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Acetylation as a major determinant to microtubule-dependent autophagy: Relevance to Alzheimer's and Parkinson disease pathology☆

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ABSTRACT

Protein post-translational modifications (PTMs) that potentiate protein aggregation have been implicated in several neurological disorders, including Alzheimer's (AD) and Parkinson's disease (PD). In fact, Tau and alpha-synuclein (ASYN) undergo several PTMs potentiating their aggregation and neurotoxicity.

Recent data posits a role for acetylation in Tau and ASYN aggregation. Herein we aimed to clarify the role of Sirtuin-2 (SIRT2) and HDAC6 tubulin deacetylases as well as p300 acetyltransferase in AD and PD neurodegeneration. We used transmitochondrial cybrids that recapitulate pathogenic alterations observed in sporadic PD and AD patient brains and ASYN and Tau cellular models.

We confirmed that Tau protein and ASYN are microtubules (MTs)-associated proteins (MAPs). Moreover, our results suggest that α-tubulin acetylation induced by SIRT2 inhibition is functionally associated with the improvement of MT dynamic determined by decreased Tau phosphorylation and by increased Tau/tubulin and ASYN/tubulin binding. Our data provide a strong evidence for a functional role of tubulin and MAPs acetylation on autophagic vesicular traffic and cargo clearance. Additionally, we showed that an accumulation of ASYN oligomers imbalance mitochondrial dynamics, which further compromise autophagy. We also demonstrated that an increase in Tau acetylation is associated with Tau phosphorylation. We found that p300, HDAC6 and SIRT2 influences Tau phosphorylation and autophagic flux in AD. In addition, we demonstrated that p300 and HDAC6 modulate Tau and Tubulin acetylation.

Overall, our data disclose the role of Tau and ASYN modifications through acetylation in AD and PD pathology, respectively. Moreover, this study indicates that MTs can be a promising therapeutic target in the field of neurodegenerative disorders in which intracellular transport is altered.

1. Introduction

Neurodegenerative diseases are a heterogeneous group of chronic, progressive disorders characterized by the gradual loss of neurons in the central nervous system, which leads to deficits in specific brain functions (memory, movement, cognition) [1]. The mechanism that drives chronic progression of AD and PD age-related neurodegenerative disorders remains elusive and effective treatments are still lacking.

Remarkably, both diseases share a common neuropathological feature such as the deposition of specific misfolded proteins [2]. In PD there is the presence of intracytoplasmic inclusions (Lewy bodies, LBs) which comprise a dense core of different proteins, being the main one ASYN [3] whereas in AD there is the presence of amyloid plaques, composed of amyloid-β (Aβ), a cleavage peptide derived from amyloid precursor protein (APP), and neurofibrillary tangles (NFTs), primarily composed of hyperphosphorylated Tau [4]. Notwithstanding, Tau deposition is found in other neurodegenerative brain diseases. The fact that Tau mutations can cause familial dementia proved that aberrant Tau itself can trigger neurodegenerative processes. The mechanisms by which Tau mutations cause neurodegeneration are still controversial but alterations in PTMs are believed to underlie the demise of affected brain cells.

Abbreviations: PTMs, Post-translational modifications; AD, Alzheimer's disease; PD, Parkinson's disease; ASYN, alpha-synuclein; SIRT2, Sirtuin-2; MTs, microtubules; MAPs, microtubule-associated proteins

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The first link established between ASYN and PD come from the disclosure that mutations in the ASYN gene lead to the disease. In fact, duplications or triplications of the ASYN locus in separate families cause autosomal-dominant PD involving altered kinetics of the aggregation of the protein [5]. The accumulation of toxic oligomeric species of ASYN may be one of the key processes for PD pathology.

Understanding the molecular basis of both diseases has proven to be a major challenge in the field of neurodegenerative diseases. Although several hypotheses have been proposed to explain the molecular mechanisms underlying AD and PD pathogenesis, a growing body of evidence has highlighted the role of MT-dependent dynamics disruption as a major contributor to etiopathogenesis. MTs are labile dynamic structures that are stabilized by Tau-tubulin interactions and importantly mediate organelle transport, cell motility, division, and morphology [6]. In fact, Tau is known to control MT stability due to its degree of phosphorylation, with an increase of phosphorylation favoring the depolymerization of MT [7]. Nevertheless, it remains uncertain whether Tau hyperphosphorylation alone is enough to induce disease since it is not always accompanied by abnormal Tau aggregation and cellular dysfunction [8]. Additionally, Alim and colleagues found that ASYN is also a tubulin associated/binding protein and that *WT* ASYN induces tubulin polymerization into MTs whereas ASYN mutants lose this potential [9]. On the other hand tubulin induces ASYN fibrillization in yeast, rat and human brain [10]. The loss of MT assembly affects intraneuronal axonal transport, namely mitochondrial anterograde transport and autophagic vesicles (AVs) retrograde transport. Remarkably, mitochondrial function and axonal transport are intimately connected, in fact mitochondria supply the energy that is required to allow molecular motors to move along microtubules and transport mitochondria, autophagic and synaptic vesicles, as well as, some proteins, such as ASYN [11]. Although the mechanism whereby ASYN accumulates in LBs is not fully understood, evidence suggests that defective axonal transport of ASYN may itself contribute to the process. Supporting the association between ASYN aggregation and MT stability there is the discovery that chemical inhibitors of SIRT2 change the characteristics of the intracytoplasmatic inclusions (LBs) and are protective against ASYN-induced cytotoxicity [12]. Indeed, it was previously described by our group that in sporadic cases of PD, mitochondrial dysfunction causes the increase of cytosolic NAD+, which induces the activation of SIRT2 [13]. SIRT2 is a cytoplasmic class III histone deacetylase that interacts with and deacetylates MTs in vitro and in vivo [14]. SIRT2 co-localizes with the MT network and deacetylates lys40 of α-tubulin [14]. The same residue of α-tubulin is also deacetylated by histone acetylase 6 (HDAC6), a class II histone deacetylase, leading to changes in cellular motility [15]. Indeed, acetylation has been associated with stable MTs, however this relationship has not been clearly proven. There are some studies which support the theory that acetylation enhances MT stability [15] whereas others have suggested that acetylation occurs only on stable MTs, but the acetylation itself does not stabilize MTs [16]. Interestingly, Tau acetylation has been classified as a new potential regulatory modification implicated in AD and other neurodegenerative disorders [17]. We previously reported in AD brain's a reduction in the levels of acetylated tubulin in both cortex and hippocampus when compared to controls [18]. Moreover, mass spectrometry analysis identified specific lysine residues, including lysine 290 within the MT-binding motif as the major sites of Tau acetylation [19]. Recent data points that abnormal acetylation of Tau could play a mechanistic role in Tau-mediated neurodegeneration by decreasing MT assembly, reducing tau solubility and increasing Tau fibrillization [19]. In addition, it was identified that HDAC6 is responsible for the deacetylation of Tau residues [20]. On the other hand, histone acetyltransferase p300 (p300) was shown to be involved in Tau acetylation [21]. Moreover, because in AD there is a good correlation between Tau pathology, synaptic dysfunction and cognitive decline [22] we can hypothesize that neurodegeneration in selective brain areas, results from the loss of MT assembly due to tubulin and Tau PTM alterations causing intracellular axonal trafficking failure, which would impair the movements, interaction and function of vital organelles, such as mitochondria. Furthermore, these MT-dependent trafficking alterations can prompt the deficient mobilization of autophagosome cargos toward the lysosome contributing to a decreased proteolytic flux through autophagy culminating in the accumulation of misfolded proteins, such as ASYN and Tau.

To study our hypothesis we used cytoplasmatic hybrids with patients platelet mtDNA that recapitulate pathogenic features observed in sporadic PD and AD brains [23,24]. Furthermore a model of tauopathy, SH-5Y5Y Tau P301L cells, which are characterized by abnormal accumulation of phosphorylated Tau [25] and ASYN overexpressing cells that mimic multiplication of the ASYN locus leading to enhanced ASYN aggregation [26] were used to model familial AD and PD respectively. To modulate Tau, ASYN and tubulin acetylation we ascertained the contribution of p300, HDAC6 and SIRT2 to phenotypic changes, using the specific inhibitors C646, Tubastatin A and AK1, respectively. Using sporadic and familial cellular models we also tackled fundamental differences in tubulin, ASYN and tau acetylation levels, and its contribution to the neurodegenerative process.

Overall, this work hopes to elucidate whether and how alterations in tubulin, Tau and ASYN acetylation levels can cause MTs impairment and disruption of the intracellular trafficking. Under this context, autophagic activity and cargo degradation through autophagy-lysosomal pathway can be affected causing the accumulation of impaired mitochondria, autophagic vesicles and protein aggregates.

2. Materials and methods

2.1. Chemicals

Ammonium chloride (NH_4Cl) was obtained from Merck KGaA (Darmstadt, Germany). Leupeptin and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) were purchased from Sigma Chemical Co (St. Louis, MO, USA). AK1 was acquired from ChemBridge Corporation (San Diego, USA). C646 was obtained from Calbiochem, Merck KGaA (Darmstadt, Germany) and Tubastatin A from BioVision, Inc. (San Francisco, CA, USA). A concentration screening was performed with MTT reduction ability test to select the final concentration used in cell lines.

2.2. Human subjects

Sporadic PD and AD patients, as well as, healthy individuals were all recruited after approval by the University of Kansas School of Medicine Institutional Review Board. Individuals in the AD group were recruited from the University of Kansas Alzheimer's Disease Center (KU ADC). Each subject was determined, based on cognitive testing and by a memory disorders subspecialist clinician, to meet criteria for sporadic AD. Individuals in the PD group were followed regularly in a tertiary referral movement disorders clinic at the Kansas University Medical Center and met criteria commonly used to diagnose PD in clinical and research settings [27]. In both groups none of the patients were believed to have alternative diagnoses, degeneration of related systems, drug-induced parkinsonism, or any other serious medical illness, including another neurodegenerative disease. The control subjects were participants of a longitudinal “normal aging/normal cognition” cohort that is characterized serially by the Brain Aging Project at the University of Kansas School of Medicine and have not been diagnosed with a neurodegenerative or pre-neurodegenerative disease condition. The age of the AD subject platelet donors was 71.5 ± 9.7 years and the age of the control subject platelet donors was 73.9 ± 7.7. The age of the PD subjects who participated in this study was 64 + 12.8 years and for the control subjects was 74.3 + 5.5 years. After providing informed consent, sporadic AD (n = 8) and the respective age-matched control subjects (n = 7), sporadic PD (n = 9) and the respective age-matched

control subjects (n = 5) underwent a 10-mL phlebotomy using tubes containing acid–citrate–dextrose, as an anticoagulant, to provide the platelets needed for cell fusions. A minimal of three control (CT) and three sporadic Parkinson's disease (sPD cells) or Alzheimer's disease (sAD cells) patient-derived cybrid cells (each derived from different individuals) were used.

2.3. Preparation of platelet mitochondria

Following provision of informed consent, 60 mL of blood was collected through venipuncture in tubes containing acid–citrate–dextrose as an anticoagulant. Mitochondria were obtained from human platelets according to previously described methods [28]. Protein content was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions for plate reader.

2.4. Generation, culture and experimental treatments of cybrid cell lines

To create the cybrid cell lines for this study, we used NT2 (Ntera2/D1) cells, a teratocarcinoma cell line with neuronal characteristics (Stratagene, La Jolla, CA) for PD cells and human neuroblastoma SH-SY5Y cells (ATCC-CRL-2266) for AD cells. These cells were depleted of endogenous mtDNA (rho0 cells) via long-term ethidium bromide exposure [29]. Rho0 cells lack intact mtDNA, do not possess a functional electron transport chain, and are auxotrophic for pyruvate and uridine [30]. Consequently, platelet mitochondria from either PD, AD or control subjects were isolated from the individual blood samples and were used to repopulate NT2 rho0 cells with mtDNA as previously described [29]. Briefly, Rho0 cells were co-incubated in Polyethylene glycol (Merck Chemicals) with platelets from the human subjects, as previously described by our group [30]. After fusion, selection is performed to remove the Rho0 cells that have not repopulated with platelet mtDNA. After selection, the resultant cybrid cells were switched to cybrid growth medium and were grown in 75 cm^2 tissue culture flasks maintained in a humidified incubator at 37 °C and 5% CO_2. AD cybrids growth medium is Dulbecco's modified Eagle's medium (DMEM) whereas PD cybrids growth medium is Optimem, both obtained from Gibco-Invitrogen (Life Technologies Ltd., UK) supplemented with 10% non-dialyzed Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin solution. Non-dialyzed FBS was obtained from Gibco-Invitrogen (Life Technologies Ltd., UK). Rho0 cell growth medium (Optimem for NT2 and DMEM for SH-SY5Y) was supplemented with 10% non-dialyzed FBS, 200 μg/mL sodium pyruvate from Sigma (St. Louis, MO, USA), 100 μg/mL uridine from Sigma (St. Louis, MO, USA) and 1% penicillin-streptomycin solution. Prior to experiments, cell lines were maintained in the cybrid growth medium. For western blotting and immunoprecipitation analysis, cybrid cell lines were seeded in petri-dishes at a density of 0.25×10^6 cells/mL. For immunocytochemistry analysis, cybrid cell lines were grown on coverslips in 12-well plates at a density of 0.1×10^6 cells/mL. After 24 h the medium was refreshed and experimental treatments were performed. AK1 was prepared in DMSO and was added for 24 h to the culture medium with a final concentration of 5 μM (PD cybrids) or of 10 μM (AD cybrids). Tubastatin A was prepared in water and was added for 24 h to the culture medium with a final concentration of 5 μM. C646 was prepared in DMSO and was added for 24 h to the culture medium with a final concentration of 2.5 μM. Where indicated, 20 mM NH4Cl and/or 100 μM leupeptin (Sigma, St. Louis, MO, USA) were added for 4 h to the culture medium. The combination of NH4Cl with leupeptin blocks all types of autophagy, as it reduces the activity of all lysosomal proteases by increasing the lysosomal lumen pH without affecting the activity of other intracellular proteolysis systems [31]. For all experimental procedures, controls were performed in the absence of those agents.

2.5. Culture and experimental treatments of ASYN SH-SY5Y cells

Stable human neuroblastoma SH-SY5Y cell lines (ATCC-CRL-2266) inducibly over-expressing ASYN were grown in 75 cm^2 tissue flasks in RPMI 1640 with 10% FBS, 2 mM L-glutamine and antibiotics like 250 μg/mL Geneticin, 50 μg/mL Hygromicin B and also the selection antibiotic 2 μg/mL doxycycline hyclate (DOX). The presence of DOX represses ASYN and the absence of this antibiotic induces ASYN expression. RPMI, Geneticin, Hygromicin and DOX were obtained from Gibco-Invitrogen (Life Technologies Ltd., UK). Cells were maintained at 37 °C in a humidified incubator under an atmosphere of 95% air and 5% CO2. For Western blotting and immunoprecipitation analysis cells were plated in 6-well plates at a density $0{,}25 \times 10^6$ cells/mL. For immunocytochemistry analysis, cells were grown on coverslips in 12-well plates at density of 0.05×10^6 cells/coverslip. After 24 h the medium was changed and experimental treatments were performed. AK1 was prepared in DMSO and was added for 24 h to the culture medium with a final concentration of 5 μM. Where indicated 20 mM NH_4Cl and/or 100 μM Leupeptin, were added for 4 h to the culture medium. FCCP was prepared in DMSO and was added for 2 h to the culture medium with a final concentration of 10 μm.

2.6. Culture and experimental treatments of Tau P301L mutant cells

SH-SY5Y human neuroblastoma cells were purchased from ATCC. SH-SY5Y cells were stably transfected as described by [25]. Briefly, SHSY5Y cells were transfected with constructs encoding P301L mutant (P) human tau (using the longest human tau isoform, htau40), under the control of the CMV promoter. SH-SY5Y untransfected cells were used as control. Cells were maintained in 75 cm^2 tissue culture flasks under a humidified atmosphere of 95% air, 5% CO2, at 37 °C. Cells were grown in DMEM, supplemented with 10% non-dialyzed FBS, 1% penicillin–streptomycin solution and 44 mM sodium bicarbonate. Cells with the P301L mutation construct were selected with the antibiotic blasticidin at a final concentration of 3 μg/mL. For Western blotting analysis cells were plated in 6-well plates at a density $0{,}25 \times 10^6$ cells/mL. After 24 h the medium was changed and experimental treatments were performed. AK1 was prepared in DMSO and was added for 24 h to the culture medium with a final concentration of 20 μM. Tubastatin A was prepared in water and was added for 24 h to the culture medium with a final concentration of 5 μM. C646 was prepared in DMSO and was added for 24 h to the culture medium with a final concentration of 2.5 μM. Where indicated 20 mM NH_4Cl and/or 100 μM leupeptin, were added for 4 h to the culture medium.

2.7. Preparation of cellular extracts

For western blot analysis the preparation of cytosolic cell extracts were carried out as followed. Individual cell lines were washed in ice-cold PBS (1×) and lysed in 1% Triton X-100 containing hypotonic lysis buffer (25 mM HEPES, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA, pH 7.5 supplemented with 2 mM DTT, 0.1 mM PMSF, and a 1:1000 dilution of a protease inhibitor cocktail from Sigma (St. Louis, MO, USA). For phosphorylated proteins, the buffer is further supplemented with 2 mM sodium orthovanadate and 50 mM of sodium fluoride. Cell suspensions were frozen three times in liquid nitrogen and centrifuged at 20,000 ×*g* for 10 min. The resulting supernatants were removed and stored at −80 °C. Protein content was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions for plate reader.

2.8. Preparation of mitochondrial and cytosolic extracts

In order to obtain pure mitochondrial and cytosolic protein extracts, protein subcellular extraction was performed in cell culture using ProteoExtract® Subcellular Proteome Extraction Kit from Calbiochem®,

according to the manufacturer's protocol. Briefly, each extraction buffer is sequentially incubated with cell pellet taking advantage of the differential solubility of certain subcellular compartments in the specific buffer. After incubation, the subcellular compartment solubilized in the respective extraction buffer is separated from cell pellet by appropriate centrifugation force. Subsequently cytosolic and mitochondrial fractions were frozen three times on liquid nitrogen. Protein content was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions for plate reader.

2.9. Immunoprecipitation assay

Cells were scraped and lysed on ice in a non-denaturing lysis buffer (20 mM Tris-HCl (pH 7.0), 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, supplemented with 0.1% SDS, 1% Triton X-100, 2 mM DTT, 0.1 mM PMSF and a 1:1000 dilution of a protease inhibitor cocktail). Cellular suspensions were centrifuged at 20,000 ×*g* for 10 min at 4 °C and whole lysates were assayed for protein concentration using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions for plate reader. 500 μg of each sample were precleared with Protein A Sepharose beads (GE Healthcare Bio-Sciences, Uppsala, Sweden) for 1 h, 4 °C, and then incubated with primary antibody, overnight at 4 °C and with nutation. The primary antibodies used were: 1:100 monoclonal anti-ASYN (211) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and 1:1000 monoclonal anti-Tau from Sigma (St. Louis, MO, USA). Protein A–Sepharose beads were then added to samples followed by 2 h incubation. The beads were spun down and washed seven times in washing buffer (1% Triton X-100, 500 mM NaCl, 2 mM EDTA, 2 mM EGTA, 20 mM Tris-HCl (pH 7.0)). The last supernatant was collected and 25 μl of 2× sample buffer was added. The samples were boiled at 95–100 °C for 5 min to denature the protein and to separate it from the protein-A beads. The boiled proteins were centrifuged at 20,000 ×*g* for 5 min at room temperature and the supernatants collected. Samples were separated by SDS–PAGE and subjected to Western blotting as aforementioned.

2.10. Western blot analysis

For western blot analysis equal amounts of protein (from 30 to 50 μg) were resuspended in 6× sample buffer (4× Tris·Cl/SDS, pH 6.8, 30% glycerol, 10% SDS, 0,6 M DTT, 0.012% bromophenol blue) and separated under reducing conditions. Phospho-Tau Thr 181, Phospho-Tau Ser396, acetylated Tau, acetylated α-tubulin, p62, OPA1, Mitofusin 1, Mitofusin 2, Phospho-Drp1 and Drp1 samples were loaded onto 10% SDS-PAGE. LC3B and Fis1 samples were loaded onto 15% SDS-PAGE. The samples from the immunoprecipitation assay were loaded onto 12% SDS-PAGE (for ASYN) and onto 10% SDS-PAGE (for Tau). After samples being resolved by electrophoresis in SDS polyacrylamide gels an transferred to PVDF membranes, non-specific binding was blocked by gently agitating the membranes in 5% non-fat milk or 5% BSA for phosphorylated proteins and 0,1% Tween in TBS for 1 h at room temperature. The blots were subsequently incubated with the respective primary antibodies overnight at 4 °C with gentle agitation: 1:1000 monoclonal anti-Tau from Sigma (St. Louis, MO, USA); 1:250 polyclonal anti-p-Tau (Thr 181) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); 1:750 polyclonal anti-p-Tau (Ser 396) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); 1:20,000 monoclonal anti-acetylated Tubulin from Sigma (St. Louis, MO, USA); 1:1000 polyclonal anti-LC3B from Cell Signaling Technology (Danvers, MA, USA); 1:1000 polyclonal anti-acetylated-lysine from Cell Signaling Technology (Danvers, MA, USA); 1:750 anti-Tau (Acetyl K280) from AnaSpec (Fremont, CA, USA); 1:1000 anti-SQSTM1/p62 from Sigma (St. Louis, MO, USA); 1:1000 polyclonal rabbit anti-phospho-Drp1 (Ser616) antibody from Cell Signaling Technology (Danvers, MA, USA); 1:750 polyclonal rabbit anti-Fis1 antibody from Imgenex (Littleton, CO, USA); 1:520 polyclonal rabbit anti-Opa1 antibody from Abcam (Cambridge, UK); 1:10,000 monoclonal anti-alpha-tubulin antibody from Sigma (St. Louis, MO, USA); 1:200 polyclonal rabbit anti-Drp1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); 1:200 polyclonal rabbit anti-Mfn1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); 1:500 monoclonal mouse anti-Mfn2 antibody from Abnova; 1:1000 polyclonal anti-acetylated-lysine from antibody from Cell Signaling Technology (Danvers, MA, USA). 1:10,000 monoclonal anti-α-tubulin from Sigma (St. Louis, MO, USA); 1:5000 monoclonal anti-β-actin from Sigma (St. Louis, MO, USA); 1:200 polyclonal rabbit anti-Tom20 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); 1:500 monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase from Millipore (Millipore, Billerica, MA, USA) were also used for loading control. Membranes were washed with TBS containing 0,1% non-fat milk and 0,1% Tween three times (each time for 10 min), and then incubated with the appropriate horseradish peroxidise-conjugated secondary antibody for 2 h at room temperature with gentle agitation. After three washes specific bands of interest were detected by developing with an alkaline phosphatase enhanced chemical fluorescence reagent (ECF from GE Healthcare). Fluorescence signals were detected using a Biorad Versa-Doc Imager, and band densities were determined using Quantity One Software.

2.11. Immunocytochemistry and confocal microscopy analysis

After incubation, cells were washed twice with PBS 1× and fixed for 30 min at room temperature using 4% paraformaldehyde. The fixed cells were washed again with PBS 1× three times and permeabilized with 0.2% Triton X-100 for 2 min. Subsequently cells were washed three times with PBS 1× and blocked with 3% BSA for 30 min. Cells were then washed again three times with PBS 1× and then incubated with primary antibody 1:100 polyclonal anti-Tom20 from Santa Cruz Biotechnology (Santa Cruz, CA., USA) or 1:100 anti-ASYN LB509 from Zymed Laboratories Inc. (South San Francisco, CA, USA) overnight and then with the appropriate secondary antibody 1:250 alexa fluor 594 or 488 from Molecular Probes (Eugene, OR, USA). After that cells were incubated with Hoescth 15 μg/μL for 5 min at RT and protected from light. Cells were then washed twice in PBS and the coverslips were immobilized on a glass slide with mounting medium DakoCytomation (Dako, Glostrup, Denmark). Images were acquired on a Zeiss LSM510 META confocal microscope (63× 1.4 NA plan-apochromat oil immersion lens) by using Zeiss LSM510 v3.2 software (Carl Zeiss Inc., Thornwood, NY, USA) and analyzed using Zeiss LSM Image Examiner.

2.12. Data analysis

All data result from the analysis of duplicates per experimental condition in at least three independent experiments and are expressed as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Differences between two data sets were evaluated by two tailed unpaired Student's *t*-test. Statistical tests between multiple data sets and conditions were carried out using a one way analysis of variance (ANOVA) with pair-wise multiple comparison procedures using the post hoc Bonferroni's test to determine statistical significance, as appropriate. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. The role of ASYN and Tau acetylation levels on MT impairment in PD

Firstly, as a control we performed immunocytochemistry against ASYN and it was evident that SHSY-5Y cell line overexpressing ASYN contained aggregated ASYN (Fig. 1A). We then evaluated α-tubulin acetylation levels, since this PTM is a marker of stable microtubules [32]. We observed that α-tubulin, under basal conditions, in the ASYN

overexpressing cells is less acetylated than in *WT* cells. Treatment with AK1 partially restored the acetylation of α-tubulin on ASYN overexpressing cells (Fig. 1B, C). As aforementioned, acetylation has been associated with stable MTs, therefore, we considered that the specific inhibition of SIRT2 could contribute to prevent MT network impairments. Moreover, ASYN overexpressing cells showed increased phospho-Tau levels relative to *WT* cells. Treatment with AK1, shows a trend to reduce the levels of phospho-Tau thr 181 (Fig. 1B, D). To complete the study on the affinity of Tau protein to α-tubulin under the treatment with AK1 we immunoprecipitated Tau and then performed a Western Blot to detect α-tubulin levels (Fig. 1E–F). Under basal conditions, ASYN overexpressing cells showed decreased interaction between Tau protein and α-tubulin when compared to *WT* cells. Treatment with AK1 increased the interaction between Tau protein and α-tubulin in both, ASYN overexpressing cells and *WT* cells (Fig. 1E–F). Tau protein is a MAP with the function of stabilizing the MT,

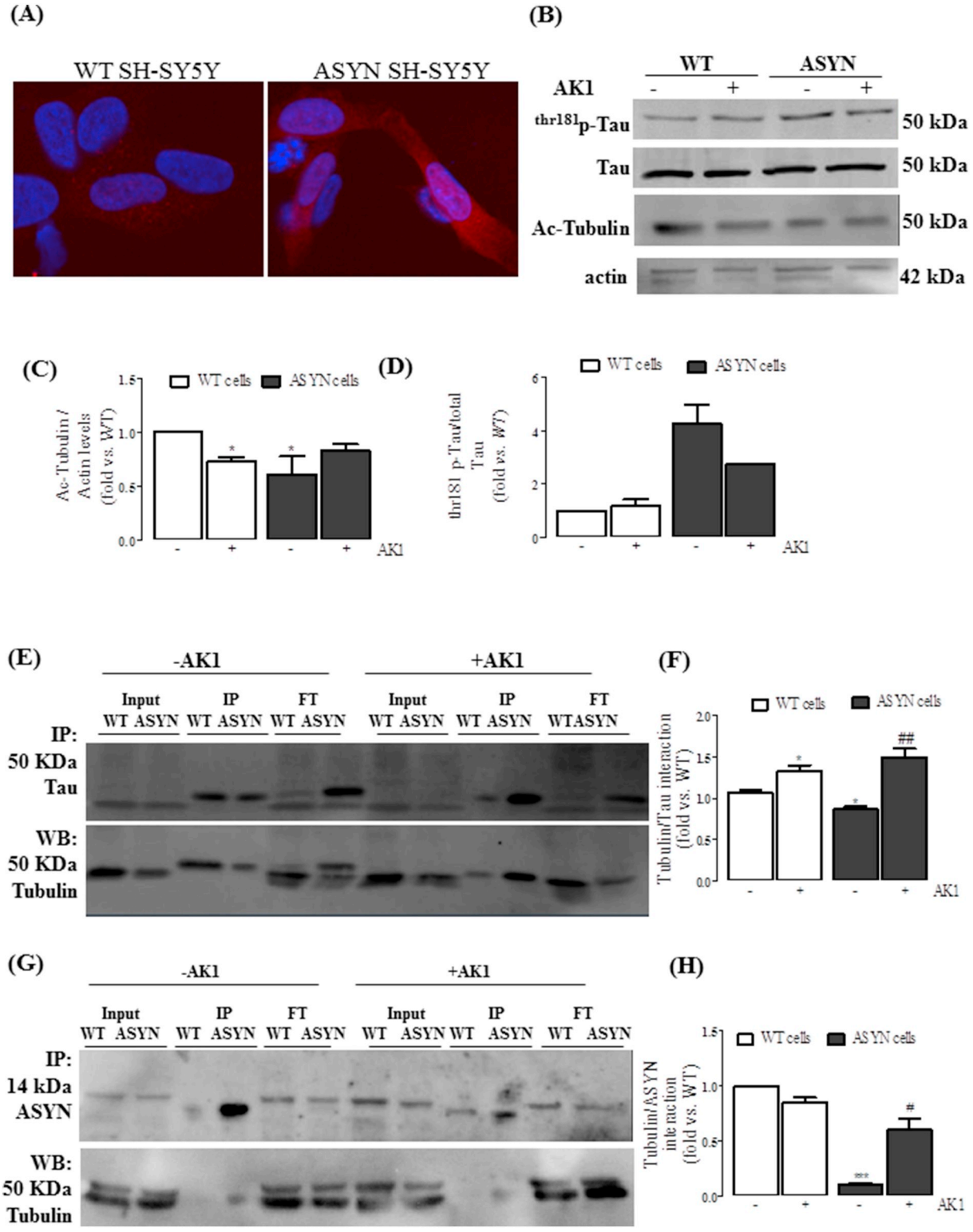

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Fig. 1. The effect of specific inhibition of SIRT2 on tubulin and MAP proteins in ASYN overexpressing cells. (A) Immunocytochemistry against ASYN. The data show that SHSY-5Y wild-type cell line had less ASYN levels compared with SHSY-5Y cell line overexpressing ASYN (n = 2) Red: ASYN, Blue: Hoechst; (B) *WT* and ASYN overexpressing cells were treated with or without AK1 (5 μM, 24 h) and then a western blot analysis was performed using the primary antibodies for phospho-Tau Thr 181 and acetylated α-tubulin. The phospho-Tau Thr 181 blot was reprobed for Tau and acetylated α-tubulin blot was reprobed for β-actin to confirm equal protein loading; (C) densitometry showing that the treatment with AK1 significantly restored the acetylation of α-tubulin in ASYN overexpressing cells (n = 3); (D) densitometry showing that the treatment with AK1 significantly reduced the levels of phospho-Tau Thr 181 in ASYN overexpressing cells (n = 3); (E) immunoprecipitation of Tau from *WT* and ASYN overexpressing cells that were treated with or without AK1 (5 μM, 24 h). Levels of Tau (top) and α-tubulin (bottom) in the input, immunoprecipitate (IP) and flow through (FT) are shown; (F) determination of α-tubulin/Tau physical interaction (n = 3). Treatment with AK1 significantly increased the interaction between Tau protein and α-tubulin in both, SHSY-5Y cell lines and *WT* cells; (G) immunoprecipitation of ASYN from *WT* and ASYN overexpressing cells that were treated with or without AK1 (5 μM, 24 h). Levels of ASYN (top) and α-tubulin (bottom) in the input, immunoprecipitate (IP) and flow through (FT) are shown; (H) determination of α-tubulin/ASYN physical interaction (n = 3). Treatment with AK1 significantly increased the interaction between ASYN protein and α-tubulin. Data is reported as the fold increase over untreated *WT* cells. $^{*}p < 0.05$ and $^{***}p < 0.001$, when compared to untreated *WT* cells. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ when compared to untreated ASYN cells. (Bonferroni's *t*-test; two-tailed unpaired Student's *t*-test).

consequently the increase in Tau/α-tubulin interaction promoted by AK1 may be a protective factor for PD. Finally, we determined the degree of interaction between ASYN and tubulin, and used AK1to know if ASYN is binding more or less to α-tubulin upon increased acetylation. We immunoprecipitated ASYN and then performed a Western Blot to detect α-tubulin levels (Fig. 1G–H). Under basal conditions, ASYN overexpressing cells showed significantly decreased interaction between ASYN and α-tubulin when compared to *WT* cells. Treatment with AK1 increased the interaction between ASYN and α-tubulin in ASYN overexpressing cells (Fig. 1G–H).

As previously reported under basal conditions, sPD cybrids showed decreased acetylation levels at lys40 of α-tubulin relative to CT cybrids [13]. Treatment with AK1 significantly restored the acetylation of α-tubulin in sPD cybrids indicating that AK1 could contribute to prevent MT network impairments. In order to establish the effects of the inhibition of SIRT2 on MT stability, we determined the degree of affinity of Tau protein to α-tubulin, based on the principle that the binding of Tau to α-tubulin contributes to MT structural integrity. It's already known that there's a correlation between the levels of phospho-Tau and the degree of affinity of tau to α-tubulin, in fact, the higher amount of phospho-Tau lowers the binding of Tau to α-tubulin [33]. Taking into account, we measured the levels of tau phosphorylated at ser396 and at thr181. Under basal conditions, sPD cybrids showed increased phospho-Tau levels relative to CT cybrids (Fig. 2A–C). Treatment with AK1 reduced the levels of phospho-Tau ser 396 and phospho-Tau thr 181 in PD cells. We also wanted to know if ASYN could act similarly to Tau. We observed that in sPD cells ASYN is less acetylated (Fig. 2D–E), which could imply a decreased interaction between ASYN and α-tubulin. Indeed, we found that in sPD cybrids ASYN binds less to α-tubulin (Fig. 2F–G). Treatment with AK1 tends to increase the interaction between ASYN and α-tubulin in both, sPD and CT cybrids (Fig. 4F–G). Assuming that the binding of ASYN, a MAP, to α-tubulin promotes MT stabilization, this is another argument that corroborates the fact that the inhibition of SIRT2 prevents disruption of the intracellular trafficking by increasing the acetylation levels of ASYN in both, sPD and CT cybrids.

3.2. MT dynamics affect autophagic turnover in PD models

Autophagic turnover relies on stable MT, in fact MTs have been implicated in the initiation and maturation of autophagosomes and mature autophagosomes transport along these tracks in order to be fused with lysosomes allowing their content degradation [34]. Our former studies revealed that autophagy failure stemming from mitochondrial dysfunction is not translated into defects in autophagosomes formation but mainly in the autophagosomes trafficking along the MT network toward the lysosomal compartment [23,31,35].

Bearing in mind that SIRT2 deacetylase regulates MT assembly and knowing that increasing the acetylation at lys 40 of α-tubulin enhances the affinity of MAPs, such as Tau and possibly ASYN, we wanted to know if the inhibition of SIRT2 activity could improve the autophagic turnover in ASYN overexpressing cells as it did in sPD cybrids [13]. We evaluated autophagosome accumulation by detecting LC3-II, an autophagosome marker, and its turnover. Since LC3-II is partially degraded by lysosomal hydrolases, following autophagosome-lysosome fusion, increased LC3-II might represent a reduced rate of fusion and LC3-II clearance rather than increased autophagy. To address this question we monitored the autophagic flux, the process from autophagosome formation to degradation, comparing the accumulation of autophagosomes after inhibition of lysosomal function (with NH_4Cl and leupeptin) to the steady-state levels. With this approach we observed that under basal conditions ASYN overexpressing cells have lower autophagic flux (Fig. 3) when compared to *WT* cells. Treatment with AK1 increased the autophagic flux in ASYN overexpressing cells. Similarly to autophagosomes, ASYN is actively transported by microtubules from its site of synthesis in the cell body along axons to synaptic termini. Several data indicated that defective axonal transport of ASYN is a potential pathogenic event in alpha-synucleinopathies [36]. In fact, work from our group reported that in PD cybrids AK1 significantly reduced both ASYN triton-soluble low weight oligomers and triton-insoluble high weight oligomers accumulation [13].

3.3. MT disruption affects mitochondria dynamics in PD models

Mitochondrial function, transport and degradation rely on mitochondrial fission and fusion events and are dependent of MT assembly [37]. It has already been described in previous works that an alteration in mitochondrial network is associated with sPD [13,23,38]. Therefore we wanted to evaluate whether mitochondrial network and dynamics is affected in our cellular models with compromised MTs. Tom 20 is a subunit of the TOM (translocase of outer membrane) receptor complex responsible for the recognition and translocation of cytosolically synthesized mitochondrial pre-proteins and can be used to evaluate mitochondrial morphology [39].

In previous work we observed a more perinuclear distribution of the mitochondrial network, a decrease in mitochondrial interconnectivity and elongation in PD cybrids, as compared to CT cybrids [38]. Interestingly, ASYN overexpressing cells contained a more interconnected mitochondrial network as compared to *WT* cells (Fig. 4A). As a positive control, we exposed cells to FCCP, an uncoupling agent that disrupts mitochondria membrane potential by transporting hydrogen ions through the membrane before they can be used to provide the energy for oxidative phosphorylation (OXPHOS). As expected, in *WT* cells, we observed a more dramatically fragmented mitochondria network, resulting in the appearance of round-shaped disconnected mitochondria (Fig. 4A).

The observed abnormal mitochondrial network could be due to a disturbance of the proteins involved in the mitochondrial fission/fusion process. To distinguish between these possibilities we evaluated the proteins involved in the mitochondrial fission and fusion process. Given the slight differences in the protein levels between ASYN overexpressing cells and *WT* cells (data not shown), we next asked whether the subcellular localization of the proteins was dissimilar. For this purpose, cells were fractionated in mitochondrial and cytosolic extracts.

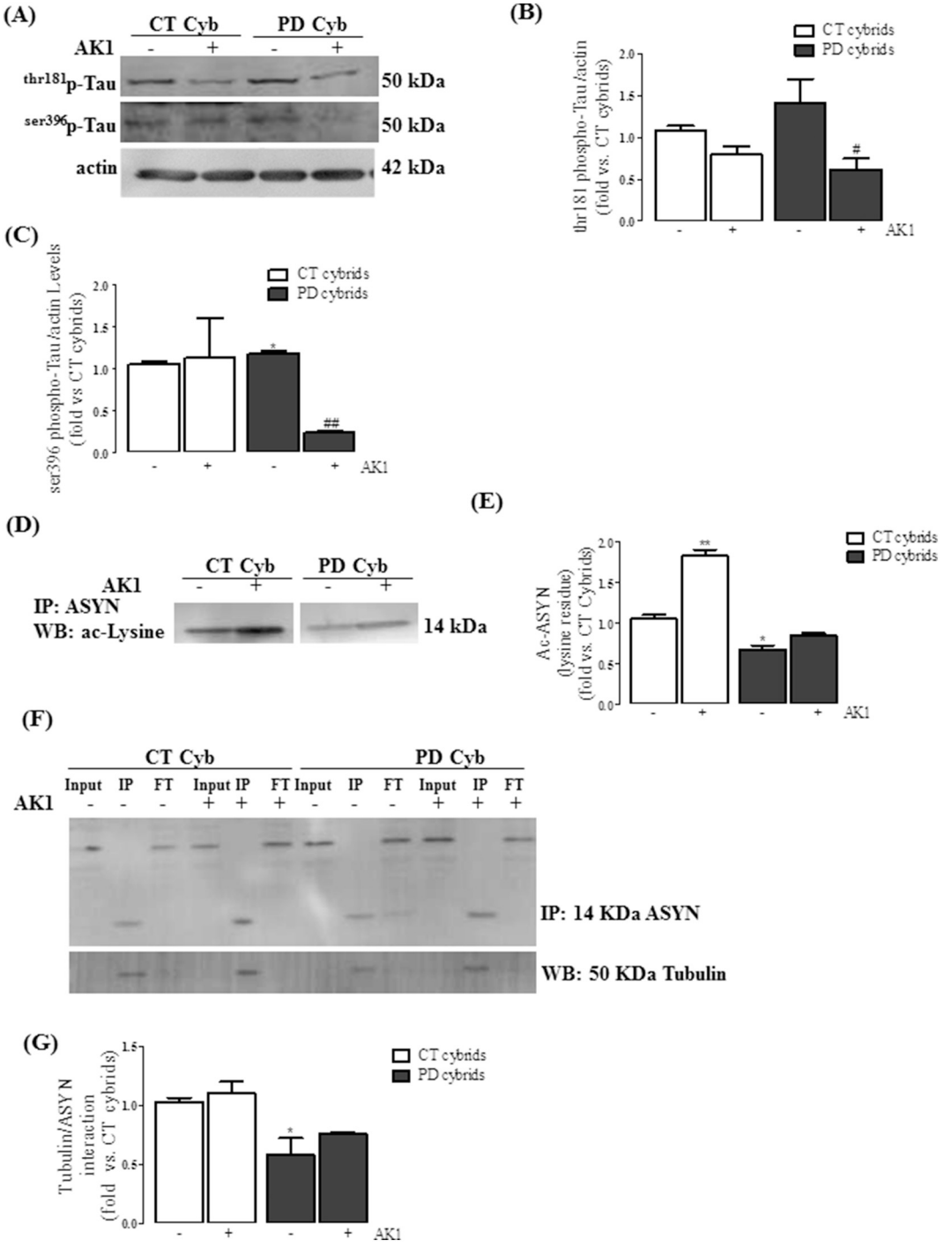

Fig. 2. The effect of specific inhibition of SIRT2 on tubulin and MAP proteins in sporadic PD cybrids. (A) CT and sPD cybrids were treated with or without AK1 (5 μM, 24 h) and then a western blot analysis was performed using the primary antibodies for phospho-Tau Thr 181 and phospho-Tau Ser396. The blots were reprobed for actin to confirm equal protein loading; (B) densitometry showing that the treatment with AK1 significantly reduced the levels of phospho-Tau Thr 181 in sPD (n = 1–2); (C) densitometry showing that the treatment with AK1 significantly reduced the levels of phospho-Tau Ser 396 in sPD (n = 3); (D–E) western blot analysis and respective densitometry showing that after the treatment with AK1 there was an increased in the acetylation levels of ASYN in both, sPD and CT cybrids. Data is reported as the fold increase over untreated CT cybrids (n = 2); (F) immunoprecipitation of ASYN from CT and sPD cybrids that were treated with or without AK-1 (5 μM, 24 h). Levels of ASYN (top) and α-tubulin (bottom) in the input, immunoprecipitate (IP) and flow through (FT) are shown; (G) determination of α-tubulin/ASYN physical interaction (n = 2). Treatment with AK1 significantly increased the interaction between ASYN and α-tubulin in both, sPD and CT cybrids. *$p < 0.05$ and **$p < 0.01$, when compared to untreated CT cybrid group and $^{\#}p < 0.05$, when compared to untreated PD cybrid (Bonferroni's *t*-test; two-tailed unpaired Student's *t*-test).

The results from the fractionation process and the quantification of the proteins involved in mitochondrial dynamic revealed that ASYN overexpressing cells show an increase in mitochondrial levels of the pro-fusion proteins Opa1 and Mfn1 when compared *WT* cells (Fig. 4B–C). Moreover, a decrease in mitochondrial ser616-phospho-Drp1 was observed in ASYN overexpressing cells (Fig. 4B and D). Our results strongly suggest that ASYN overexpression induces a decrease in mitochondrial fission or stimulates mitochondrial fusion, which points

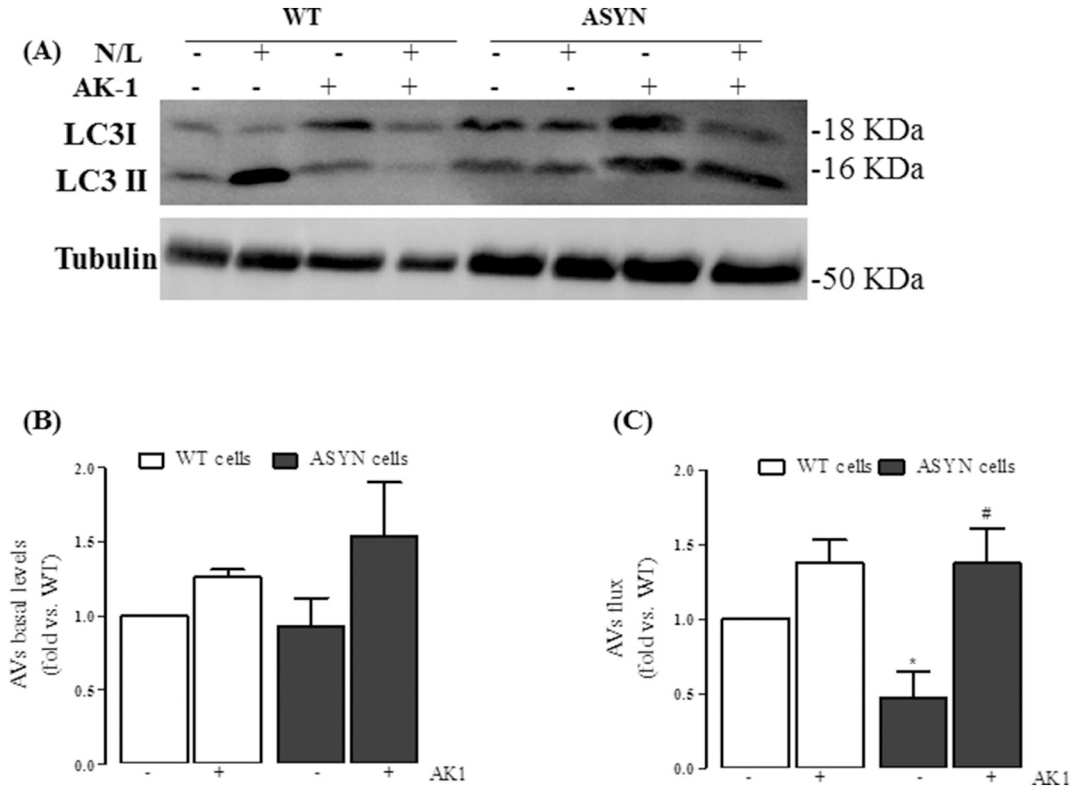

Fig. 3. Evaluation of the autophagic turnover in ASYN overexpressing cells under the specific inhibition of Sirt2. (A) Immunoblot for endogenous LC3-II from *WT* and ASYN overexpressing cells treated with or without AK1 (5 μM, 24 h) following culture in the presence or absence of lysosomal inhibitors (N/L - NH4Cl and Leupeptin) for the last 4 h. The blots were reprobed for α-tubulin to confirm equal protein loading (n = 2); (B) densitometric analysis of endogenous levels of LC3-II, data is reported as absolute values (n = 3); (C) assessment of autophagic flux, calculated as the ratio of LC3-II densitometric value of N/L treated samples over the corresponding untreated samples (n = 1–2). AK1-treated ASYN overexpressing cells showed an increase in autophagic flux. Data is reported as the fold increase over untreated CT cybrids or *WT* cells. *p < 0.05, when compared to untreated *WT* cells and #p < 0.05, when compared to untreated ASYN cells. AVs stands for autophagic vacuoles (Bonferroni's *t*-test; two-tailed unpaired Student's *t*-test).

toward an increase in mitochondrial elongation (more fused mitochondria).

3.4. MT modifications in Tau mutant cells

Pro301-to-leu (TauP301L) is the most common Tau missense mutation associated with neurodegenerative tauopathies, such as AD. Indeed, neuroblastoma cells infected with TauP301L cDNA are characterized by the accumulation of hyperphosphorylated Tau and development of NFTs mimicking what happens in FTD and AD [25]. Moreover, Tau P301L has reduced MT binding affinity and less efficient MT assembly properties as compared with *WT* Tau [40]. It is then plausible that overexpression of Tau P301L could lead to less stable microtubules due to Tau hyperacetylation.

As expected we observed that P301L cells show increased phosphorylation levels at Ser396 relatively to *WT* cells (Fig. 5A–B). Interestingly, these mutant cells have increased acetylation levels at K280 of Tau and less acetylated tubulin when compared to *WT* cells (Fig. 5C–F), which indicate that P301L cells have instable MTs [41]. We found that only AK1 reduced phospho-Tau levels at ser396 in P301L cells. Nevertheless, both tubastatin A and C646 indicate the same trend. Moreover, we detected that tau acetylation levels were increased in P301L cells but only C646 significantly reverted this increase (Fig. 5C–D). We observed that tubastatin A increased tubulin acetylation levels in both control and P301L cells whereas AK1 only increased tubulin acetylation levels in P301L cells (Fig. 5E–F). These results indicate that both AK1 and tubastatin A promote MT stability in mutant cells. On the other hand C646 had no effect on tubulin acetylation.

3.5. Modulation of MT acetylation in sAD cells

Recent studies indicated that acetylation plays an important role in the phosphorylation state of Tau protein [42]. The importance of acetylation has also been widely studied in the MT network since it improves its stability, although this relationship is not clear-cut [15]. First, we evaluated the levels of Tau phosphorylation at ser396 residue and of Tau acetylation at K280 residue and observed a significant increase of both phospho and acetylated-Tau (Fig. 6A–D). This increase was correlated with a decrease in tubulin acetylation indicating MT instability, similar to what was observed in Tau mutant cells (Fig. 6E–F). Treatment of AD cybrids with AK1, tubastatin A or C646 promoted a significant decrease in the levels of phospho-Tau (Fig. 6A–B). As expected, tubastatin A and AK1 significantly augmented tubulin acetylation whereas only C646 significantly decreased Tau acetylation suggesting that SIRT2 do not deacetylate Tau and that p300 does not acetylate tubulin (Fig. 6C–F).

3.6. Alterations in autophagic activity in AD models

As previously mentioned, autophagic flux is highly dependent on MT network [34]. Remarkably, MT acetylation causes the recruitment of molecular motor dyneins and kinesins to MTs therefore holding a role for the dynein-dependent retrograde trafficking of either lysosomes or autophagosomes [43]. More interestingly, Xie and colleagues revealed that acetylated MTs are required for the fusion of autophagosomes with lysosomes [44]. Taking into account that HDAC6 and p300 enzymes are recognized as modulators of Tau acetylation [21,45] we evaluated the role of HDAC6 and p300 enzymes in autophagic turnover

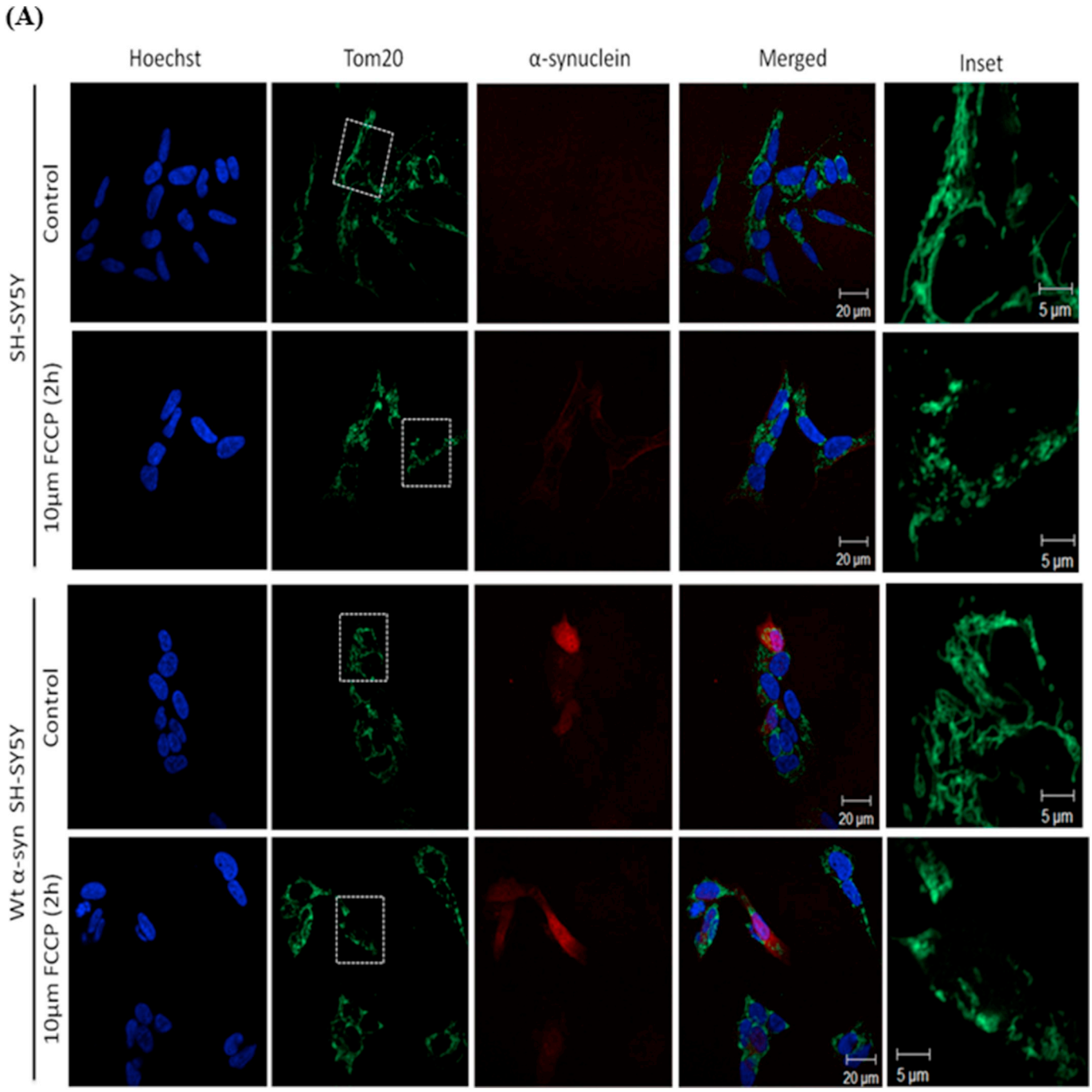

Fig. 4. Mitochondrial dynamics and network alterations in ASYN overexpressing cells. (A) Mitochondrial fragmentation in *WT* ASYN overexpressing cells. Representative immunofluorescence pictures evidencing mitochondrial network in SH-SY5Y cells, overexpressing or not *WT* ASYN, under basal conditions, or treated with FCCP (10 μM, 2 h). Immunostaining was performed against the mitochondrial protein Tom20 and α-synuclein. Green: Tom20, Red: ASYN, Blue: Hoechst. (B–C) Fusion and Fission protein expression levels in *WT* ASYN overexpressing cells. Cells were harvested and fractionated into mitochondrial and cytosolic fractions. Representative immunoblot (B) and quantification analysis (C–D) of the proteins levels involved in mitochondrial fusion (C) and fission (D). Equal protein amounts (50 μg) were loaded and confirmed with GAPDH and Tom20 for the cytosolic and mitochondrial fractions, respectively. The purity of the mitochondrial and cytosolic fractions is confirmed by the negligible contamination each fraction with the marker of the other fraction. Data represent mean ± SEM values derived from 3 independent determinations. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, when compared to untreated *WT* cells (Bonferroni's *t*-test; two-tailed unpaired Student's *t*-test).

in Tau P301L cells. We started by monitoring the autophagic flux and found a decrease in the autophagic flux of untreated Tau P301L cells (Fig. 7A–B). As a long-lived protein, Tau is expected to be degraded in lysosomes. Taking this into account, abnormal processing and altered clearance by autophagic-lysosomal machinery of Tau protein have been implicated in AD pathology [18]. Remarkably, we observed that p300 inhibition significantly increase the autophagic flux (Fig. 7), probably due to a decrease in Tau acetylation and phosphorylation levels. Likewise, AD cybrids have reduced autophagic flux (Fig. 8). Indeed, in AD cells, all inhibitors led to an improvement of the autophagic flux (Fig. 8).

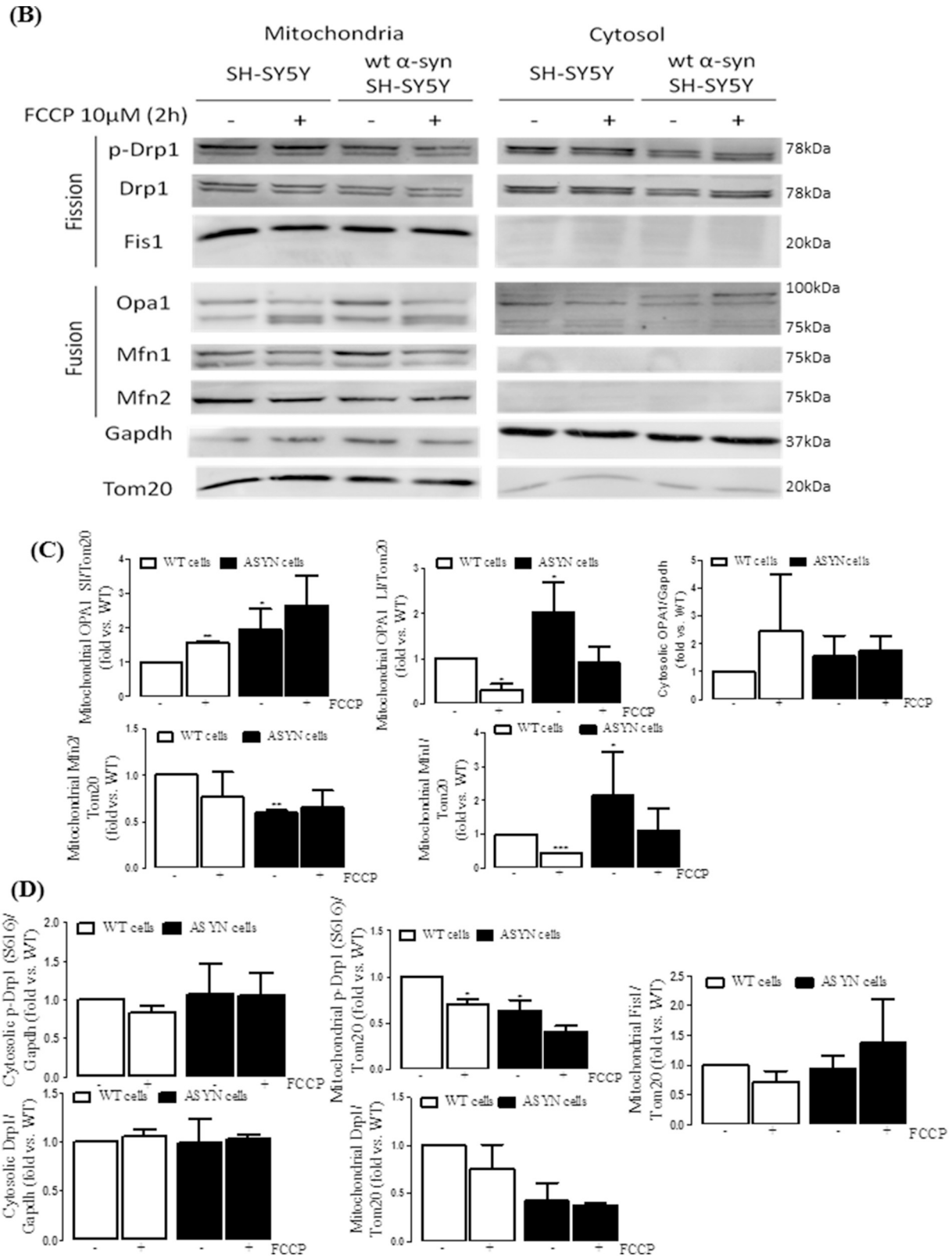

Fig. 4. (*continued*)

4. Discussion

With this work we further clarified the role of Tau and ASYN acetylation elucidating how acetylation modulation (with AK1, tubastatin A and C646) impact autophagy/lysosomal and mediate the potential pathogenic mechanisms of neurodegeneration in PD and AD.

Given that axonal transport is disrupted in PD and knowing that Tau protein is a MAP with the function of stabilizing the MT, this research aimed to clarify the role of Tau protein in PD. Interestingly ASYN cells and sPD cells show enhanced phospho-Tau levels at ser396 and thr181 indicating that ASYN accumulation leads to Tau hyperphosphorylation and MT disassembly as confirmed by reduced tubulin acetylation.

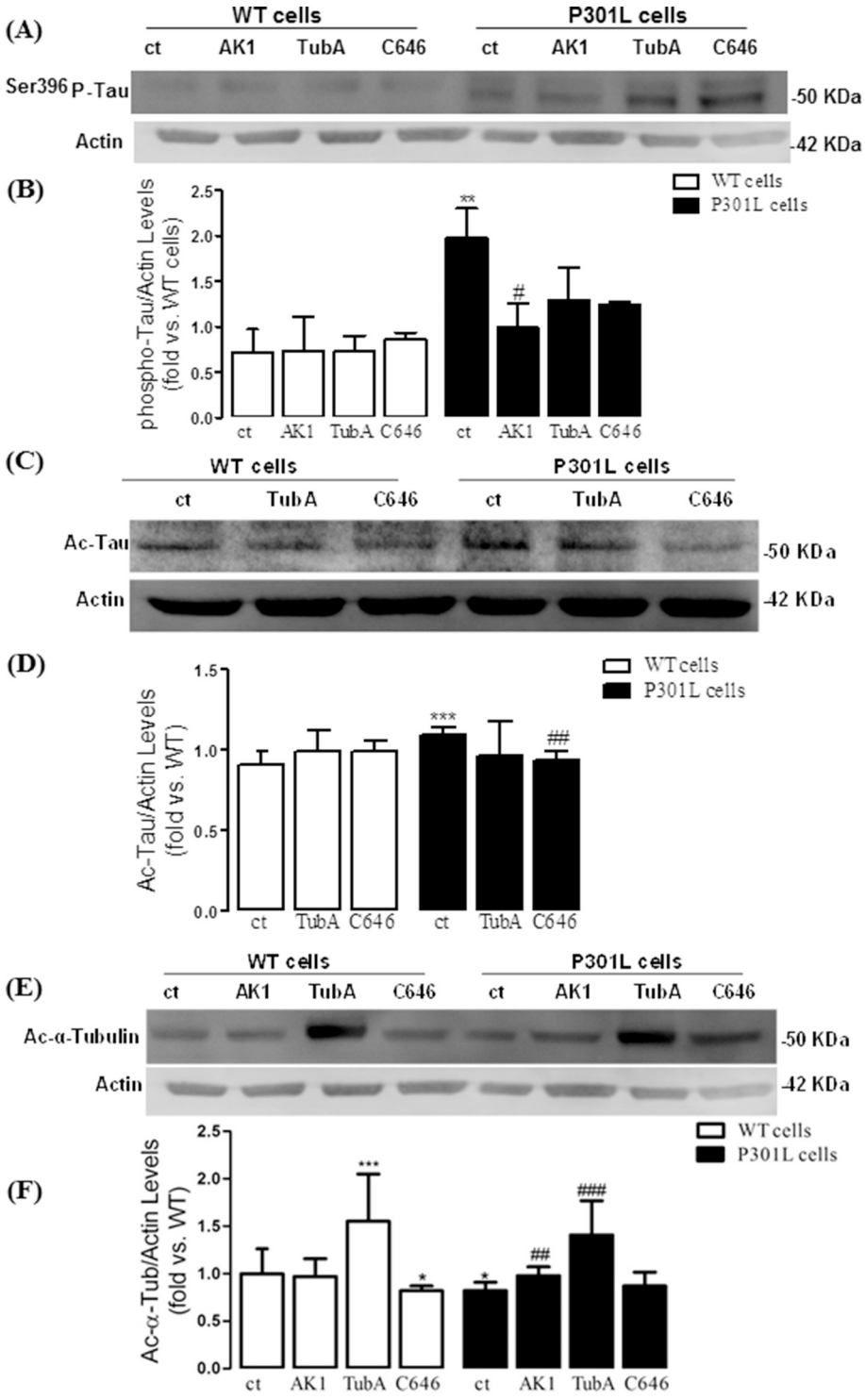

Fig. 5. Acetylation modulation effect of MT proteins in P301L cells. SH and P301L cells were incubated with 20 μM AK1; 5 μM Tub A; 2.5 μM C646. (A–B) Representative immunoblot for phospho-Tau (S396) and respective densitometry analysis. (C–D) Representative immunoblot for acetylated-Tau and respective densitometry analysis. (E–F) Representative immunoblot for acetylated-alpha-tubulin and respective densitometry analysis. Data represent the mean ± SEM values derived from 4 to 7 independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, significantly different as compared to untreated *WT* cells. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ significantly different as compared untreated P301L cells (Bonferroni's *t*-test; two-tailed unpaired Student's *t*-test).

Surprisingly, with this work we showed that there is a direct link between Tau dysfunction and PD. In fact, mutations in the Tau gene are associated to parkinsonism linked to chromosome 17 (FTDP-17) [46]. Corroborating these data we also found reduced co-localization of Tau with MTs. Tau hyperphosphorylation leads to a reduced ability of Tau to interact with MTs inducing the assembly of Tau into toxic filaments. Similarly we found that in ASYN cells and in cells harboring sPD patient mitochondria ASYN also binds poorly to tubulin. The aforementioned results raise the possibility that in PD, MT network disruption can lead or can result in decreased association of ASYN and Tau to tubulin. Our results clearly support that ASYN accumulation underlies MT disruption by affecting MAP function of ASYN leading to Tau phosphorylation, which contributes to impaired Tau-MT interactions. Interestingly another PTM of ASYN have been identified namely N-terminal acetylation, which seems to contribute to ASYN oligomerization and cytotoxicity [47]. However, herein in conjunction with decreased ASYN interaction with MTs we found that ASYN is less acetylated in PD cells. Taking into account that PD cells with mitochondrial DNA from sporadic PD patients show cytoskeleton alterations, which manifest as MT depolymerization and increased formation of ASYN oligomers [48], we can hypothesize that ASYN acetylation protects ASYN from oligomerization. Remarkably a recent report showed that N-terminal

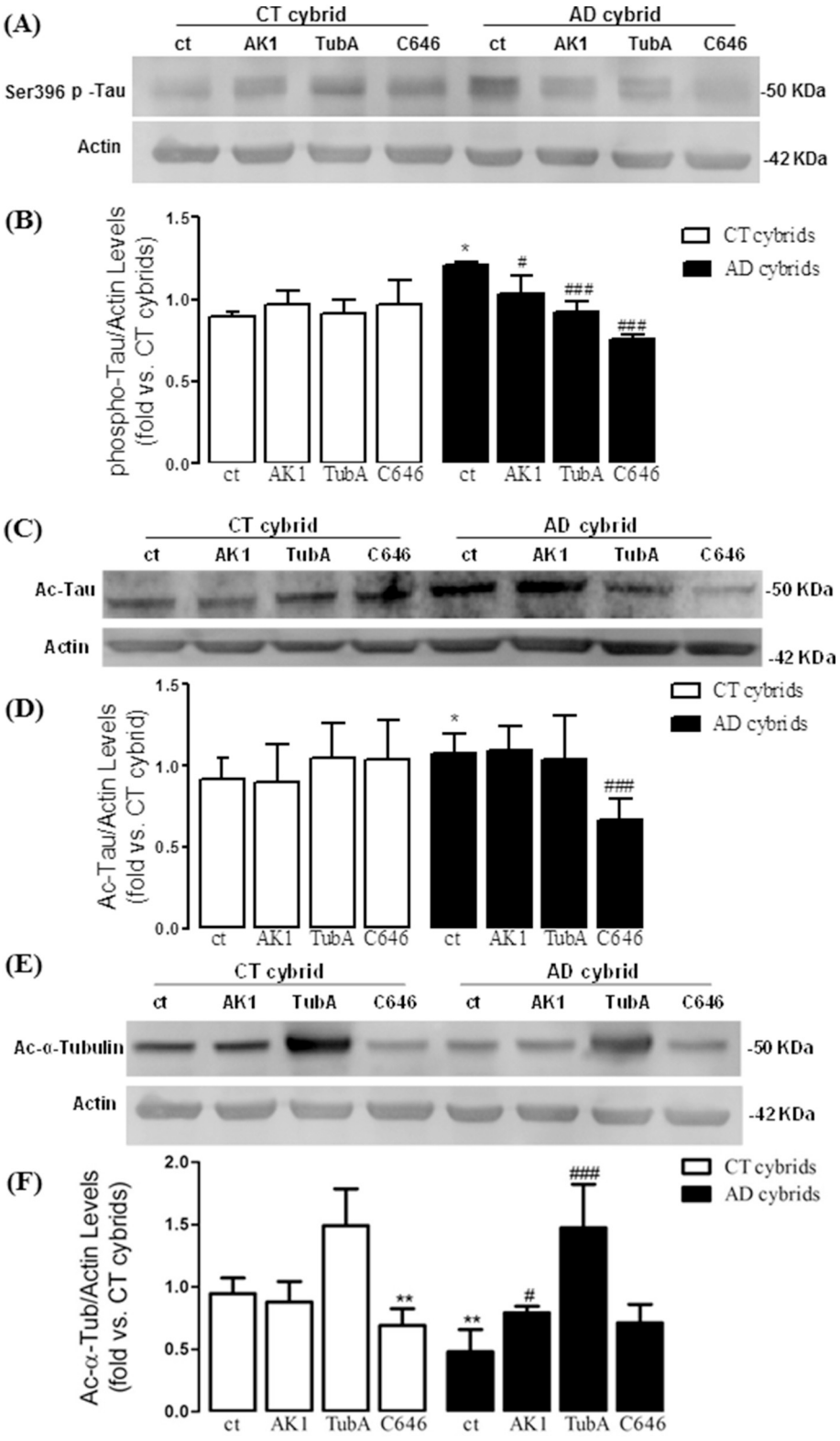

Fig. 6. Acetylation modulation effect of MT proteins in AD cybrid cells. Cells from CT and AD cybrids were treated 10 μM AK1, 5 μM Tub A, 2.5 μM C646. (A) Western blot analysis of phospho-Tau levels (S396) and respective densitometry analysis (B). (C) Western blot analysis of acetylated Tau levels and respective densitometry analysis (D). (E) Western blot analysis of acetylated α-tubulin levels and respective densitometry analysis (F). Data represent the mean ± SEM values derived from 5 to 7 independent experiments. *$p < 0.05$ and **$p < 0.01$ significantly different as compared to untreated CT cybrid. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ significantly different as compared to untreated AD cybrid (Bonferroni's *t*-test; two-tailed unpaired Student's *t*-test).

acetylation protected ASYN from oligomerization by preserving its native conformation against pathological aggregation [49]. In this study we demonstrated the role of SIRT2 in MT instability via α-tubulin deacetylation and Tau hyperphosphorylation. We provide evidence that α-tubulin acetylation via SIRT2 inhibition is functionally associated with the improvement of intracellular trafficking, providing a better autophagic turnover in PD cybrids and ASYN overexpressing cells. These data are consistent with previous reports demonstrating that ASYN-mediated neurotoxicity in several models of PD is partially due to deacetylation of α-tubulin by SIRT2 [12]. Additionally, dysfunctional mitochondria degradation by autophagy is dependent on a differential regulation of fusion-fission events that seems to be altered in sPD and ASYN overexpressing cells [37,38].

It is well described that Tau is a MAP and that its phosphorylation state regulates Tau binding to MTs, which correlates with Tau aggregation and MT damage in AD [50,51]. In AD brains it was reported

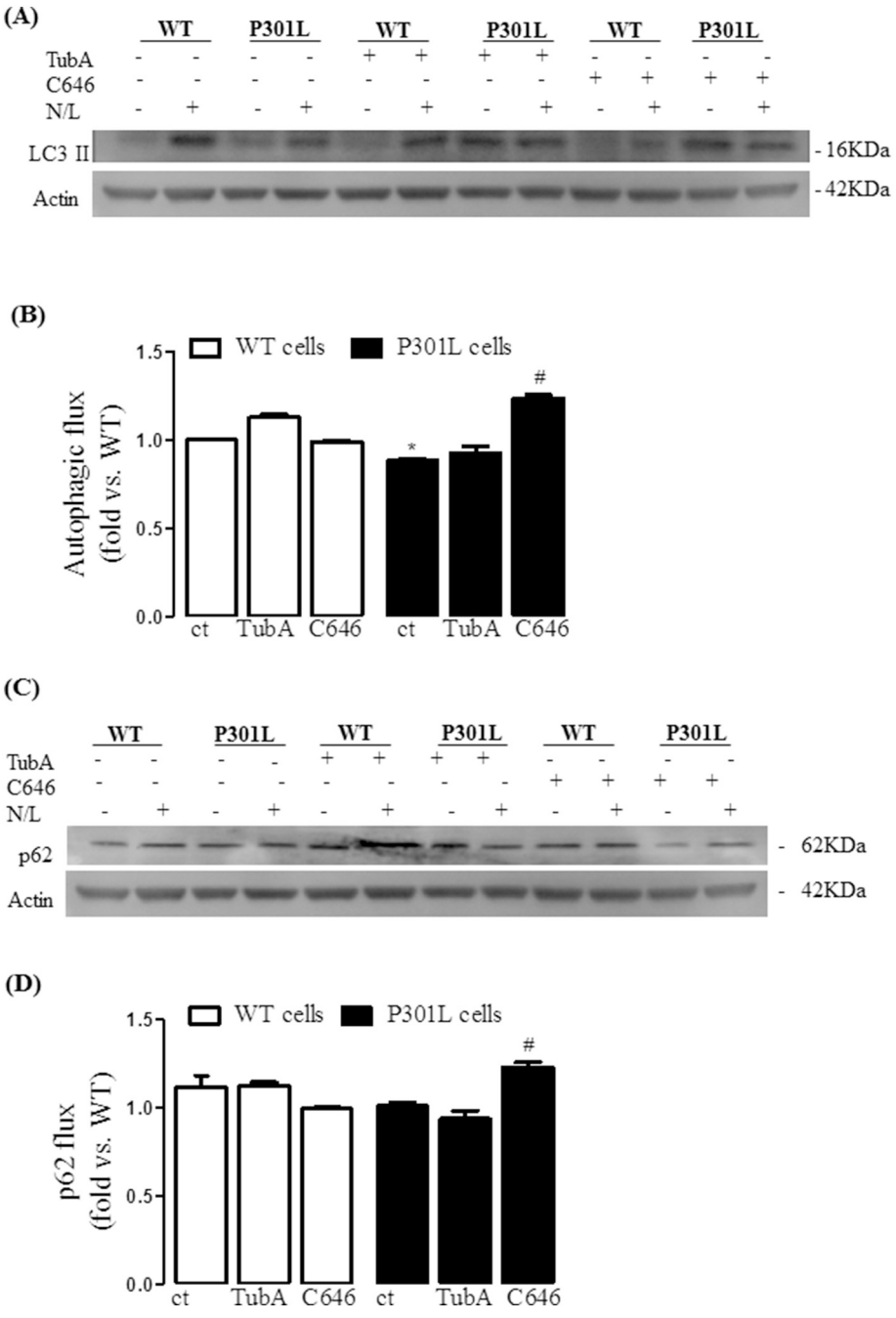

Fig. 7. MT acetylation modulation impact on autophagic markers in P301L cells. Cells from SH and P301L were treated 5 μM Tubastatin A, 2.5 μM C646 and were examined by immunoblotting using the anti-LC3-II and anti p62. Representative immunoblot for LC3 II and respective densitometric analysis (A–B). Representative immunoblot for p62 and respective densitometric analysis (C–D). Data represent the mean ± SEM values derived from 2 to 4 independent experiments. *$p < 0.05$, significantly different relatively to untreated *WT* cells. #$p < 0.05$, significantly different as compared untreated P301L cells (Bonferroni's *t*-test; two-tailed unpaired Student's *t*-test).

that Tau phosphorylation at ser396 is significantly increased when compared with other phosphorylation sites, and occurs prior to the appearance of NFTs [52]. Tau P301L mutation is characterized by the accumulation of phosphorylated Tau (ser396) and is widely used to study tauopathies. As expected, we found that in SH-SY5Y cells transfected with Tau constructs that carry P301L mutation, phosphorylated Tau protein levels are increased relatively to the parental SH cells. Interestingly, this was also observed in hybrid cells harboring sAD patient mitochondria. Compelling evidence suggests a preponderant involvement of MTs derived-axonal transport failure in AD and PD [53]. Acetylation of MT-α-tubulin plays a role in the maintenance of stable populations of MTs [14]. In line with this, we observed in Tau and sAD cells, as well as, in ASYN cells that acetylated-tubulin levels were decreased indicating MT instability and axonal transport defects.

Recently, studies have shown that Tau is also post-translationally modified by lysine acetylation being a disease-specific modification in AD, likely representing a major regulatory tau modification [21,42,54]. However, data from literature on this topic is contradictory. It was described that Tau is acetylated and that this acetylation prevents the degradation of phosphorylated Tau [21]. On the other hand, it was reported that the acetylation of Tau on KXGS motifs (in the MT-binding domain) inhibits phosphorylation and also prevents Tau aggregation [45]. In this study we show that Tau phosphorylation increase is positively correlated with Tau acetylation in sAD and fAD models which underlines MT instability characterized by a decrease in α-tubulin acetylation leading to a reduction in the autophagic flux [55].

We demonstrated that tubulin deacetylation by SIRT2 or HDAC6 activation in the cytosol leads to MT loss of stability and depolymerization, which facilitates Tau dissociation and consequent phosphorylation in AD models. In fact, in 3xTg-AD mice SIRT2 inhibition was shown to increase tubulin acetylation [56]. Surprisingly, p300 inhibition did not change tubulin acetylation in any AD models. Although

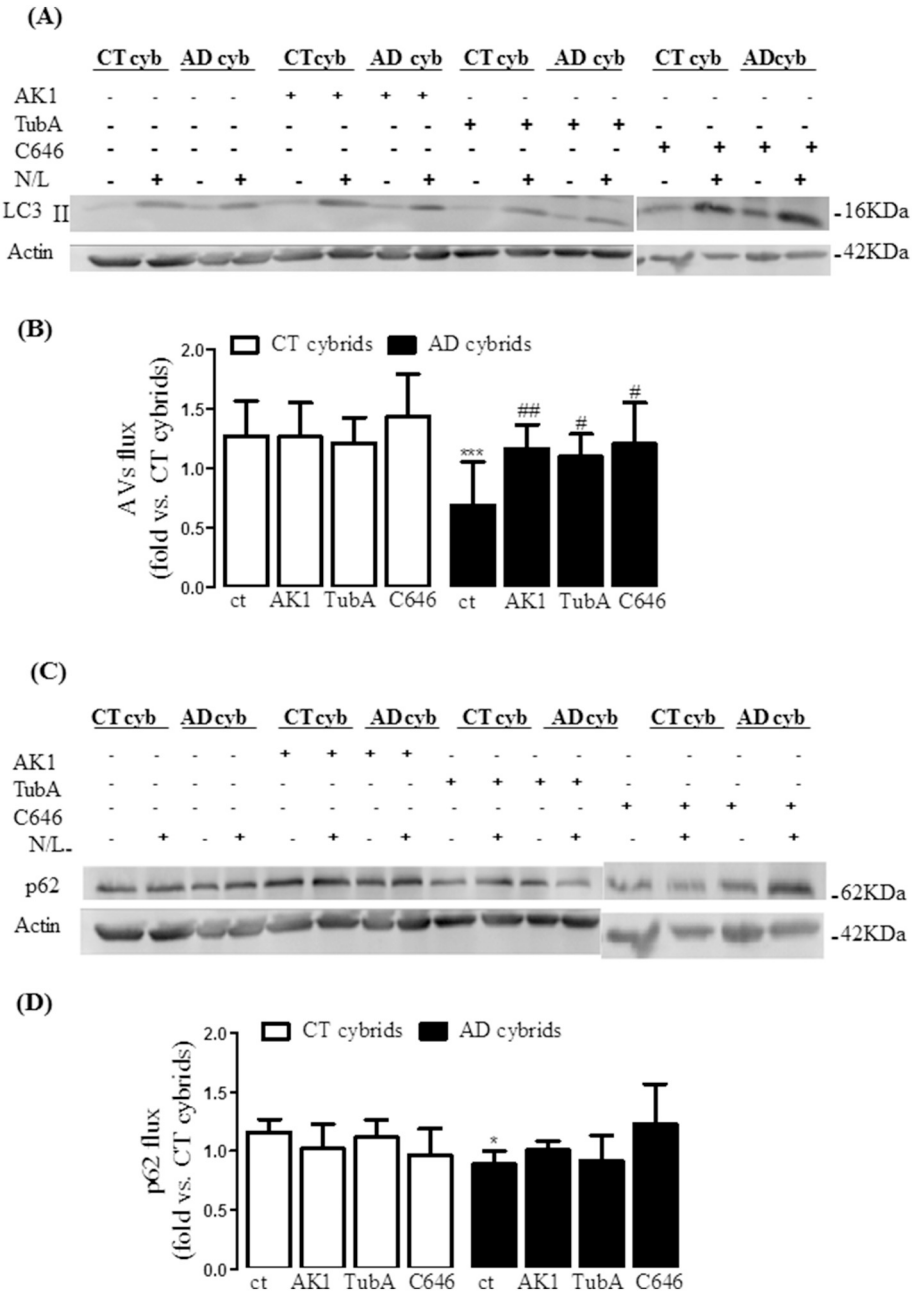

Fig. 8. MT acetylation modulation impact on autophagic markers in AD cybrids. Cells from CT and AD cybrids were treated 10 μM AK1, 5 μM Tub A, 2.5 μM C646 and were examined by immunoblotting using the anti-LC3-II (A) and anti-p62 (C) antibodies. Representative immunoblot for LC3 II and respective densitometry analysis (A–B). Representative immunoblot for p62 levels and respective densitometry analysis (C–D). Data represent the mean ± SEM values derived from five independent experiments. *$p < 0.05$, ***$p < 0.001$ significantly different as compared to untreated CT cybrid. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significantly different as compared to untreated AD cybrid (Bonferroni's *t*-test; two-tailed unpaired Student's *t*-test).

there is no described evidence in the literature in respect to tubulin as a p300 substrate, we observed a significant decrease in acetylated tubulin in the treated C646 *WT* SH-SY5Y and CT cybrid cells. Until now the exact mechanism by which C646 decreases acetylated tubulin is not clear, but it was described that p300 can modulate the function of HDAC6 and SIRT2. It was reported that p300 interacts with and acetylates HDAC6 resulting in the down-regulation of HDAC6 deacetylase activity [57]. Similarly p300 was reported to mediate SIRT2 deacetylase activity [57]. These results show that p300 can regulate the acetylation status of tubulin indirectly through the action of SIRT2 and HDAC6. Moreover, p300 appears to work directly by decreasing Tau acetylation levels and indirectly as a modulator of Tau phosphorylation. Corroborating this data, Tau acetylation by p300 was seen in the early stages of AD and hyperacetylation impairs Tau degradation, promoting the accumulation of abnormally phosphorylated Tau [21,58]. We found that in fAD and sAD models both SIRT2 and HDAC6 inhibition improved tubulin acetylation but only C646 decreased Tau acetylation. Additionally, we found that the inhibition of p300, HDAC6 and SIRT2 contributed to the reduction of phosphorylated Tau in AD probably due to a decrease in acetylated Tau or increase in tubulin acetylation favoring autophagic cargo removal in both models. This result is interesting taking into account that HDAC6 tubulin deacetylase activity has been shown to be required for autophagy progression and clearance. However, others have demonstrated that inhibition of this enzyme increases the acetylation of MTs and contributes to an improvement of autophagy. On the other hand, SIRT2 over-activation has been associated with reduction of autophagy efficiency [59].

Although the present findings strongly suggest that SIRT2 and p300 can hold potential therapeutic effects in PD and AD respectively, further studies are required to better understand the function of each enzyme on disease pathologic features. Moreover, a better understanding of the effects of Tau and ASYN acetylation are crucial since it have been documented contradictory roles for these PTMs in PD and AD disorders progression. Our study supports growing evidence that MTs acetylation modulation may be a clinically beneficial target of therapeutic interventions to counteract AD and PD pathology.

Transparency document

The Transparency document associated with this article can be found, in online version.

Author contributions

SMC and ARE designed the experiments; ARE executed the immunoprecipitation and autophagy related experiments; AP performed the western blot experiments in ASYN cells; RG executed the western blot experiments in P301L cells; DS carried out the immunocytochemistry experiments. ARE, DFS and SMC carried out the data analysis; ARE wrote the paper.

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Conflict of interest

The authors declare that they have no conflict of interest.

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