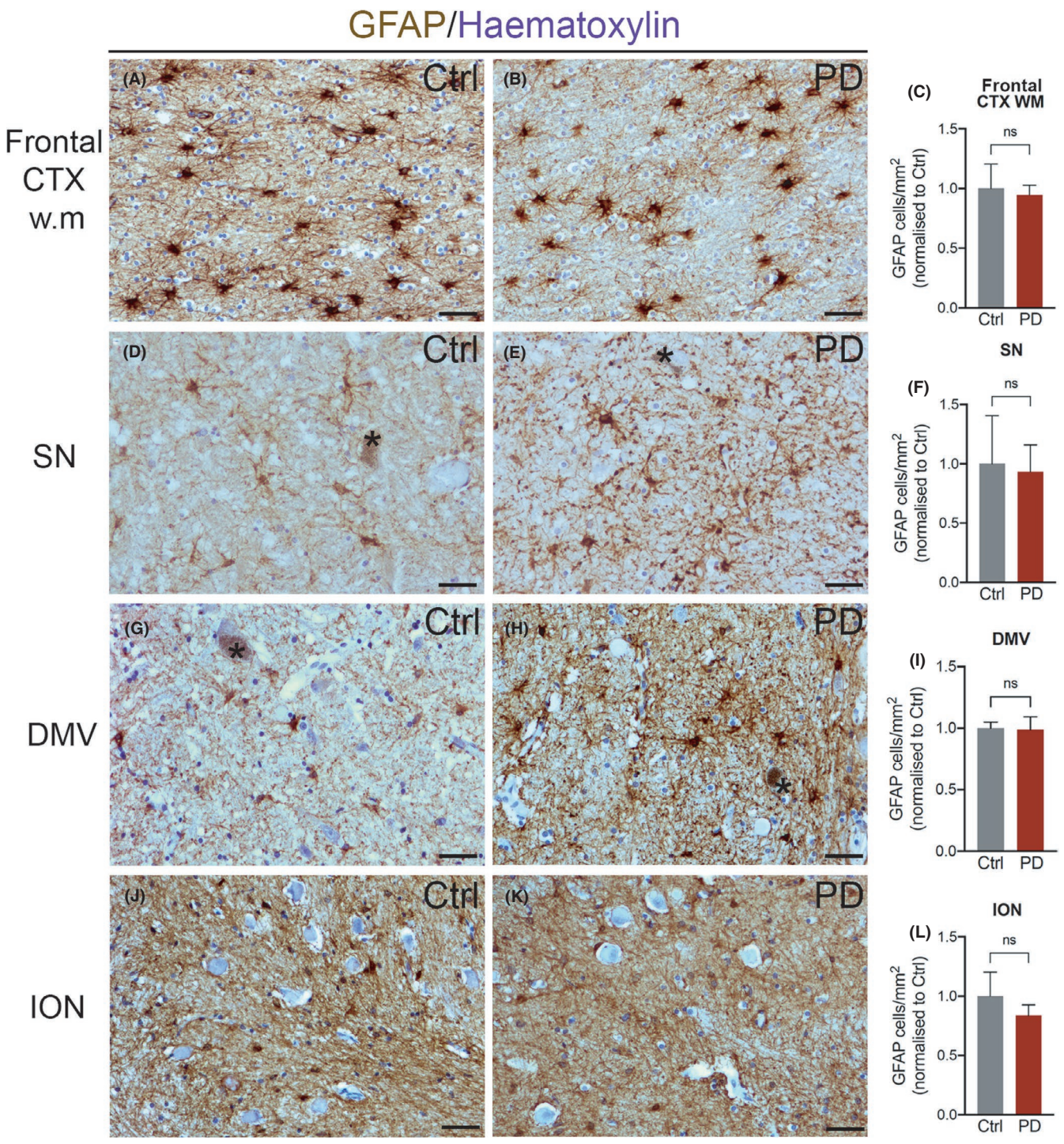

FIGURE 3 GFAP distribution in *post-mortem* human brain. In frontal cortex white matter (Frontal CTX w.m.; A and B), *substantia nigra* (SN; D and E), dorsal motor nucleus of vagus (DMV; G and H), and inferior olivary nucleus (ION; J and K), GFAP staining is localized in human astrocytes in control and PD samples. Nuclei are stained with hematoxylin. Black asterisks: dopaminergic neurons containing neuromelanin. Scale bar, 40 μm. Graphs show the density of GFAP positive astrocytes in controls and PD patients Frontal Cortex WM (C, 1527 vs 3363), SN (F, 776 vs 897), DMV (I, 850 vs 1029), and ION (L, 1281 vs 1895). Data in graphs are reported as mean ± standard error; Mann-Whitney test (n.s.)

or Tyrosine Hydroxylase 1:200; or LAMP1 1:100 overnight at RT) followed by highly pre-adsorbed secondary antibodies (2 h). A control was performed using CYP27B1 diluted 1:1500 revealed with Alexa Fluor 488 donkey anti-rabbit and gave no signal. Proximity ligation assay was used for α-Synuclein oligomers using red amplification reagent, as previously described.[22] In order to visualize total α-Synuclein, Alexa Fluor 647 donkey anti-rabbit (1:200) was added during polymerase step. Hoescht 33342 (1:5000 for 10 min) was used for nuclei

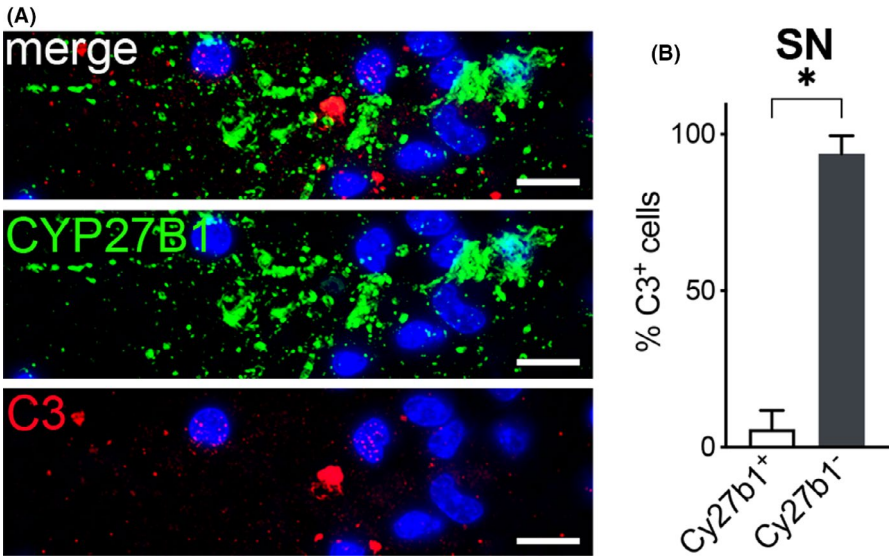

FIGURE 4 CYP27B1 positive astrocytes do not display neurotoxic signature. CYP27B1 positive astrocyte (green) and C3 (red) staining in PD samples (A). Nuclei are counterstained with Hoechst (blue). Scale bar, 10 μm. Graph (B) shows the percentage of C3 cells containing CYP27B1 (200 cells analyzed, $n = 4$ patients). Data in graph are reported as mean ± standard error; Mann-Whitney test (* $p < 0.05$)

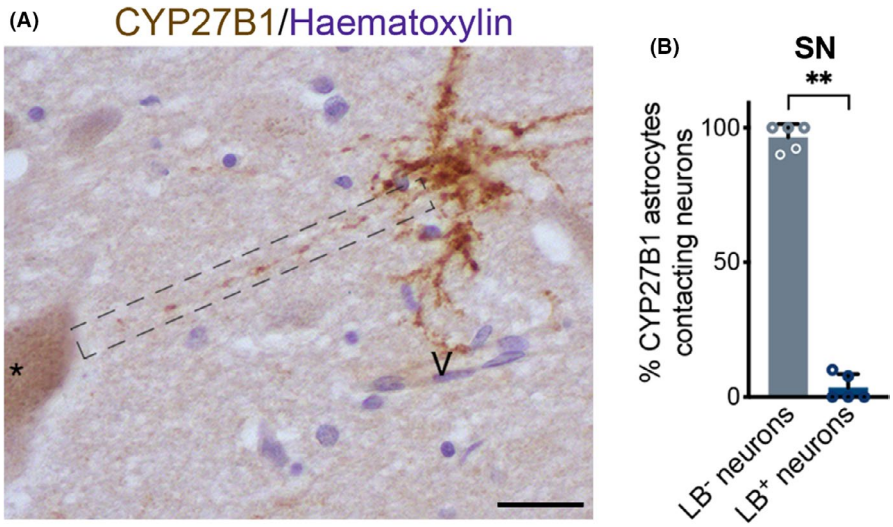

FIGURE 5 Correlation between CYP27B1 astrocytes and Lewy body pathology. CYP27B1 positive astrocyte characterized by a long varicose process (dashed rectangle) contacting a dopaminergic neuron containing neuromelanin (black asterisk) and a blood vessel (V) in PD sample (A). Nuclei are counterstained with hematoxylin. Scale bar, 30 μm. Graph (B) shows that most neurons contacted by CYP27B1 positive astrocytes do not contain Lewy bodies (530 neurons analyzed, n = 5 patients). Data in graph are reported as mean ± standard error; Mann-Whitney test (** $p < 0.01$)

counterstaining. Samples were mounted using Mowiol-DABCO and examined with Nikon spinning disk confocal microscope, equipped with CSI–W1 confocal scanner unit.

2.5 | Quantification and statistical analysis

All quantifications were independently performed using ImageJ (cell counter) by two different operators (FG, SM, or CR). Statistical comparisons were conducted by Mann-Whitney and one-way ANOVA tests. Statistical significance was assessed using GraphPad Prism software. Significance was established at $p < 0.05$.

2.6 | Data availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

3 | RESULTS

3.1 | Vitamin D-activating enzyme is overexpressed in astrocytes of PD patients

We tested whether vitamin D signaling components are altered in PD. In *substantia nigra*, VDR localized in the nucleus and cytoplasm of neuromelanin containing neurons with no differences between controls and PD patients (Figure S1 A-C). Besides, vitamin D degrading enzyme CYP24A1 was not altered in PD and was mainly expressed in neurons as in controls (Figure S1 D-E″).[23]

In *substantia nigra*, CYP27B1 was mainly present in dopaminergic neurons as revealed by the presence of neuromelanin (Figure 1 A) and the staining with TH (Figure 2 A-A‴). The percentage of dopaminergic neurons positive for CYP27B was significantly decreased in PD (Figure 2 B-C) compared to control subjects (Figure 2 A-A‴). This result could suggest that a less activation of vitamin D is implicated in the disease. Furthermore, in PD, CYP27B1 labeled a subpopulation of parenchymal astrocytes with long processes and varicosities

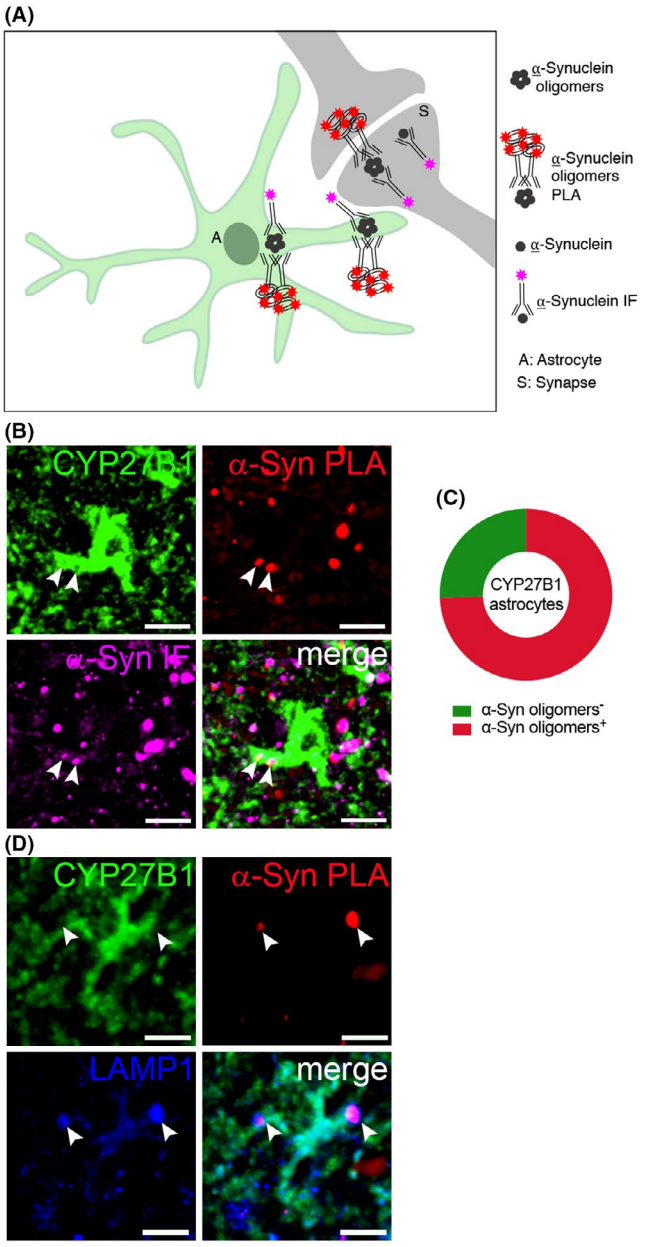

FIGURE 6 CYP27B1 astrocytes contain α-Synuclein oligomers. (A) Scheme representing the experimental procedure to visualize the relationship between α-Synuclein oligomers (proximity ligation assay, PLA) and total α-Synuclein (immunofluorescence, IF) with CYP27B1 positive astrocyte. (B) CYP27B1 positive astrocytes (green) containing total α-Synuclein staining (purple) and α-Synuclein oligomers (red) in PD samples Scale bar, 25 μm. Graph (C) shows that 74.4% of CYP27B1 positive astrocytes ($n = 43$) bears α-Synuclein oligomers. (D) CYP27B1 positive astrocytes (green) containing vesicles positive for both α-Synuclein oligomers (red) and LAMP1 (blue). Scale bar, 40 μm

that resembled the varicose projection astrocytes exclusively found in human brain (Figure 1 B,C, Figure 2 E-E‴ and Figure 5 A).[24,25] CYP27B1 positive astrocytes increased also in other areas involved in the pathology, including the frontal cortex (gray matter, Figure 1 D-F; white matter, Figure 7 A,B,D) and the dorsal motor nucleus of vagus (Figure 1 G-I), while no differences were observed in the inferior olivary nucleus that is instead spared by the disease (Figure 1 J-L). We confirmed that CYP27B1 positive cells in PD are astrocytes expressing the astrocytic markers S100β, Apolipoprotein E, and GFAP (Figure 2 D-F; Figure S2 A-B‴). Interestingly, the increase in CYP27B1 positive astrocytes was not the result of a general expansion of astrocytes in PD, as the number of GFAP positive cells was unchanged between control subjects and PD patients (Figure 3 A-L), but it could be the result of a neuroprotective response. In summary, CYP27B1 overexpressing astrocytes are enriched in PD patients.

3.2 | CYP27B1 positive astrocytes and α-Synuclein pathology

We tested whether CYP27B1 positive astrocyte accumulation is beneficial or detrimental for PD neuropathology. During PD, astrocytes can acquire a neurotoxic phenotype that exacerbate neuronal damage.[6] To determine whether CYP27B1 positive astrocytes have a neurotoxic signature, we analyzed the expression of complement component 3 (C3).[6] We found that among all C3 positive cells in PD *substantia nigra*, only 4% were CYP27B1, thus indicating that they did not acquire a neurotoxic state (Figure 4 A,B).

Next, we investigated the contribution of CYP27B1 positive astrocytes in α-Synuclein pathology. Braak stage 6 dopaminergic neurons accumulate Lewy bodies in their cytoplasm.[26,27] We examined the relationship between dopaminergic neurons and CYP27B1 positive astrocytes in the *substantia nigra* (Figure 5 A). Interestingly, the majority of CYP27B1 positive astrocytes were in direct contact with neurons that did not contain Lewy bodies (Figure 5 B). Consistently with this data, we did not observe CYP27B1 positive astrocytes in *locus coeruleus*, where almost all the neurons we analyzed contained Lewy bodies (Figure S3 A-D‴).

We therefore hypothesized that CYP27B1 positive astrocytes can be directly linked to α-Synuclein uptake and clearance. We performed proximity ligation assay to detect α-Synuclein oligomers *in vivo* and found that 74.4% of CYP27B1 positive astrocytes were able to uptake the toxic α-Synuclein aggregates (Figure 6 A-C and S4 A-B‴). Then, we used LAMP1, a marker for degradative autophagy-lysosomal organelles,[28] in order to discover whether autophagy could be the mechanism involved in α-Synuclein clearance in CYP27B1 positive astrocytes. Notably, we found that α-Synuclein oligomers that are detected inside CYP27B1 positive astrocytes co-localize with LAMP1-positive vesicles (Figure 6 D and S4 C-D‴), thus suggesting the involvement of an autophagy pathway.

Finally, we examined whether the increase in CYP27B1 positive astrocytes correlated with clinical aspects of PD patients (Table 1). We observed that CYP27B1 expression in the frontal cortex white matter was significantly increased in patients with Lewy body pathology, but without white matter alteration or dementia (Figure 7 A,B,D). Conversely, PD patients with white matter alterations and concomitant dementia had significantly fewer CYP27B1 positive astrocytes in the cortex (Figure 7 C,D), despite the total GFAP positive cells were unchanged (Figure 3 C).

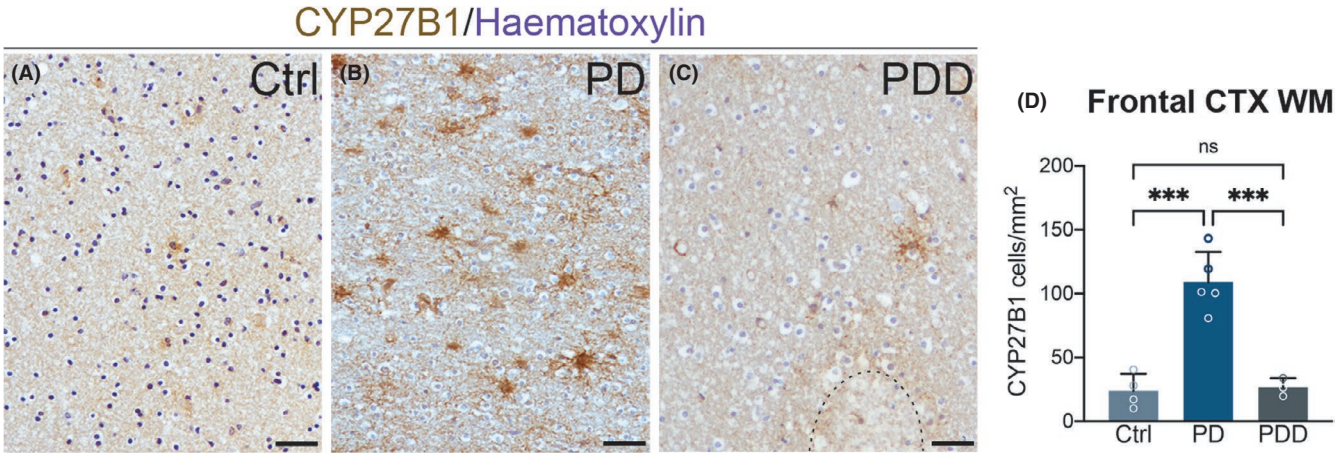

FIGURE 7 Comparison of the CYP27B1 positive astrocytes distribution in frontal cortex (white matter) between control (A), PD (B), and PD dementia (C) samples. In (C) is visible a white matter lesion (black outline). Graph (D) shows the number of CYP27B1 astrocytes normalized on area unit (807 astrocytes analyzed for controls, $n = 4$; 4594 for PD, $n = 5$, and 647 for PD dementia, $n = 3$). Nuclei counterstained with hematoxylin (blue). Scale bar, 40 μm. Data in the graph are mean ± standard error; one-way ANOVA with Tukey's multiple comparison test (*** $p < 0.001$)

4 | DISCUSSION

Our study unravels for the first time the alterations of vitamin D pathway in human brain of PD patients. While CYP24A1 and VDR did not display different expression between patients and controls, we showed that CYP27B1 increased in a subpopulation of astrocytes with neuroprotective features exclusively in brain areas involved in PD pathology (dorsal motor nucleus of vagus, *substantia nigra* and frontal cortex). Moreover, we found that CYP27B1 positive astrocytes are involved in an autophagy mediated-α-Synuclein uptake. These findings suggest an unprecedented role for CYP27B1 positive astrocytes in the pathology of PD.

Reactive astrocytes heavily contribute to neurodegeneration, and recent evidence suggests that neurotoxic astrocytes accumulate also in the *substantia nigra* of PD patients.[6] The exact mechanism that drives astrocytes into detrimental alterations (eg, the secretion of neurotoxins) is unknown. Vitamin D prevents these astrocytes alterations in PD mouse model,[29] while vitamin D signaling is unknown in PD patients. We found that activation of vitamin D pathway might be a novel mechanism to prevent the neurotoxic switch of astrocytes. Indeed, CYP27B1 positive astrocytes did not upregulate C3, thus protecting neurons from the complement component-driven synapse degeneration.

Recent evidence suggests that astrocytes participate in α-Synuclein oligomer clearance *in vitro* in primary cultures and patient-derived iPSC model.[2,5,30] Starting from these previous papers on cellular model, showing that α-Synuclein fibrils co-localized with LAMP1, a marker for degradative autophagy-lysosomal organelles,[28] we looked for the involvement of the autophagy pathway in α-Synuclein clearance occurring in our samples. The strategy of staining with LAMP1 allowed us to highlight the involvement of autophagy in α-Synuclein clearance that occurs in the complexity and uniqueness of the human brain. Hence, we show for the first time that an astrocyte subpopulation that is positive for CYP27B1 can internalize α-Synuclein oligomers through autophagy in PD patients. Interestingly, these cells morphologically resemble varicose projection astrocytes that are known to be fast conduit in neuron-vascular unit and are characterized by high speed calcium waves.[25] In addition, the overexpression of wild-type or familial mutant α-Synuclein triggers calcium homeostasis alterations.[31] Given the key role of vitamin D in calcium buffering,[32] we speculate that the increase of CYP27B1 in PD astrocytes could be a positive strategy to counteract the calcium alterations. In line with that, CYP27B1 positive astrocytes are exclusively in contact with dopaminergic neurons without Lewy bodies suggesting their role in α-Synuclein oligomers clearance and osmoregulatory function.

Vitamin D deficiency may lead to cognitive impairments.[14-17] Vitamin D supplementation can be beneficial in slowing down PD progression, but the underlying mechanisms are still unknown.[13,33] We found that the brains of PD patients without dementia have a threefold higher content of CYP27B1 positive astrocytes in the frontal cortex and do not have white matter degeneration, thus suggesting that vitamin D could exert its neuroprotective role through astrocytes.[11] Although it would be appropriate to assess serum 25(OH)D and start supplementation to prevent PD or to slow its progression, the exact mechanisms underlying aberrant vitamin D pathways still need to be established. Considering that PD patients are usually characterized by low serum 25(OH)D,[12] data in the different study groups presented low heterogeneity and VDR and CYP24A1 are similarly expressed in brains of PD patients and controls, a noteworthy question raised by our findings is how CYP27B1 can prevent neurotoxic alterations and promote α-Synuclein uptake by astrocytes? A limitation of our study is that we could not assess 25(OH)D levels and genetic status of the analyzed patients, and thus, we cannot exclude the potential contribution neither of concomitant vitamin D deficiency nor of polymorphisms in VDR.[13] Further studies are warranted to clarify the complex relationship between vitamin D activation in astrocytes and PD.

Finally, our neuropathological investigation links for the first time vitamin D to the clearance of α-Synuclein aggregates and demonstrates that the presence of CYP27B1 positive astrocytes distinguishes PD patients.

ACKNOWLEDGMENTS

The authors thank all patients and families for their contribution, and "Fondazione Grigioni per il Morbo di Parkinson" (Milan-Italy) for SM and FG salary and for long-lasting support to GC. Part of this work was carried out at Unitech-NOLIMITS, an advanced imaging facility established by the Università degli Studi di Milano.

CONFLICT OF INTEREST

The authors reported no conflict of interest.

AUTHOR CONTRIBUTIONS

Samanta Mazzetti, Chiara Rolando, Federica Giampietro, Michela Barichella, and Graziella Cappelletti contributed equally to the original research idea conception, study design, literature search, data collection, data analysis and interpretation, figure and table formatting, and writing of the manuscript. Angelica Giana, Alessandra M Calogero, Giorgio Giaccone, Viviana Cereda, and Emanuele Cereda contributed to data collection and analysis and assisted in the writing of the text. Alida Amadeo, Nicola Palazzi, Alessandro Comincini, Manuela Bramerio, and Serena Caronni contributed to data collection. Graziella Cappelletti and Gianni Pezzoli supervised the study and assisted in the writing of the text. All authors provided intellectual content and critical review of the manuscript.

ETHICS STATEMENT

The study procedures were in accordance with the principles outlined in the Declaration of Helsinki and approved by the Ethics Committee of University of Milan (protocol code 66/21, 15.06.2021).

DATA AVAILABILITY STATEMENT

The dataset of this research is deposited in the official computer archive of the Cappelletti's laboratory (Department of Biosciences, University of Milan), and it is available upon request.

ORCID

Samanta Mazzetti https://orcid.org/0000-0002-2944-8357
Federica Giampietro https://orcid.org/0000-0002-4000-8934
Alessandra M Calogero https://orcid.org/0000-0003-2262-4992
Alida Amadeo https://orcid.org/0000-0002-7581-8339
Nicola Palazzi https://orcid.org/0000-0001-9681-3695
Alessandro Comincini https://orcid.org/0000-0002-8690-4224
Giorgio Giaccone https://orcid.org/0000-0002-4803-0802
Viviana Cereda https://orcid.org/0000-0003-4818-0963
Emanuele Cereda https://orcid.org/0000-0002-0747-1951
Graziella Cappelletti https://orcid.org/0000-0003-0903-5392
Chiara Rolando https://orcid.org/0000-0003-2418-8346
Gianni Pezzoli https://orcid.org/0000-0003-4665-6710

REFERENCES

1. Loria F, Vargas JY, Bousset L, et al. α-Synuclein transfer between neurons and astrocytes indicates that astrocytes play a role in degradation rather than in spreading. *Acta Neuropathol*. 2017;134(5):789-808. Available from http://www.ncbi.nlm.nih.gov/pubmed/28725967
2. Miyazaki I, Asanuma M. Neuron-Astrocyte Interactions in Parkinson's Disease. *Cells*. 2020;9(12):2073–4409. Available from: https://www.mdpi.com/2073-4409/9/12/2623
3. Kam T-I, Mao X, Park H, et al. Poly(ADP-ribose) drives pathologic α-synuclein neurodegeneration in Parkinson's disease. *Science* (80-). 2018;362(6414):eaat8407. 10.1126/science.aat8407
4. Goedert M, Jakes R, Spillantini MG. The Synucleinopathies: Twenty Years On. *J Parkinsons Dis*. 2017;7(s1):S51-69. 10.3233/JPD-179005
5. Lindström V, Gustafsson G, Sanders LH, et al. Extensive uptake of α-synuclein oligomers in astrocytes results in sustained intracellular deposits and mitochondrial damage. *Mol Cell Neurosci*. 2017;82:143-156. Available from https://linkinghub.elsevier.com/retrieve/pii/S1044743116301841
6. Liddelow SA, Guttenplan KA, Clarke LE, et al. Neurotoxic reactive astrocytes are induced by activated microglia. *Nature*. 2017;541(7638):481-487. Available from http://www.nature.com/articles/nature21029
7. Yun SP, Kam T-I, Panicker N, et al. Block of A1 astrocyte conversion by microglia is neuroprotective in models of Parkinson's disease. *Nat Med*. 2018;24(7):931-938. Available from http://www.nature.com/articles/s41591-018-0051-5
8. Moretti R, Morelli ME, Caruso P. Vitamin D in Neurological Diseases: A Rationale for a Pathogenic Impact. *Int J Mol Sci*. 2018;19(8):2245. Available from: http://www.mdpi.com/1422-0067/19/8/2245
9. Goltzman D, Hendy GN, Karaplis AC, Kremer R, Miao D. Understanding Vitamin D From Mouse Knockout Models. In: *Vitamin D*. Elsevier. 2018;613-631.Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780128099650000367
10. Eyles DW, Smith S, Kinobe R, Hewison M, McGrath JJ. Distribution of the Vitamin D receptor and 1α-hydroxylase in human brain. *J Chem Neuroanat*. 2005;29(1):21-30. Available from https://linkinghub.elsevier.com/retrieve/pii/S0891061804001176
11. Cui X, Gooch H, Petty A, McGrath JJ, Eyles D. Vitamin D and the brain: Genomic and non-genomic actions. *Mol Cell Endocrinol*. 2017;453:131-143. Available from https://linkinghub.elsevier.com/retrieve/pii/S0303720717303040
12. Luo X, Ou R, Dutta R, Tian Y, Xiong H, Shang H. Association Between Serum Vitamin D Levels and Parkinson's Disease: A Systematic Review and Meta-Analysis. *Front Neurol*. 2018;9:1–10. 10.3389/fneur.2018.00909
13. Suzuki M, Yoshioka M, Hashimoto M, et al. Randomized, double-blind, placebo-controlled trial of vitamin D supplementation in Parkinson disease. *Am J Clin Nutr*. 2013;97(5):1004-1013. Available from https://academic.oup.com/ajcn/article/97/5/1004/4577000
14. Balion C, Griffith LE, Strifler L, et al. Vitamin D, cognition, and dementia: A systematic review and meta-analysis. *Neurology*. 2012;79(13):1397-1405. Available from http://www.neurology.org/cgi/doi/10.1212/WNL.0b013e31826c197f
15. Llewellyn DJ. Vitamin D and Risk of Cognitive Decline in Elderly Persons. *Arch Intern Med*. 2010;170(13):1135. 10.1001/archinternmed.2010.173
16. Peterson AL, Mancini M, Horak FB. The relationship between balance control and vitamin D in Parkinson's disease-a pilot study. *Mov Disord*. 2013;28(8):1133-1137. 10.1002/mds.25405
17. Barichella M, Cereda E, Pinelli G, et al. Muscle-targeted nutritional support for rehabilitation in patients with parkinsonian syndrome. *Neurology*. 2019;93(5):e485-e496. 10.1212/WNL.0000000000007858

18. Fasano A, Cereda E, Barichella M, et al. COVID-19 in Parkinson's Disease Patients Living in Lombardy, Italy. *Mov Disord*. 2020;35(7):1089-1093. 10.1002/mds.28176
19. Hughes AJ, Daniel SE, Kilford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: A clinico-pathological study of 100 cases. *J Neurol Neurosurg Psychiatry*. 1992;55:181-184.
20. Hughes AJ, Daniel SE, Lees AJ. Improved accuracy of clinical diagnosis of Lewy body Parkinson's disease. *Neurology*. 2001;57(8):1497-1499. 10.1212/WNL.57.8.1497
21. Alafuzoff I, Ince PG, Arzberger T, et al. Staging/typing of Lewy body related α-synuclein pathology: a study of the BrainNet Europe Consortium. *Acta Neuropathol*. 2009;28(117):635-652. 10.1007/s00401-009-0523-2
22. Mazzetti S, Basellini MJ, Ferri V, et al. α-Synuclein oligomers in skin biopsy of idiopathic and monozygotic twin patients with Parkinson's disease. *Brain*. 2020;143(3):920–931.
23. Landel V, Stephan D, Cui X, Eyles D, Feron F. Differential expression of vitamin D-associated enzymes and receptors in brain cell subtypes. *J Steroid Biochem Mol Biol*. 2018;177:129-134. Available from https://linkinghub.elsevier.com/retrieve/pii/S096007601730256X
24. Verkhratsky A, Nedergaard M. Physiology of Astroglia. *Physiol Rev*. 2018;98(1):239-389. 10.1152/physrev.00042.2016
25. Sosunov AA, Wu X, Tsankova NM, Guilfoyle E, McKhann GM, Goldman JE. Phenotypic heterogeneity and plasticity of isocortical and hippocampal astrocytes in the human brain. *J Neurosci*. 2014;34(6):2285-2298. 10.1523/JNEUROSCI.4037-13.2014
26. Braak H, De Vos RAI, Bohl J, Del Tredici K. Gastric α-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology. *Neurosci Lett*. 2006;396(1):67-72.
27. Mazzetti S, De Leonardis M, Gagliardi G, et al. Phospho-HDAC6 Gathers Into Protein Aggregates in Parkinson's Disease and Atypical Parkinsonisms. *Front Neurosci*. 2020;14:1-11. 10.3389/fnins.2020.00624/full
28. Cheng X-T, Xie Y-X, Zhou B, Huang N, Farfel-Becker T, Sheng Z-H. Revisiting LAMP1 as a marker for degradative autophagy-lysosomal organelles in the nervous system. *Autophagy*. 2018;14(8):1472–1474. 10.1080/15548627.2018.1482147
29. Calvello R, Cianciulli A, Nicolardi G, et al. Vitamin D Treatment Attenuates Neuroinflammation and Dopaminergic Neurodegeneration in an Animal Model of Parkinson's Disease, Shifting M1 to M2 Microglia Responses. *J Neuroimmune Pharmacol*. 2017;12(2):327-339. 10.1007/s11481-016-9720-7
30. di Domenico A, Carola G, Calatayud C, et al. Patient-Specific iPSC-Derived Astrocytes Contribute to Non-Cell-Autonomous Neurodegeneration in Parkinson's Disease. *Stem Cell Reports*. 2019;12:213-229.
31. Paillusson S, Gomez-Suaga P, Stoica R, et al. α-Synuclein binds to the ER–mitochondria tethering protein VAPB to disrupt Ca2+ homeostasis and mitochondrial ATP production. *Acta Neuropathol*. 2017;134(1):129-149. 10.1007/s00401-017-1704-z
32. Garcion E, Wion-Barbot N, Montero-Menei CN, Berger F, Wion D. New clues about vitamin D functions in the nervous system. *Trends Endocrinol Metab*. 2002;13(3):100-105. Available from https://linkinghub.elsevier.com/retrieve/pii/S1043276001005471
33. Hribar CA, Cobbold PH, Church FC. Potential Role of Vitamin D in the Elderly to Resist COVID-19 and to Slow Progression of Parkinson's Disease. *Brain Sci*. 2020;10(5):2076–3425. Available from: https://www.mdpi.com/2076-3425/10/5/284

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Mazzetti S, Barichella M, Giampietro F, et al. Astrocytes expressing Vitamin D-activating enzyme identify Parkinson's disease. *CNS Neurosci Ther*. 2022;28:703–713. doi:10.1111/cns.13801