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Melatonin directly binds and inhibits death-associated protein kinase 1 function in Alzheimer's disease

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Abstract

Death-associated protein kinase 1 (DAPK1) is upregulated in the brains of human Alzheimer's disease (AD) patients compared with normal subjects, and aberrant DAPK1 regulation is implicated in the development of AD. However, little is known about whether and how DAPK1 function is regulated in AD. Here, we identified melatonin as a critical regulator of DAPK1 levels and function. Melatonin significantly decreases DAPK1 expression in a post-transcriptional manner in neuronal cell lines and mouse primary cortical neurons. Moreover, melatonin directly binds to DAPK1 and promotes its ubiquitination, resulting in increased DAPK1 protein degradation through a proteasome-dependent pathway. Furthermore, in tau-overexpressing mouse brain slices, melatonin treatment and the inhibition of DAPK1 kinase activity synergistically decrease tau phosphorylation at multiple sites related to AD. In addition, melatonin and DAPK1 inhibitor dramatically accelerate neurite outgrowth and increase the assembly of microtubules.

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AUTHOR CONTRIBUTIONS

DC designed the studies, analyzed the data, and wrote the manuscript. DC and YM performed most of experiments. NK performed qPCR and in vitro kinase assay, and edited the manuscript. GL performed immunohistochemical analysis and edited the manuscript. FF and CL helped with brain slice experiments and edited the manuscript. C.-LG and FK helped with biotin-melatonin experiments and edited the manuscript. TZ provided advice and edited the manuscript. YX and LW provided advice and technical assistance. XZZ and KPL provided human specimens and wrote the manuscript. THL conceived and supervised the project, designed the studies, and wrote the manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Mechanistically, melatonin-mediated DAPK1 degradation increases the activity of Pin1, a prolyl isomerase known to play a protective role against tau hyperphosphorylation and tau-related pathologies. Finally, elevated DAPK1 expression shows a strong correlation with the decrease in melatonin levels in human AD brains. Combined, these results suggest that DAPK1 regulation by melatonin is a novel mechanism that controls tau phosphorylation and function and offers new therapeutic options for treating human AD.

Keywords

Alzheimer's disease; death-associated protein kinase 1 (DAPK1); melatonin; Pin1; tau

1 | INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is mainly generated in the pineal gland and critically involved in various physiological processes, including regulating circadian rhythms.[1,2] Melatonin has been demonstrated to have neuroprotective effects in neurodegenerative diseases, including Alzheimer's disease (AD).[3-7] Neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau and amyloid-β (Aβ) plaques derived from amyloid precursor protein (APP) processing are two major molecular neuropathological features of AD.[8,9] It has been reported that melatonin levels are decreased in human AD patients.[10-12] Moreover, melatonin has been shown to decrease tau phosphorylation and Aβ secretion in cellular and animal models.[13-17] In particular, melatonin efficiently reduces chemical-induced tau hyperphosphorylation in neuronal cells and rats.[18-20] Furthermore, melatonin decreases the level of phosphorylated tau by inhibiting tau kinases or activating phosphatases.[7,21,22] Although several melatonin-related kinases that regulate tau hyperphosphorylation have been identified, the molecular mechanisms of the melatonin-mediated attenuation of their kinase activity and tau phosphorylation are poorly understood because only a handful of direct melatonin targets and interacting proteins have been identified.

Death-associated protein kinase 1 (DAPK1) is a calcium/calmodulin-dependent serine/threonine kinase that plays an important role in regulating neuronal function and cell death.[23-25] DAPK1 expression levels are relatively upregulated in the postmitotic regions within the cerebral cortex and hippocampus and in cerebellar Purkinje cells compared with that of other brain regions, suggesting that DAPK1 might play an important role in neurogenesis.[26] Interestingly, the overall expression of DAPK1 mRNA in the brain gradually declines during postnatal stages, but DAPK1 expression has become restricted to the hippocampus and cortical region of the adult brain.[26] This different expression pattern implies that DAPK1 might have other functions and that it may affect synaptic transmission and plasticity. Emerging evidence suggests that DAPK1 might regulate neuronal cell death at multiple levels.[27] The overexpression and activation of DAPK1 impair neural cell viability, and neurons that do not express DAPK1 are more resistant to apoptotic stimuli, as shown by in vitro and in vivo research.[25] Moreover, DAPK1 physically interacts with and phosphorylates the NR2B subunit of the N-methyl-D aspartate receptor and mediates brain damage induced by ischemic stroke, and inhibiting DAPK1 activity confers neuroprotection in a stroke

mouse model.[28,29] These results suggest that the inhibition of DAPK1 expression protects neurons from neuronal injuries.

Recent studies have also indicated that DAPK1 might be critically implicated in late-onset AD (LOAD).[27,30] DAPK1 has been identified to be a potential LOAD contributing gene by single nucleotide polymorphism analysis.[31,32] and modulate DAPK1 allele-specific gene expression.[31] Moreover, DAPK1 kinase activity-deficient mice show improved cognitive ability compared with wild-type (WT) mice, suggesting that the inhibition of DAPK1 activity has cognitive benefits.[33,34] Recently, we discovered that the expression of DAPK1 is dramatically upregulated in the hippocampi of 75% of human AD patients in comparison with age-matched nondemented controls.[35-37] Furthermore, DAPK1 controls tau function by modulating the assembly of microtubule and neuronal differentiation,[35,38] and overexpressing DAPK1 enhances tau expression and its phosphorylation at multiple amino acid residues associated with AD in cells and animal models in a kinase activity- and age-dependent manner.[35,39,40] In addition, DAPK1 significantly increases Aβ secretion and promotes Aβ-induced neuronal cell death in AD.[36,37] These results strongly suggest that DAPK1 deregulation contributes to AD progression and that DAPK1 inhibition might have novel therapeutic implications in AD. However, it is not known whether and how DAPK1 expression or activity is regulated in AD, including tau hyperphosphorylation.

In this manuscript, we report the surprising findings that melatonin induces DAPK1 ubiquitination and increases its degradation via direct binding to the ankyrin repeat domain of DAPK1. Moreover, the inhibitory effects of melatonin on tau hyperphosphorylation are potently amplified by cotreatment with a pharmacological DAPK1 activity inhibitor. Consequently, melatonin and this DAPK1 inhibitor work cooperatively to ablate DAPK1 expression and/or activity, thereby suppressing tau expression and hyperphosphorylation and increasing microtubule assembly and neurite outgrowth. The negative correlation between the expression of DAPK1 and the melatonin levels in human AD brains further substantiates the significance of these findings. Taken together, our study demonstrates for the first time a novel role of melatonin in the direct regulation of DAPK1, and synergistic targeting of DAPK1 by melatonin and DAPK1 inhibitors offers an attractive approach to block tau hyperphosphorylation-induced neurodegeneration in AD.

2 | MATERIALS AND METHODS

2.1 | Materials

(4Z)-4-(3-pyridylmethylene)-2-styryl-oxazol-5-one was obtained from Millipore-Sigma and was used to specifically suppress the kinase activity of DAPK1. Paclitaxel, nocodazole, luzindole, 4-P-PDOT, serotonin, and tryptamine were purchased from Millipore-Sigma. Cycloheximide (CHX) was purchased from Cell Signaling Technology and was used to inhibit the synthesis of proteins to measure the stability of DAPK1 protein. Paclitaxel was used to stabilize microtubules, and nocodazole was applied to disassemble microtubules. Luzindole and 4-P-PDOT were used to inhibit the activity of melatonin receptors.

2.2 | Cell culture

The human cervix carcinoma cell line HeLa was obtained from the American Type Culture Collection. The human neuroblastoma cell lines SH-SY5Y and SK-N-BE(2) were obtained from the Stem Cell Bank/Stem Cell Core Facility, SIBCB, CAS. HeLa cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). SH-SY5Y and SK-N-BE(2) cells were cultured in DMEM Nutrient Mix F12 containing 10% FBS. All media contained 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco; Thermo Fisher Scientific). The cultures were maintained at 37°C under 5% CO_2.

2.3 | Plasmid transfection

The 5'-end-Flag tagged and CMV promoter-driven mammalian expression vector pRK5 was used to clone the cDNA fragment encoding human DAPK1. The full-length cDNA of human WT tau was cloned into the mammalian expression vector pEGFP. HeLa and SK-N-BE(2) cells were transiently transfected with different plasmids using TurboFect transfection reagent (Thermo Fisher Scientific) by following the manufacturer's protocols.

2.4 | Quantitative RT-PCR assay

Total RNA was isolated from SH-SY5Y, SK-N-BE(2), and primary neuronal cells using NucleoZOL (Macherey-Nagel) according to the description of the manufacturer. Real-time qRT-PCR was carried out on the QuantStudio Real-Time PCR System (Applied BioSystems) under the following thermocycling conditions: 95°C for 3 min followed by 40 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 30 s. Each sample was analyzed in duplicate. To amplify human DAPK1, the primers hDAPK1-F (5'-TCAAGACAGGCACGGCAAT-3') and hDAPK1-R (5'-CCACCACGATAGGCATGTTG-3') were used. For mouse DAPK1 amplification, the primers mDAPK1-F (5'-GCACCCAAATGTCATCACCCT-3') and mDAPK1-R (5'-AAACAGCTCACCTCCTGCAAC-3') were used. For mouse melatonin receptor type 1a (MT1) amplification, the primers mMT1-F (5'-AAGAAGCTCAGGAACTCAGGGAA-3') and mMT1-R (5'-GTAGCAGTAACGGTTCATAGCGA-3') were used. For mouse melatonin receptor type 1b (MT2) amplification, the primers mMT2-F (5'-ACTTCGTGGGGAACCTGCTTG-3') and mMT2-R (5'-TTGAAGACAGAGCCAATGACACT-3') were used. For human and mouse GAPDH amplification, the primers hGAPDH-F (5'-AGCCTCAAGATCATCAGCAATG-3'), hGAPDH-R (5'-TGATGGCATGGACTGTGGTCAT-3'), mGAPDH-F (5'-AAGGTCGGTGTGAACGGATTTG-3'), and mGAPDH-R (5'-GTTGAGGTCAATGAAGGGGTCGT-3') were used. For the amplification of human and mouse 18S rRNA, the primers 18S rRNA-F (5'-TGTCTCAAAGATTAAGCCATGCA-3') and 18S rRNA-R (GCGACCAAAGGAACCATAACTG-3') were used.

2.5 | Primary cortical neuronal cell cultures in mice

Primary cultures of mouse embryonic cortical neurons were conducted according to the literature.[41,42] Briefly, embryonic neurons were dissociated from embryonic day 16 pregnant C57BL/6 mice and resuspended in minimal essential medium supplemented with 5% FBS,

5% horse serum, 100 μM L-glutamine, and 28 mM D-glucose. Cells were plated onto poly-D-lysine-coated 6-well plates and maintained in a serum-free medium system with neurobasal media supplemented with cytosine β-D-arabinofuranoside to prevent the proliferation of glial cells. Cortical neuronal cells were used to determine the expression or protein stability of endogenous DAPK1 (at 1 week) and neurite outgrowth (at 2 days).

2.6 | Organotypic brain slice cultures

The culture of organotypic mouse brain slice was prepared as described previously[43] with minor modifications. Briefly, brains were collected from 3- or 4-month-old htau mice and cut into 300-μm-thick coronal slices by using a vibratome (Leica VT1000s). The expression and phosphorylation of endogenous tau in the hippocampus were measured for each brain slice. The slices were cultured on organotypic Millicell cell culture inserts (Millipore-Sigma) in 6-well culture plates with culture medium.

2.7 | Ubiquitination assay

HeLa cells were transiently transfected with Flag-DAPK1 and His-ubiquitin using TurboFect transfection reagent (Thermo Fisher Scientific) according to the description of the manufacturer for 24 h. After washing with PBS, the cells were treated with or without 12.5 μM MG-132 for 30 min followed by melatonin (0, 0.5 or 1.0 μM) treatment for 9 h. After the PBS washing, the cells were frozen with liquid nitrogen and kept at −80°C overnight. The cells were incubated with lysis urea buffer (40 mM Tris-HCl (pH 7.5), 6 M urea, 20 mM imidazole, and 0.5% Triton X-100) at 4°C for 30 min with rotation. Cell lysates were incubated on ice for sonication every 10 min and centrifuged at 18 000 *g* at 4°C for 10 min. The supernatant was collected, and 40 μL of Ni-NTA agarose (Invitrogen; Thermo Fisher Scientific) was added. The lysates with beads were rotated for 3 h at 4°C. After washing 3 times with lysis urea buffer and once with washing buffer (40 mM Tris-HCl and 50 mM NaCl, pH 7.5), the lysates were eluted in 50 μL of SDS sample buffer, boiled for 10 min, and analyzed by immunoblotting assay.

2.8 | Synthesis of biotin-melatonin

A dried 100-mL two-neck flask equipped was charged with biotin (1.00 mmol) and dichloromethane (5.0 mL). The mixture was stirred in nitrogen at room temperature for 10 min. Subsequently, $POCl_3$ (1.0 mL) was added using a glass syringe, and the reaction mixture was stirred under reflux conditions for 4 h at 40°C. After the reaction was completed, the solution of the crude product was concentrated in a vacuum drier. Then, the crude product was dissolved in tetrahydrofuran (THF). The reaction mixture was stirred in an ice bath, and trimethylamine (1.5 mL) was added within 5 min. Then, melatonin solution (1.00 mmol melatonin dissolved in THF) was added within 5 min. The solution was stirred at room temperature for 2 h. After the reaction was completed, the solvent was removed in vacuum. The residue was purified by silica gel column chromatography to obtain the corresponding product.

2.9 | DAPK1 pull-down assay using biotin-melatonin

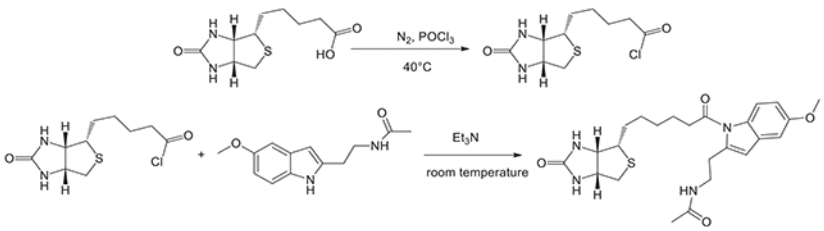
For competition assay of biotin-melatonin and melatonin, HeLa cells transfected with DAPK1 were incubated with melatonin for 12 h at 4°C. After lysis with pull-down buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, and 0.5% NP40), the lysates were incubated with 4 μM biotin-melatonin at 4°C for 2 h, and then, 15 μL of 50% NeutrAvidin beads (Pierce, Thermo Fisher Scientific) that had preincubated overnight with the pull-down buffer containing 0.1 mg/mL BSA was added. After 1 h rotation, the beads were washed 5 times with the pull-down buffer with 0.1 mg/mL BSA. DAPK1 proteins were eluted in SDS sample buffer, boiled for 10 min, and analyzed by immunoblotting assay.

2.10 | Immunohistochemical and immunoblot analyses

Immunohistochemical and immunoblot analyses were conducted as described previously.[35-37] The antibodies used in the current study are detailed in Table 1, and each antibody validation has been performed using various cell lines and mice (Figure S1).

2.11 | Neurite outgrowth assay

Primary embryonic neurons were treated with melatonin (0.2 μM) and DAPK1 inhibitor (0.5 μM) for 12 h before neuronal differentiation. Once neurons developed neurites, they were fixed with 4% PFA for 15 min at room temperature. For immunofluorescence analysis, the slides were blocked with 7% serum in PBS at room temperature for 1 h and then incubated with anti-Tuj1 antibody at a 1:250 dilution overnight at 4°C. After extensive washing with PBS, the slides were incubated with Alexa 546-labeled anti-rabbit IgG (Invitrogen; Thermo Fisher Scientific) at a 1:200 dilution and Hoechst 33 342 at a 1:2000 dilution for 2 h at room temperature. The levels of neurite outgrowth were evaluated by counting the number of neurites per cell and the average neurite length per differentiated cell. These parameters were quantified in ten images randomly chosen from three independent experiments. Cell with at least one neurite with a length equal to the cell body diameter was defined as differentiated neurons.[35] Neurites were defined as branches derived from the soma and having a length longer than the diameter of the cell body.[35]



2.12 | In vivo microtubule assembly assay

Primary neurons were incubated with paclitaxel (0.2 μM) and nocodazole (2 μM) for 12 h and melatonin (0.2 μM) and DAPK1 inhibitor (0.5 μM) for 48 h. After the treatment, cells were harvested and lysed at 37°C for 5 min with hypotonic buffer (20 mM Tris-HCl (pH 6.8), 2 mM EGTA, 1 mM $MgCl_2$, and 0.5% NP-40) in the presence of protease inhibitor cocktail. Cell lysates were then subjected to centrifugation at 18 000 × *g* at 25°C for 10 min. The unpolymerized tubulins were remained soluble in the supernatants and collected to a

fresh tube. The pellets were resuspended using an identical volume of hypotonic buffer and sonicated in ice bath for 2 min to dissolve polymerized (insoluble) tubulin. SDS-PAGE was performed to separate protein samples (10 μg) from both fractions.

2.13 | Animals and human brain samples

Pregnant female C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center. Htau mice were purchased from the Jackson Laboratory (No. 005491). All animal generation, husbandry, and experimental procedures were approved by the Experimental Animal Ethics Committee of Fujian Medical University according to the Association for Assessment and Accreditation of Laboratory Animal Care International regulations. Brain hippocampal tissues from human Alzheimer's patients and controls were obtained from the Harvard Brain Tissue Resource Center (Boston, MA, USA). All studies on human samples were reviewed and approved by the Institutional Review Boards at the Beth Israel Deaconess Medical Center. Experiments were carried out in accordance with the regulations in the Federal Policy for the Protection of Human subjects. In most cases, brain hippocampal tissues obtained within 30 h *postmortem* were used.

2.14 | Statistical analysis

Experimental data are presented as the means ± standard error of three independent experiments. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison *post hoc* test was applied to calculate the statistical significance using the GraphPad Prism 7, and $P < .05$ were considered statistically significant.

3 | RESULTS

3.1 | Melatonin reduces endogenous DAPK1 protein levels in a dose- and time-dependent manner

Since it has been reported that melatonin levels are decreased and DAPK1 expression is increased in human AD patient samples, we first investigated the effect of melatonin on the expression of DAPK1 protein in cells. Different concentrations of melatonin were administered to human neuroblastoma cells, that is, SH-SY5Y and SK-N-BE(2) cells, and DAPK1 expression was measured by an immunoblotting assay. While DAPK1 expression was not changed after the administration of DMSO and 0.01 or 0.1 μM melatonin, the expression levels of DAPK1 were decreased after the administration of melatonin at concentrations of 0.2, 0.5, and 1 μM (Figure 1A-D). We further examined DAPK1 protein expression after melatonin treatment in mouse primary cortical neurons. In contrast, with that in DMSO control-treated cells, endogenous DAPK1 expression was dramatically decreased in melatonin-treated cells in a dose-dependent manner (Figure 1E,F). In addition, melatonin effectively reduced DAPK1 levels in a time-dependent manner (Figure 1G-L). Moreover, other indoleamines such as serotonin or tryptamine did not affect DAPK1 expression (Figure 1M,N). These results suggest that melatonin might specifically regulate DAPK1 expression at the transcriptional or post-transcriptional level. However, DAPK1 mRNA levels were not decreased in neuronal cells or primary cortical neurons treated with melatonin, serotonin, or tryptamine compared to DMSO control cells by 18S (Figure 2) and GAPDH control mRNAs (Figure S2), suggesting that melatonin affects DAPK1 at the

protein level rather than at the level of transcription. Taken together, our data show that melatonin reduces the protein level of DAPK1 in cells.

3.2 | Melatonin decreases DAPK1 protein stability and promotes its ubiquitination

Given that melatonin decreased endogenous DAPK1 protein levels without changing its mRNA levels, we asked whether melatonin might affect the stability of DAPK1 protein. To examine whether melatonin promotes the turnover of DAPK1 in vivo, we administered melatonin to primary cortical neurons and then monitored DAPK1 protein stability after treatment with CHX to terminate the synthesis of new protein, as previously mentioned.[35] The half-life of endogenous DAPK1 was significantly decreased to less than 6 h in the presence of melatonin, but DAPK1 protein was stable in the absence of melatonin (Figure 3A,B). We measured whether reduced DAPK1 levels affect its enzymatic activity using a well-known DAPK1 substrate. We previously showed that DAPK1 directly phosphorylates Pin1 Ser71.[35,44] Indeed, phosphorylated Pin1 Ser71 levels were reduced along with DAPK1 reduction, suggesting that melatonin decreases DAPK1 enzymatic activity through regulating DAPK1 stability (Figure 3A). It has been reported that DAPK1 is degraded by the ubiquitin-mediated proteasome pathway to regulate DAPK1 protein stability.[45-49] To examine whether melatonin can decrease DAPK1 protein stability via a proteasome-dependent degradation pathway, we added MG-132 to suppress the activity of proteasome and then administered melatonin. In the presence of MG-132, DAPK1 protein was stable after treatment with melatonin compared to DMSO control (Figure 3C,D), indicating that melatonin regulates DAPK1 protein stability through the proteasome pathway. To investigate whether melatonin promotes the ubiquitination of DAPK1 in cells, we examined the effects of melatonin on the ubiquitination of DAPK1 by cotransfecting DAPK1 and His-tagged ubiquitin into cells and then adding melatonin and MG-132. In the presence of melatonin, His-ubiquitin, and MG-132, DAPK1 was substantially polyubiquitinated (Figure 4A,B). However, ubiquitinated DAPK1 was barely detected in the absence of His-ubiquitin or MG-132 (Figure 4A,B), indicating the specificity of the in vivo ubiquitination assay. Moreover, the ubiquitination of DAPK1 was completely abrogated in the absence of melatonin (Figure 4C,D). Furthermore, melatonin increased DAPK1 ubiquitination in a dose-dependent manner (Figure 4E,F). These results indicate that melatonin not only reduces the half-life of DAPK1 but also promotes the ubiquitination of DAPK1 in cells.

3.3 | Melatonin directly binds to DAPK1

It has been shown that melatonin exerts its cellular effects by interacting with several intracellular proteins such as quinone reductase 2, calreticulin, vitamin D receptor, and tau.[50-54] To examine whether melatonin interacts with DAPK1 in cells, we synthesized a biotinylated arsenate compound (biotin-melatonin). Then, a biotin-melatonin pull-down assay was performed in cells transfected with DAPK1. Biotin-melatonin efficiently bound to DAPK1, and the binding was competed by melatonin (Figure 5A). Next, to identify which domain in DAPK1 is important for its interaction with melatonin, we tested a series of truncated DAPK1 variants in a pull-down experiment (Figure 5B). DAPK1 fragments of 637 to 1423 and 848 to 1423 were unable to bind to melatonin. In contrast, the DAPK1 fragments of 1 to 1423 and 1 to 636 efficiently bound to melatonin, suggesting that the kinase domain (KD) or ankyrin repeat (AR) domain of DAPK1 is likely bound to melatonin

(Figure 5C). To determine whether melatonin directly binds to the KD or AR domain, we purified GST-DAPK1-KD and GST-DAPK1-AR and further examined the in vitro interaction using GST pull-down experiments. GST-DAPK1-AR, but not control GST or GST-DAPK1-KD, directly bound to biotin-melatonin (Figure 5D). To examine whether melatonin directly inhibits DAPK1 activity, we performed in vitro kinase assay. (4Z)-4-(3-pyridylmethylene)-2-styryl-oxazol-5-one (DAPK1-i) was chosen to inhibit the activity of DAPK1 as a positive control. DAPK1-i binds to DAPK1 kinase domain and selectively inhibits the activity and function of DAPK1.[35,55] Our previous research has demonstrated the efficacy and specificity of this compound as it alleviates DAPK1 activity-dependent neuronal death.[37] Recombinant DAPK1 (1-666) was incubated with GST-Pin1 in the presence of DMSO, melatonin, or DAPK1-i and ATP, followed by immunoblotting analysis. While phosphorylated Pin1 Ser71 levels were reduced with the DAPK1-i, the levels of phosphorylated Pin1 Ser71 were not changed with DMSO or melatonin, indicating that melatonin is not a direct inhibitor of DAPK1 activity and might regulate its function through DAPK1 protein stability (Figure 5E). Thus, melatonin directly binds to DAPK1, increases DAPK1 ubiquitination, and promotes its degradation through the proteasome pathway.

3.4 | DAPK1 levels are regulated by melatonin, but not melatonin receptors

To further characterize the molecular mechanisms underlying the melatonin-mediated suppression of DAPK1 expression we tested the effects of luzindole, which is an antagonist of MT1 and MT2, and 4P-PDOT, which is an antagonist of MT2. First, both melatonin receptors were expressed in mouse primary cortical neurons (Figure 5F). DAPK1 levels after melatonin treatment were reduced compared to those after DMSO control treatment (Figure 5G,H). However, treatment with luzindole plus melatonin did not abolish the effects of melatonin-mediated DAPK1 expression in mouse primary cortical neurons (Figure 5G,H). Similarly, treatment with 4P-PDOT plus melatonin decreased DAPK1 levels (Figure 5I,J). Phosphorylated Pin1 Ser71 levels were also reduced with melatonin plus melatonin receptor antagonists (Figure 5G,I). Thus, these results indicate that melatonin directly affects DAPK1 levels independent of melatonin receptors.

3.5 | Melatonin and a DAPK1 inhibitor synergistically reduce tau hyperphosphorylation

Given the specific regulation of DAPK1 protein stability by melatonin, we next aimed to clarify whether this interaction is relevant to the pathogenesis of AD. Aberrantly, phosphorylated tau protein aggregates into fibrils with abnormal conformations during tau-related pathologies, including AD.[9,56] DAPK1 plays important roles in regulating tau phosphorylation and is implicated in tau-related pathologies in AD.[35,38-40] Previous research from our group and others demonstrated that Pin1 modulates the stability and phosphorylation of tau and alters its function in tau-related pathology[43,57-60] and that the activity of Pin1 is negatively regulated by DAPK1 through phosphorylating the Ser71 residue.[44] To unravel the underlying mechanisms by which DAPK1 regulates tau phosphorylation by melatonin, we used ex vivo brain slice cultures from htau mice,[61] which were generated by crossing mice expressing a human PAC, H1 haplotype-derived tau transgene with tau knockout mice, and treated them with both melatonin and a DAPK1 inhibitor. Htau mice develop robust tau-related pathologies with tau hyperphosphorylation in an age-dependent manner.[61,62] In contrast to DMSO control, melatonin or the DAPK1-i

reduced total tau levels as well as the phosphorylation of tau in htau brain slices, as measured by Thr231-, Ser262-, and Ser396- specific antibodies against specific tau phosphorylation sites and/or abnormal NFT-specific conformations in AD (Figure 6A,B). Moreover, simultaneous treatment with melatonin and the DAPK1-i dramatically decreased all tau phosphoepitopes detected (Figure 6A,B), suggesting that melatonin and the DAPK1 inhibitor synergistically reduced total tau and phosphorylated tau in htau brain slices. Furthermore, compared with DMSO, melatonin and/or the DAPK1-i reduced the Ser71 phosphorylation of Pin1 (Figure 6A,C), indicating that the decreased phosphorylation of tau in melatonin- or DAPK1-i-treated slices was due to the phosphorylation of Pin1. The effects of DAPK1 inhibition by melatonin and the DAPK1 inhibitor on the phosphorylation of tau and Pin1 in human neuronal cells were further evaluated using an immunoblotting assay with Thr231-, Ser262-, Ser396-, and pSer71-Pin1 antibodies. Compared with DMSO control, melatonin plus the DAPK1-i significantly reduced the phosphorylation levels of tau and Pin1 in tau-overexpressing SK-N-BE(2) cells (Figure 6D-F). Thus, these results showed that combined treatment with melatonin and the DAPK1 inhibitor significantly reduced tau and Pin1 phosphorylation in human neuronal cells as well as brain slice cultures, an in situ model that resembles and retains many physiological properties of living brain tissue.

3.6 | Melatonin promotes neurite outgrowth and microtubule assembly

The site-specific phosphorylation of tau is important in regulating microtubule dynamics and neuronal differentiation.[63-67] Considering that melatonin suppresses tau phosphorylation by decreasing DAPK1 protein stability, we investigated the biological significance and possible application of the combined treatment of melatonin and DAPK1 inhibitor on the function of tau by determining neurite outgrowth and microtubule assembly. Treatment with DMSO resulted in low-level neurite outgrowth and maturation, whereas treatment with melatonin or the DAPK1-i moderately increased neurite outgrowth and the maturation of mouse primary cortical neurons (Figure. However, the simultaneous addition of both melatonin and the DAPK1-i to mouse primary cortical neurons significantly increased neurite outgrowth and maturation (Figure 7A-C). Since tau is primarily involved in modulating the stability and function of cytoskeletal microtubule[63-65] and since the abnormal phosphorylation of tau disrupts the normal organization of microtubule,[68-70] we also investigated the effect of melatonin and the inhibition of DAPK1 on microtubule organization. While melatonin or the DAPK1-i alone moderately increased the protein level of polymerized tubulin in the pellet, simultaneous treatment with melatonin and the DAPK1-i at significantly increased these levels, showing that greater effects were induced by microtubule-stabilizing paclitaxel (Figure 7D,E). However, microtubule-disrupting nocodazole inhibited tubulin polymerization (Figure 7D,E). Therefore, these results suggest that the suppression of DAPK1 expression and activity by melatonin and the DAPK1 inhibitor greatly increases neuronal differentiation and microtubule polymerization by affecting tau functions.

3.7 | Melatonin levels are inversely correlated with DAPK1 expression in human AD brains

It has been reported that the expression of DAPK1 is highly elevated in human AD brains[35-37] and that melatonin contents are decreased in AD patients.[10-12,71-75] Given that melatonin inhibits DAPK1 function through a direct association and that DAPK1 inhibition

reduces tau hyperphosphorylation and promotes neurite outgrowth and microtubule assembly, we asked whether melatonin levels correlate with DAPK1 levels in human normal and AD brain tissues (Table 2). To investigate the in vivo correlation between endogenous melatonin and DAPK1 expression in human tissues, immunohistochemistry staining was conducted on serial sections of brain tissue from normal individuals and AD patients using specific antibodies against melatonin and DAPK1. Based on the immunohistochemical assay, DAPK1 levels are increased markedly in the hippocampal neurons of Alzheimer's patients compared with age-matched normal brains (Figure 8A,C). Interestingly, we found that melatonin levels are decreased in AD brains compared to control brains (Figure 8B,D). Moreover, there were also inverse correlations between DAPK1 and melatonin levels in human brains, including the brains 8 normal subjects and 8 AD patients, as indicated by the Pearson correlation coefficient ($r = -0.7680$) (Figure 8E). Thus, these results show that melatonin levels are downregulated in human AD brains and that these levels might be inversely correlated with DAPK1 expression.

4 | DISCUSSION

Although cumulative research indicates that melatonin and DAPK1 may have essential roles in the development of AD, the molecular link between melatonin and DAPK1 remains elusive. In this study, we discovered that melatonin critically regulates DAPK1 by reducing its protein stability, thereby attenuating tau hyperphosphorylation in AD. We first found that melatonin, but not other indoleamines, downregulates DAPK1 protein levels rather than its transcriptional levels, suggesting that melatonin might specifically regulate DAPK1 protein stability. We demonstrated that melatonin-mediated DAPK1 degradation is regulated by the ubiquitination via the proteasome pathway. Since melatonin directly binds to the AR domain of DAPK1 that facilitates protein-protein interactions and is involved in the degradation of DAPK1 through the ubiquitination[30,45] and does not inhibit DAPK1 activity in vitro kinase assay system, melatonin might destabilize DAPK1 protein through the ubiquitin-proteasome pathway, thereby decreasing DAPK1 function. Interestingly, three E3 ubiquitin ligases, carboxyl terminus of HSC70-interacting protein (CHIP), DAPK-interacting protein 1 (DIP1), and KLHL20, have been shown to target and degrade DAPK1 through the ubiquitin-proteasome pathway.[45-49] Further studies are required to determine whether melatonin regulates DAPK1 degradation through known DAPK1 ubiquitin ligases.

Tau is important in regulating neurite outgrowth and microtubule structural integrity and the neuronal transportation of proteins and other molecules.[76] In AD brains, hyperphosphorylated tau is aggregated into paired helical filaments (PHFs) with abnormal conformations and eventually forms NFTs.[9,56] We found that the suppression of DAPK1 expression by melatonin and the inhibition of its activity by a DAPK1 inhibitor synergistically reduces tau phosphorylation at AD-related sites, including Thr231, Ser262, and Ser396, and tau expression, suggesting that the inhibition of DAPK1 might offer promising options for reducing tau hyperphosphorylation. Moreover, melatonin-mediated decreases in tau protein expression and phosphorylation are accompanied by decreased Pin1 phosphorylation at Ser71. We previously reported that DAPK1 phosphorylates Ser71 in the catalytic site of Pin1 and inhibits its cellular function.[44] Pin1 plays a key role in protecting against neurodegeneration by restoring the conformation and function of phosphorylated tau

or by promoting its dephosphorylation.[43,57-60] Altogether, these results suggest that melatonin regulates the phosphorylation of tau through the DAPK1-mediated Pin1 phosphorylation pathway.

We were particularly interested in the potential association between DAPK1 and melatonin expression in human AD brains. It has been reported that kinases which are responsible for tau hyperphosphorylation are increased in AD.[77] In human AD, GSK3β, CDK5, p38, Erk1/2, and JNK1/2/3 activity or levels are increased.[78-84] We previously reported that DAPK1 is highly upregulated in human AD brains, and DAPK1 expression is accompanied by hyperphosphorylated tau or Aβ deposits.[35-37] Moreover, melatonin is decreased in the serum and cerebrospinal fluid (CSF) of AD patients.[10-12,71-75] We measured DAPK1 and melatonin levels in the hippocampi of human brain tissues because in AD, the hippocampus manifests earlier and more severe neuronal damages than other cortical regions. Our data showed that melatonin levels are inversely correlated with DAPK1 expression in AD, suggesting that melatonin might be an upstream regulator or DAPK1. However, loss of melatonin may not be enough to increase DAPK1 protein stabilization in AD brain. DAPK1 expression and/or stability has been regulated by multiple mechanisms such as transcription, microRNAs, ubiquitination, and post-translational modifications.[30] Therefore, multiple factors including melatonin might be involved in the regulation of DAPK1 levels in the development of AD. It has not yet been determined what molecular steps and how much melatonin contribute to DAPK1 protein stability in AD. Moreover, the inverse relationship between melatonin and DAPK1 could be affected by disease stages, age, gender, race, sample preparation, and the time of patients' death (daytime vs nighttime). A large number of postmortem normal and human AD brain samples and the newly developed brain molecular imaging techniques in AD patients will facilitate investigation of this possibility.

Although DAPK1 expression is not affected by melatonin receptors, it cannot be ruled out that they might be connected in the regulation of mechanisms of AD progression. Indeed, melatonin receptors have been shown to decrease Aβ levels by reducing nuclear Pin1 levels in neuronal cells.[85] Additional studies are needed to determine whether DAPK1 has a play in melatonin receptor-mediated mechanisms and whether melatonin also regulates Aβ secretion through DAPK1. Since tau accumulation and phosphorylation are critical events in AD, our finding of the effect of melatonin in reducing DAPK1-mediated tau accumulation and phosphorylation substantiates the need for further studies on the effects of melatonin in attenuating AD via the downregulation of DAPK1 using AD animal models. Moreover, it might be interesting to determine whether treatment with both melatonin and a DAPK1 inhibitor synergistically rescue age-related cognitive impairment in AD transgenic mouse models. Thus, intensive cross-talk between melatonin and DAPK1 needs to be studied in animal models and human AD patients.

In summary, our data propose a model in which melatonin directly binds to DAPK1 and promotes DAPK1 degradation via the ubiquitin-mediated proteasome pathway, which results in decreased DAPK1-mediated Ser71 phosphorylation of Pin1. Combined treatment with melatonin and a DAPK1 inhibitor synergistically reduces tau accumulation and phosphorylation and promotes neurite outgrowth and microtubule assembly (Figure 9). Our work has thus identified a critical role of melatonin in regulating DAPK1 stability, tau

phosphorylation, and AD and may offer a potential novel therapeutic approach for human AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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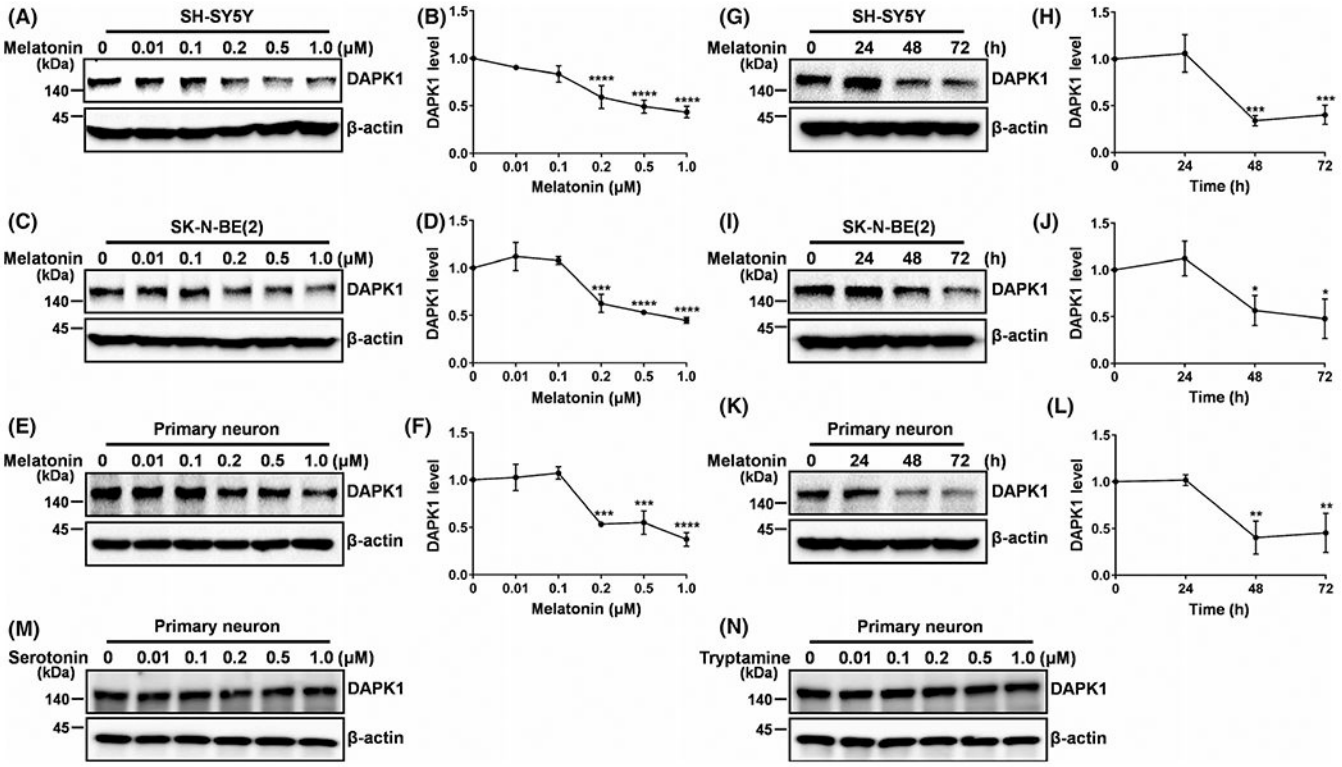

FIGURE 1.

Melatonin decreases DAPK1 protein levels. SH-SY5Y cells (A, B), SK-N-BE(2) cells (C, D), and primary neurons (E, F) were treated with various concentrations of melatonin (0, 0.01, 0.1, 0.2, 0.5, or 1.0 μM) for 48 h. SH-SY5Y cells (G, H), SK-N-BE(2) cells (I, J), and primary neurons (K, L) were treated with 0.2 μM melatonin for the indicated time (0, 24, 48, or 72 h). Primary neurons were treated with various concentrations (0, 0.01, 0.1, 0.2, 0.5, or 1.0 μM) of serotonin (M) and tryptamine (N) for 48 h. The cell lysates were subjected to immunoblotting analysis with an anti-DAPK1 or anti-β-actin antibody (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$ *vs.* DMSO control; ANOVA/Dunnett's test). All blots are representative of three independent experiments, and the data shown represent the means ± standard errors of three independent experiments

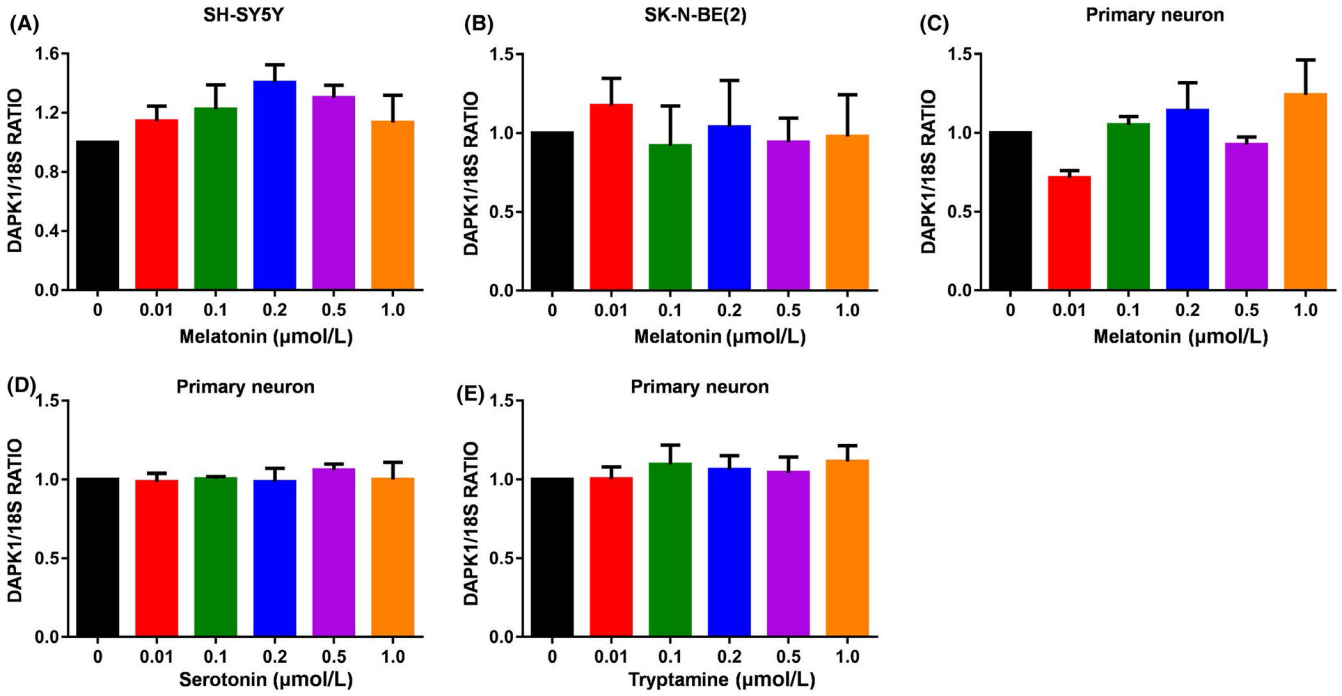

FIGURE 2.

DAPK1 mRNA levels are not changed by melatonin. SH-SY5Y cells (A), SK-N-BE(2) cells (B), and primary neurons (C) were treated with various concentrations of melatonin (0, 0.01, 0.1, 0.2, 0.5, or 1.0 μM) for 48 h. Primary neurons were treated with various concentrations (0, 0.01, 0.1, 0.2, 0.5, or 1.0 μM) of serotonin (D) and tryptamine (E) for 48 h. Quantitative RT-PCR was performed on cDNA samples to measure human or mouse DAPK1 mRNA, and the 18S gene was used as an endogenous control. Each data point represents the mean ± standard error of three independent experiments

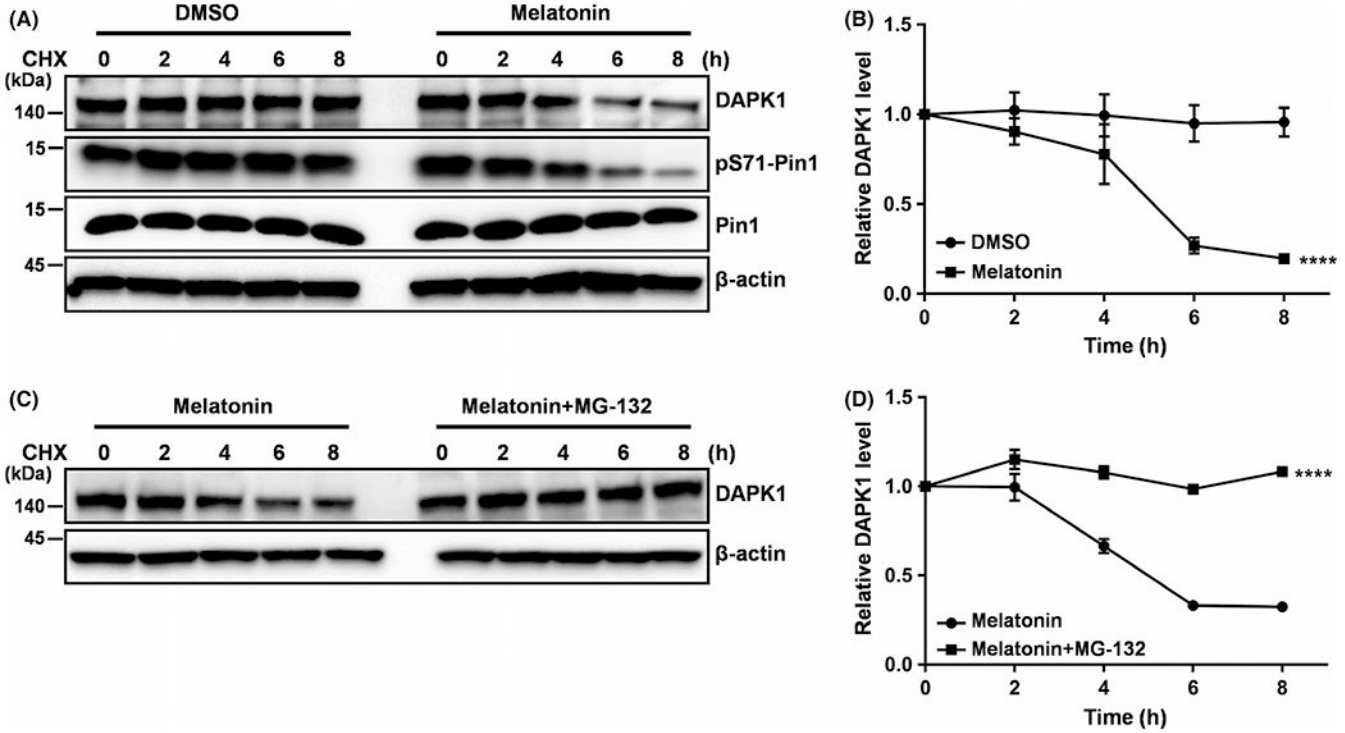

FIGURE 3.

Melatonin decreases DAPK1 protein stability. (A, B) Primary neurons were treated with 0.2 μM melatonin for 24 h and then treated with 10 μg/mL CHX for the indicated time. The cell lysates were subjected to immunoblotting analysis with an anti-DAPK1, anti-pSer71-Pin1, anti-Pin1, or anti-β-actin antibody (****$P < .0001$ vs. DMSO control; ANOVA/Dunnett's test). (C, D) Primary neurons were treated with 12.5 μM MG-132 for 30 min and then 0.2 μM melatonin for 24 h and then treated with 10 μg/mL CHX for the indicated time. The cell lysates were subjected to immunoblotting analysis with an anti-DAPK1 or anti-β-actin antibody (****$P < .0001$ *vs.* DMSO control; ANOVA/Dunnett's test). All blots are representative of three independent experiments, and the data shown represent the means ± standard errors of three independent experiments

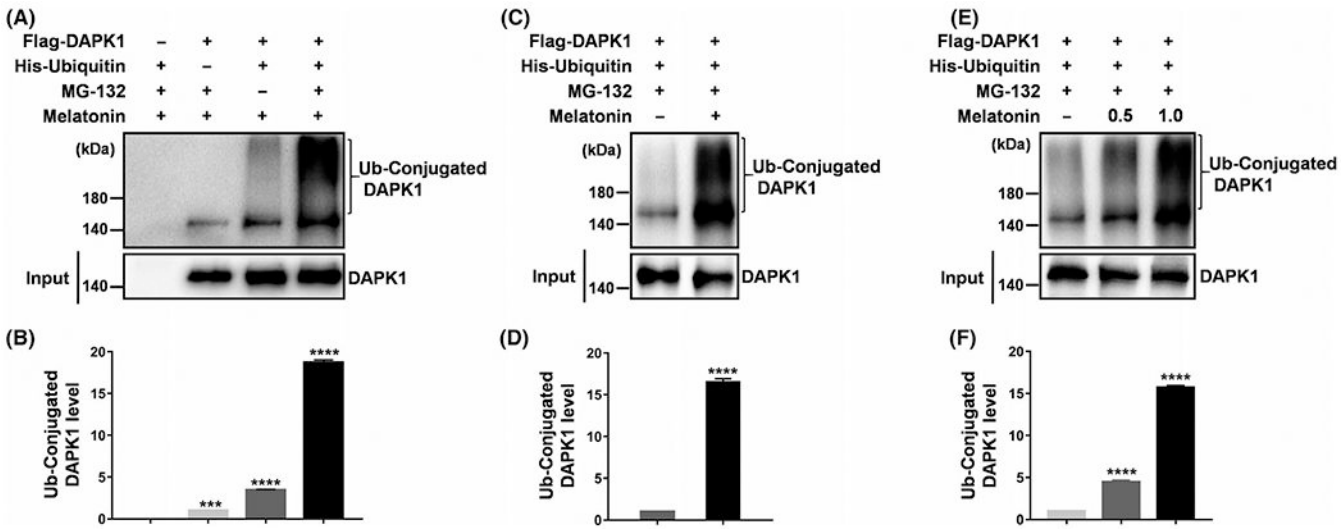

FIGURE 4.

Ubiquitination of DAPK1 by melatonin in vivo. (A, B) HeLa cells expressing DAPK1 and His-tagged ubiquitin or vector controls were treated with 0.5 μM melatonin for 12 h and then treated with 12.5 μM MG-132 or DMSO for 9 h, and ubiquitin-conjugated proteins were captured with Ni-beads and subjected to immunoblotting analysis with an anti-DAPK1 antibody (***$P < .001$, ****$P < .0001$; ANOVA/Dunnett's test). (C, D) HeLa cells expressing DAPK1 and His-tagged ubiquitin were treated with 0.5 μM melatonin or DMSO for 12 h and then treated with 12.5 μM MG-132 for 9 h, and ubiquitin-conjugated proteins were captured with Ni-beads and subjected to immunoblotting analysis with an anti-DAPK1 antibody (****$P < .0001$; ANOVA/Dunnett's test). (E, F) HeLa cells expressing DAPK1 and His-tagged ubiquitin were treated with various concentrations of melatonin (0, 0.5, or 1.0 μM) for 12 h and then treated with 12.5 μM MG-132 for 9 h, and ubiquitin-conjugated proteins were captured with Ni-beads and subjected to immunoblotting analysis with an anti-DAPK1 antibody (****$P < .0001$; ANOVA/Dunnett's test). All blots are representative of three independent experiments, and the data shown represent the means ± standard errors of three independent experiments

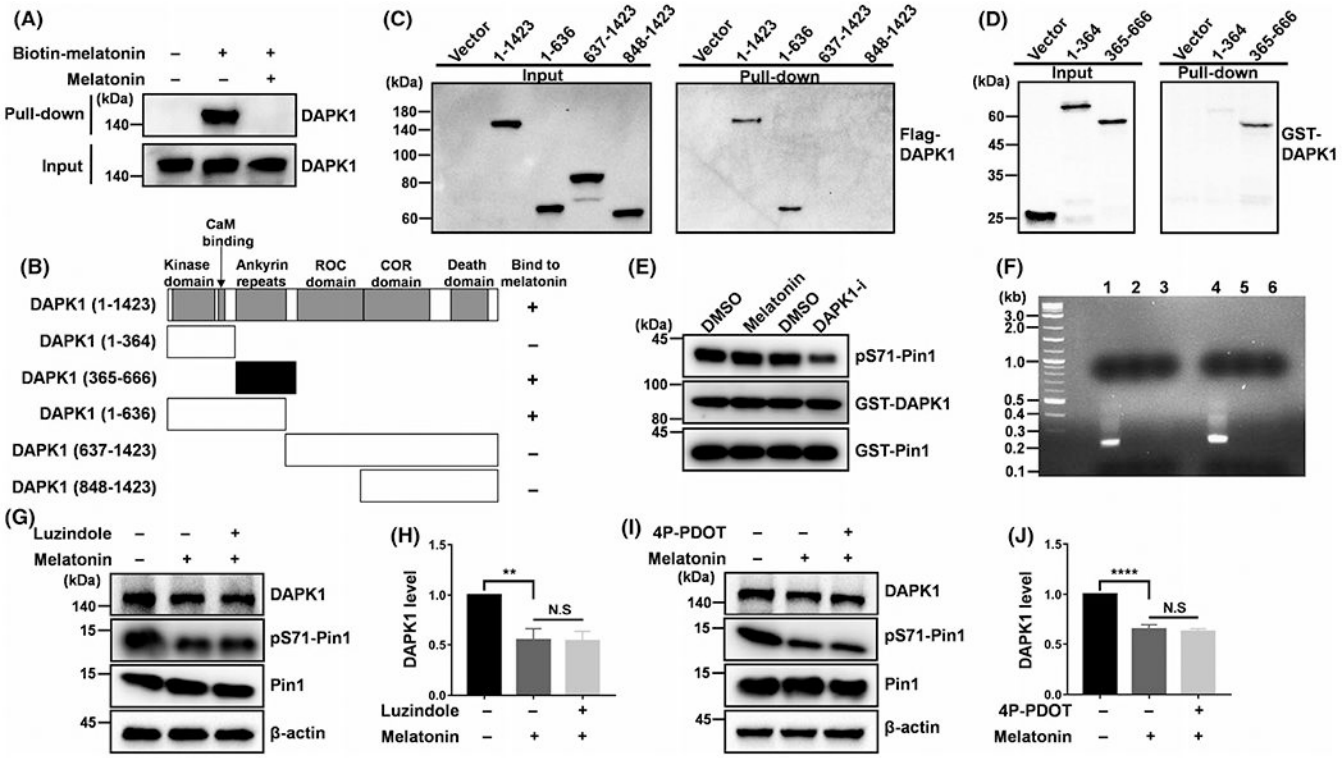

FIGURE 5.

Melatonin directly binds to DAPK1. (A) Biotin-melatonin binding to DAPK1 is competed by melatonin. DAPK1-overexpressing HeLa cells were treated with or without 10 μM melatonin for 12 h and then subjected to a biotin-melatonin pull-down assay followed by immunoblotting analysis with an anti-DAPK1 antibody. (B) Schematic representation of full-length DAPK1 and its truncated mutants. (C) Melatonin binds to the N-terminus of DAPK1. Biotin-melatonin was incubated with extracts of HeLa cells expressing Flag-DAPK1 or its truncated mutants. After washing, proteins pulled down by avidin beads were subjected to immunoblotting analysis with an anti-Flag antibody. (D) The ankyrin repeats of DAPK1 directly interact with melatonin. Purified GST-kinase or the ankyrin repeat domain of DAPK1 was incubated with biotin-melatonin. After washing, proteins pulled down by avidin beads were subjected to immunoblotting analysis with an anti-GST antibody. (E) Melatonin does not inhibit DAPK1 activity in vitro kinase assay system. Recombinant DAPK1 (1-666) and GST-Pin1 were incubated with DMSO, melatonin or a DAPK1 inhibitor, and ATP, followed by immunoblotting analysis with an anti-pSer71-Pin1, anti-DAPK1, or anti-Pin1 antibody. (F) Melatonin receptors are expressed in mouse primary cortical neurons. RT-PCR was performed on cDNA samples from primary neurons to detect mouse melatonin receptors mRNA, followed by agarose gel electrophoresis analysis with PCR products (1, primary neuron with primers of MT1;2,primary neuron without primers; 3, water with primers of MT1; 4, primary neuron with primers of MT2; 5, primary neuron without primers; and 6, water with primers of MT2). (G-J) DAPK1 expression was not changed by melatonin receptor antagonists. Primary neurons were treated with 2.0 μM luzindole (G, H) or 4P-PDOT (I, J) for 30 min and then treated with 0.2 μM melatonin for 48 h. The cell lysates were subjected to immunoblotting analysis with an anti-DAPK1, anti-

pSer71-Pin1, anti-Pin1, or anti-β-actin antibody (**$P < .01$, ****$P < .0001$ *vs.* DMSO control; ANOVA/Dunnett's test). NS, no significance. The blots are representative of three independent experiments, and the data shown represent the means ± standard errors of three independent experiments

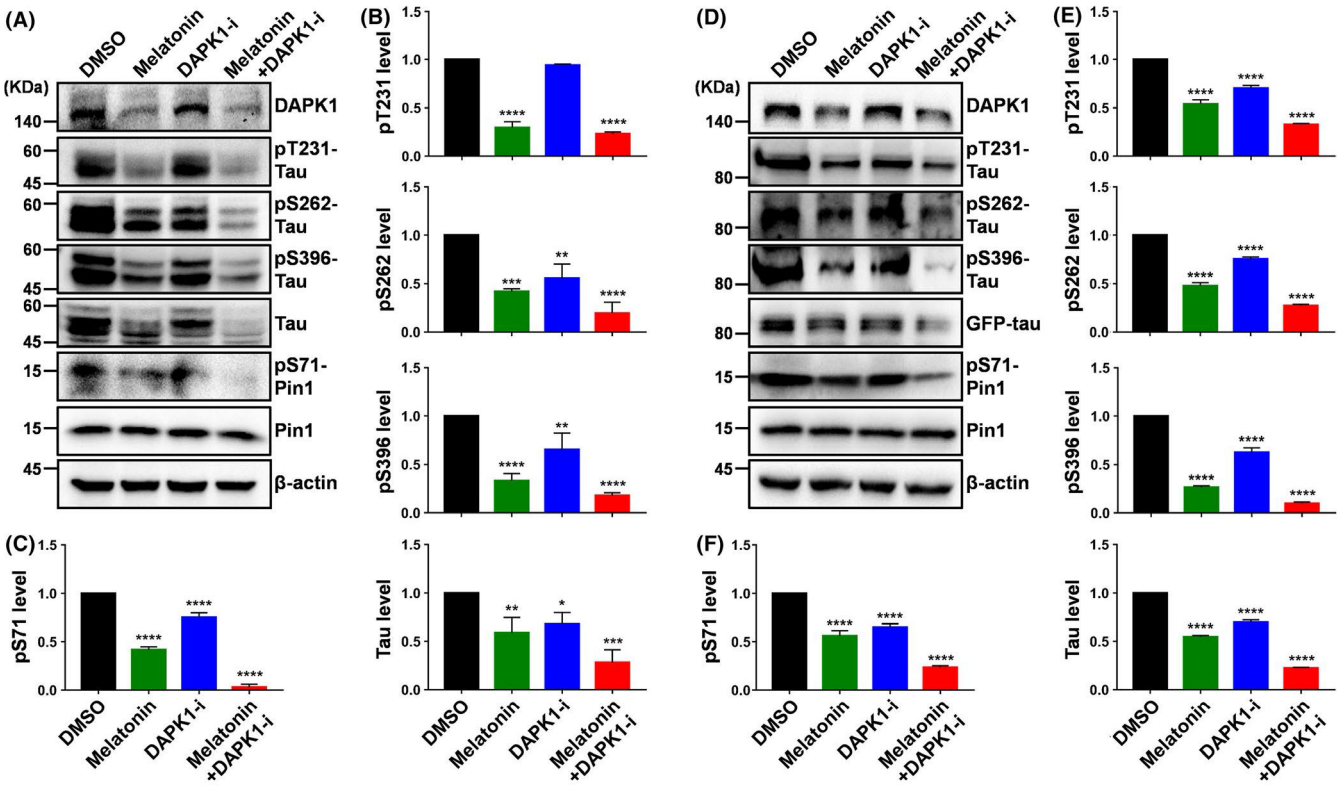

FIGURE 6.

Melatonin and a DAPK1 inhibitor synergistically reduce tau hyperphosphorylation. (A-C) Brain slices from human tau-overexpressing mice were treated with 0.75 μM melatonin or/and 4 μM DAPK1 inhibitor for 24 h. (D-F) SK-N-BE(2) cells were transfected with GFP-Tau and treated with 0.2 μM melatonin or/and 0.5 μM DAPK1 inhibitor for 48 h. The cell lysates were subjected to immunoblotting analysis with an anti-DAPK1, anti-pThr231-Tau, anti-pSer262-Tau, anti-pSer396-Tau, anti-Tau, anti-pSer71-Pin1, anti-Pin1, or anti-β-actin antibody (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$ *vs.* DMSO control; ANOVA/Dunnett's test). The blots are representative of three independent experiments, and the data shown represent the means ± standard errors of three independent experiments

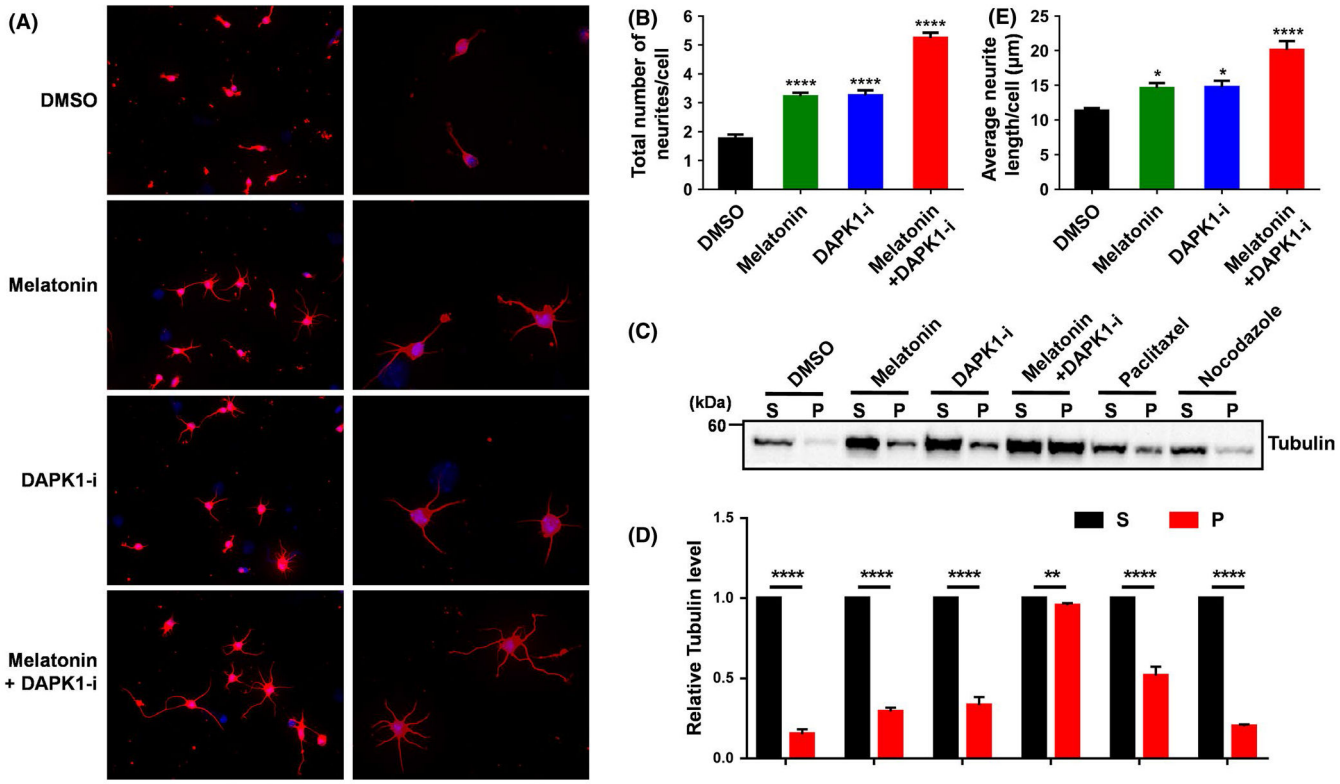

FIGURE 7.

Melatonin increases neurite outgrowth and microtubule assembly. (A) Primary neurons were treated with 0.2 μM melatonin or/ and 0.5 μM DAPK1 inhibitor for 12 h and subjected to an immunofluorescence assay with an anti-Tuj1 antibody. The total number of neurites per cell (B) and the average neurite length per cell (C) were quantified in ten random images (**P* < .05, *****P* < .0001 *vs.* DMSO control; ANOVA/Dunnett's test). The images are representative of three independent experiments, and the data shown represent the means ± standard errors of three independent experiments. (D, E) Primary neurons were treated with DMSO, 0.2 μM melatonin, or 0.2 μM melatonin plus 0.5 μM DAPK1 inhibitor for 48 h or 0.2 μM paclitaxel or 2 μM nocodazole for 12 h and then lysed with hypotonic buffer. Equal amounts of cytosolic (S, soluble) and cytoskeletal (P, polymerized) proteins were separated by SDS-PAGE and subjected to immunoblotting analysis with an anti-tubulin antibody. The tubulin expression level in the cytosolic/soluble fraction for each treatment was arbitrarily defined as '1' (***P* < .01, *****P* < .0001 *vs.* soluble control; ANOVA/Dunnett's test). The blots are representative of three independent experiments, and the data shown represent the means ± standard errors of three independent experiments

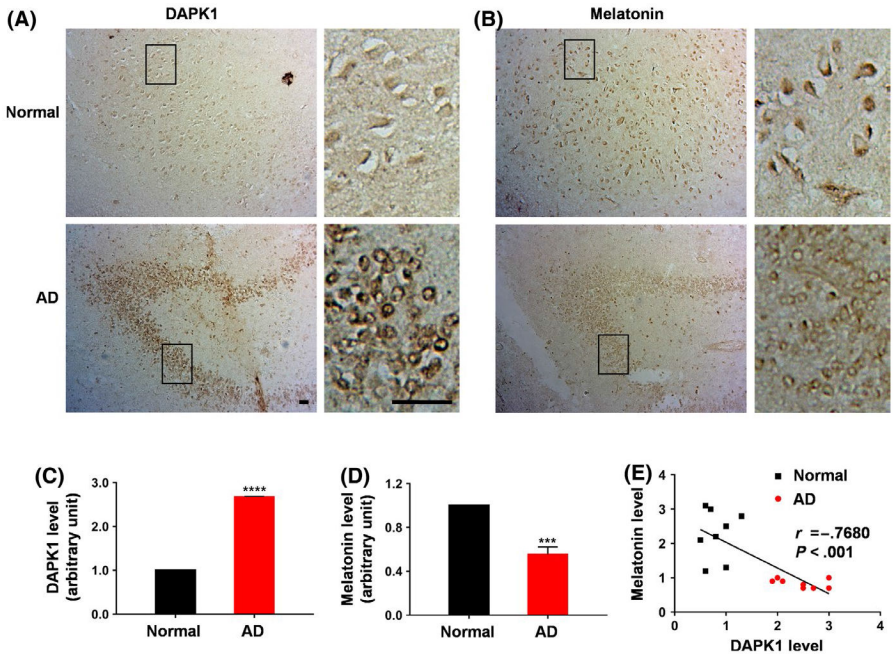

FIGURE 8.

DAPK1 levels are inversely correlated with melatonin levels in human AD brains. Immunohistochemistry using an anti-DAPK1 (A) or anti-melatonin (B) antibody was conducted on paraffin-embedded hippocampal sections from AD patients and normal controls. Scale Bar = 100 μm. (C-E) Quantitation of DAPK1 and melatonin staining intensity (arbitrary units, ***$P < .001$, ****$P < .0001$; ANOVA/Dunnett's test). The images are representative of five independent experiments, and the data shown represent the means ± standard errors of five independent experiments in a cohort of brain tissues from 8 AD patients and 8 normal subjects

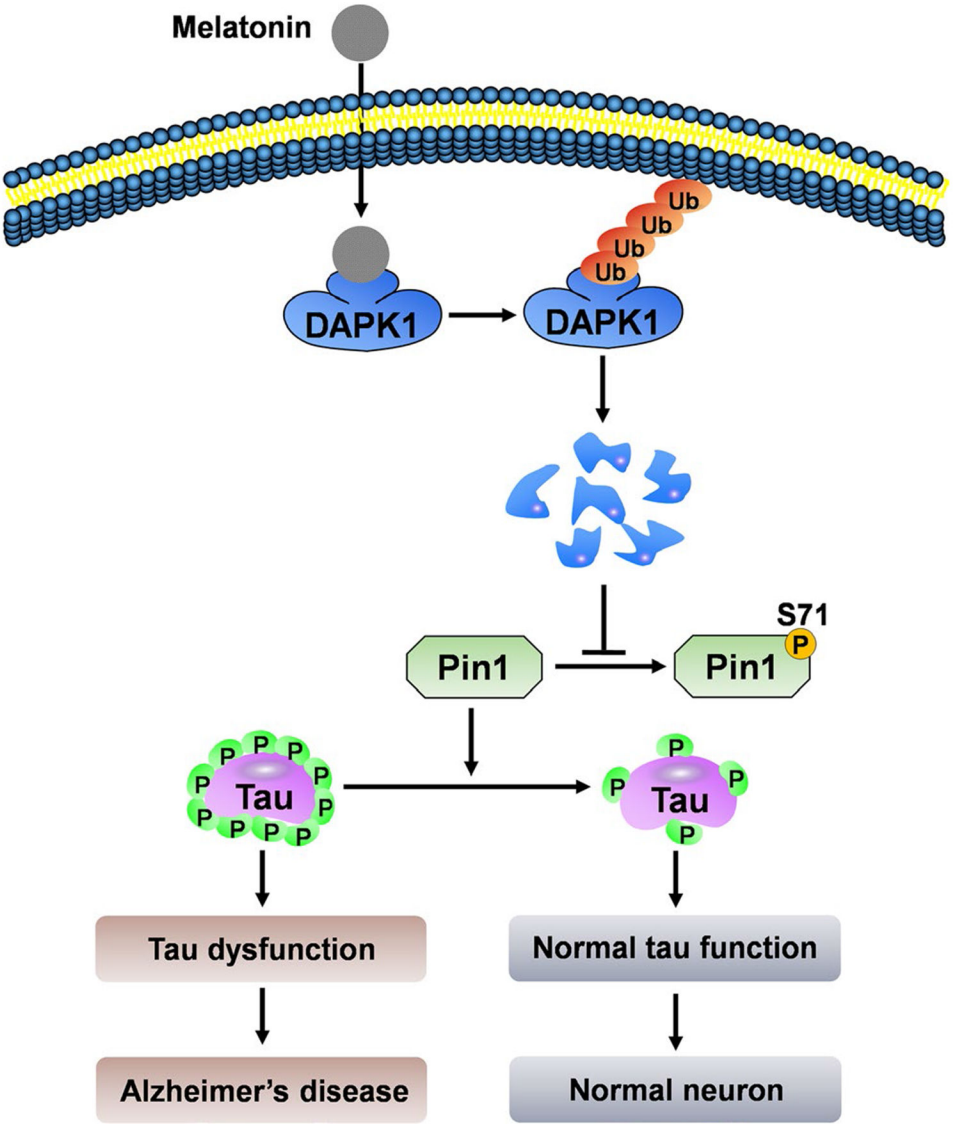

FIGURE 9.

Schematic diagram summarizing the proposed role of melatonin in the regulation of DAPK1 in AD. Under physiological conditions, melatonin directly binds to DAPK1 and increases its ubiquitin-mediated protein degradation, which results in decreased DAPK1-mediated Ser71 phosphorylation of Pin1. A reduction in Ser71 phosphorylation elevates Pin1 functions and attenuates tau hyperphosphorylation. However, the loss of melatonin induces DAPK1 levels and ultimately promotes tau hyperphosphorylation and tau-related pathology in the AD brain

TABLE 1

Primary antibodies used in this study

Antibodies	Dilutions	Source	Identifier
Mouse anti-β-actin	1:40 000 (WB)	Millipore-Sigma	Cat# A5441
Mouse anti-DAPK1	1:1000 (WB)	Millipore-Sigma	Cat# D2178
Rabbit anti-DAPK1	1:50 (IHC)	Millipore-Sigma	Cat# SAB4500620
Rabbit anti-Flag	1:1000 (WB)	Cell Signaling Technology	Cat# 2368S
Mouse anti-GST	1:2000 (WB)	Cell Signaling Technology	Cat# 2624S
Rabbit anti-melatonin	1:100 (IHC)	Abbexa	Cat# abx100179
Rabbit anti-Pin1	1:1000 (WB)	Proteintech	Cat# 10495-1-AP
Rabbit anti-pS71-Pin1	1:200 (WB)	Kun Ping Lu	44
Mouse anti-Tau	1:1000 (WB)	Cell Signaling Technology	Cat# 4019S
Rabbit anti-pT231-Tau	1:3000 (WB)	Thermo Fisher Scientific	Cat# 701 056
Rabbit anti-pS262-Tau	1:1000 (WB)	Thermo Fisher Scientific	Cat# 44750G
Rabbit anti-pS396-Tau	1:500 (WB)	Anaspec	Cat# AS-54977
Rabbit anti-α-Tubulin	1:500 (WB)	Cell Signaling Technology	Cat# 2125S
Mouse anti-Tuj1	1:250 (IF)	Beyotime	Cat# AT809

Abbreviations: IF, immunofluorescence; IHC, immunohistochemistry; WB, Western blotting.

TABLE 2

Characteristics of postmortem AD patients and age-matched control human hippocampus samples

No	NPDx	Gender	Age	PMI (h)	B&B	CERAD
1	Normal	M	64	28.60	I	A
2	Normal	F	63	26.83	I	A
3	Normal	F	82	24.42	I	A
4	Normal	F	79	17.58	II	A
5	Normal	F	91	30.10	II	A
6	Normal	F	83	29.40	I	A
7	Normal	M	83	13.00	I	A
8	Normal	F	66	25.00	II	A
9	AD	F	86	17.58	V-VI	C
10	AD	M	80	22	VI	C
11	AD	F	61	24.00	VI	C
12	AD	F	84	18.33	V	C
13	AD	F	87	29.58	V	C
14	AD	F	82	16.83	VI	C
15	AD	M	64	32.00	V	C
16	AD	F	83	22.92	VI	C

Abbreviations: CERAD, Consortium to Establish a Registry for Alzheimer's disease; F, female; M, male; NPDx, neuropsychological diagnosis; PMI, postmortem interval B&B (out of VI), Braak and Braak stage of AD.