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Effect of melatonin on calyculin A-induced tau hyperphosphorylation

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Abstract

We have found in the present study that incubation of neuroblastma N2a with calyculin A, an inhibitor of protein phosphatase-2A (PP-2A) and protein phosphatase-1 (PP-1), reduces cell viability in a dose-dependent manner, and leads to tau hyperphosphorylation at tau-1 (Ser198/199/202) and PHF-1 (Ser396/404) epitopes. In addition to inhibit PP-2A, calyculin A treatment also results in significant activation of glycogen synthase kinase-3 (GSK-3). Calyculin A induces oxidative stress manifested by elevated level of malondialdehyde and decreased activity of superoxide dismutase. When the cells were incubated simultaneously with calyculin A and melatonin (25 μM or 50 μM), the calyculin A-induced decrease in cell viability, tau hyperphosphorylation, PP-2A/GSK-3 imbalance and oxidative stress were attenuated accordingly. These results suggest (i) that calyculin A induces tau hyperphosphorylation not only by inhibition of PP-2A, but also by activation of GSK-3 in N2a cells; (ii) that melatonin efficiently attenuates the calyculin A-induced damages through not only its antioxidant effect but also its modulation to the phosphorylation system.

Keywords: Alzheimer disease; Calyculin A; Tau; Phosphorylation; Melatonin

1. Introduction

Alzheimer disease is pathologically characterized by the presence of numerous intracellular neurofibrillary tangles and extracellular β-amyloid plaques. The severity of dementia in Alzheimer disease patients correlates with the amounts of neurofibrillary tangles, which is composed of bundles of paired helical filaments formed by abnormally hyperphosphorylated tau (Grundke-Iqbal et al., 1986; Lee et al., 1991; Godert et al., 1992; Kanemaru et al., 1992); tau is a microtubule-associated phosphoprotein, abundant in neurons, and the phosphorylation of tau is regulated by protein kinases and protein phosphatases simultaneously. In Alzheimer disease brain, tau is aberrantly hyperphosphorylated (Kopke et al., 1993).

Both in vitro and in vivo studies have demonstrated that protein phosphatase-2A (PP-2A) and protein phosphatase-1 (PP-1) are two promising phosphatase responsible for Alzheimer-like tau hyperphosphorylation (Gong et al., 1993; Sontag et al., 1999; Wang et al., 1996; Yamamoto et al., 1995). Calyculin A is a phosphatase inhibitor with more potent inhibition to PP-2A than to PP-1. We have reported previously that injection of calyculin A into rat brain results in hyperphosphorylation of tau and impairment in spatial memory retention (Sun et al., 2003); and co-culture of calyculin A with SH-SY5Y neuroblastoma cells also causes neurofilament hyperphosphorylation (Li et al., 2004). However, the upstream sector leading to an imbalanced regulation of phosphorylation is still not understood, and there is no effective cure for arresting tau hyperphosphorylation.

Increasing evidence supports the involvement of oxidative stress in Alzheimer disease pathology (Markesbery and Carney, 1999; Calingasan et al., 1999; Smith et al., 1994, 2000; Zhu et al., 2000). Melatonin, a hormone secreted by the pineal gland, reduces the toxic effects caused by okadaic acid (Montilla-Lopez et al., 2002; Perez et al., 2002) in neuronal cells. Based on these reports, we speculate that oxidative stress maybe the upstream sector of calyculin A-induced lesions, and melatonin may protect tau from

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calyculin A-induced hyperphosphorylation through its anti-oxidative effect. To test this hypothesis, we in the present study cultured wild-type neuroblastoma N2a cells (N2awt) with calyculin A or melatonin plus calyculin A, and we found that calyculin A treatment not only resulted in tau hyperphosphorylation but also caused oxidative stress, whereas melatonin attenuates the oxidative stress and disturbance of tau phosphorylation system induced by calyculin A.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), Opti-MEM and fetal calf serum were purchased from Gibco BRL; Melatonin (*N*-acetyl-5 methoxytryptamine), calyculin A (Discodermia calyx), thiobarbituric acid, pyrogallol and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), were from Sigma (St. Louis, MO, USA). Monoclonal antibodies PHF-1 (1:200) to phosphorylated tau at Ser396/404, tau-1 (1:25,000) to non-phosphorylated tau at Ser198/199/202, and rabbit polyclonal antibody 111e (1:5000) against total tau were gifts from Drs. Davis P (University of Alabama in Birmingham, USA), Binder L (University of Alabama in Birmingham, USA), and Iqbal K and Grundke–Iqbal I (New York State Institute for Basic Research, USA), respectively.

2.2. Cell culture and treatment

Wild type mouse neuroblastoma N2a cell (N2awt) was obtained from Dr. Xu HX (The Burnham Institute, La Jolla, California, USA). The cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Opti-MEM containing 5% fetal bovine serum, and in a humified incubator aerated with 95% air and 5% CO_2 at 37 °C. The medium was changed every other day, and cells were plated at an appropriate density according to each experimental scale. After 24 h of subculture, cells were switched to serum-free medium for treatment.

2.3. Analysis of cell viability

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan as described previously (Yamamoto et al., 2000). In brief, N2awt cells were seeded in 96-well plates at a density of 1×10^4 cells per well. After growing for 24 h, the cells were incubated in serum-free DMEM-Opti-MEM for 12 h. Then 5 nM calyculin A or/and melatonin with final concentration of 25 μM, 50 μM or 100 μM, respectively, or 0.01% of dimethyl sulfoxide (DMSO, used as vehicle control), were added to the culture medium and incubated with the cells for 12 h. 10 μl MTT solution (5 mg/ml in phosphate-buffered saline) was added to the 96-well plates and the cells were allowed to incubate for 4 h at 37 °C. Then, cell medium was replaced with 100 μl DMSO and the absorption was measured at 570 nm with DG3022-microplate reader (TECAN, Austria). Results were expressed as the percentage of MTT reduction, assuming that the absorbance of control cells was 100%.

2.4. Western blot

After treatment, the cells were washed with phosphate-buffered saline and then lysed in sample buffer (50 mM Tris–HCl pH8.0, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxychlolate, 0.1% sodium dodecyl sulfate, 0.02% sodium azide, 100 μg/ml phenylmethysulfonyl fluoride, 1 μg/ml Aprotinin). The soluble fraction was obtained by centrifugation at 14000 *g* for 10 min at 4 °C. Equal amounts of soluble protein (20 μg) were separated in 10% sodium dodecyl sulfate–polyacrylamide gel and blotted onto nitrocellulose membranes. The blots were incubated respectively with monoclonal antibodies PHF-1 (recognizes phosphorylated tau at Ser396/404, 1:200), tau-1 (recognized non-phosphorylated tau at Ser198/199/202, 1:25,000) and rabbit polyclonal antibody 111 e (recognized total tau, 1:5000), then developed with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence substrate system (Santa Cruz Biotechnology Inc, California, USA). The protein bands were quantitatively analyzed by Kodak Digital Science 1 D software (Eastman Kodak Company, New Haven, CT, USA).

2.5. PP-2A activity assay

The activity of PP-2A towards [^{32}P]phosphorylase-a was assayed by the release of [^{32}P] as described (Gong et al., 1993). Briefly, the reaction was carried out in 20 μl reaction mixture containing 50 mM Tris, pH 7.0, 10 mM β-mercaptoethanol, 0.1 mM ethylenediaminetetra–acetic acid, 7.5 mM caffeine, 7.5 ng/μl [^{32}P]phosphorylase-a and 0.06 mg/ml cell extract. The reaction was started by addition of [^{32}P]phosphorylase-a. After incubation for 30 min at 30 °C, the reaction mixture of 7 μl was spotted on a chromatography paper already spotted with 10 μl stop solution (4 mM cold ATP in 20% trichloroacetic acid). The released [^{32}P] was separated from the substrate by ascending chromatography in 5% trichloroacetic acid in 0.2 M sodium chloride, and the radioactivity was counted by Cerenkov radiation. A PP-1 specific inhibitor, inhibitor-1 was included in the assay for PP-2A activity.

2.6. GSK-3 activity assay

GSK-3 activity in N2awt cell extracts was determined using phospho-GS peptide 2 as described previously (Pei et

al., 1997; Tsujio et al., 2000). Briefly, 7.5 μg protein was incubated for 30 min at 30 °C with 250 μM peptide substrate and 200 μM γ-[^{32}P]ATP (1,500 cpm/pmol ATP) in 30 mM Tris, pH 7.4, 10 mM magnesium chloride, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM EGTA, and 10 mM β-mercaptoethanol in a total volume of 25 μl. The reaction was stopped with 25 μl of 300 mM phosphoric acid. Then, half volume of the incubation mixture was used for liquid scintillation counting analysis. The GSK-3 activity was expressed as pmol phosphate incorporated per milligram of protein/min at 30 °C.

2.7. Measurement of malondialdehyde and superoxide dismutase

Cells were subcultured in 6-well plate, after exposure to calyculin A for 12 h with or without melatonin treatment, then cells were washed twice with phosphate-buffered saline (pH 7.4, at 4 °C) and lysed with buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM ethylenediaminetetra–acetic acid, 0.2% Triton X-100 for at least 15 min on ice. The cells were scraped from the wells and centrifuged at 17000 *g* for 15 min at 4 °C. Superoxide dismutase activity in the supernatant was determined by assessing the inhibition of pyrogallol autooxidation (Marklund and Marklund, 1974). Malondialdehyde, a metabolite of lipid peroxides, was used as an indicator of lipid peroxidation. Plasma malondialdehyde concentrations were estimated as reactive substances by thiobarbituric acid adduction (Kobe et al., 2002). In brief, N2awt cells were washed twice and collected with cold phosphate-buffered saline, centrifuged at 3000 *g* for 5 min, and then the pellets were mixed with 4 ml of 1/12 M sulfuric acid and 0.5 ml of 10% phosphotungstic acid thoroughly. After centrifugation at 3000 *g* for 10 min, the liquid phase was decanted. Four milliliter double-distilled water and thiobarbituric acid reagent (0.67% 2-thiobarbituric acid/acetic acid1:1) were then added to each sample, mixed and heated in water bath at 95 °C for 1 h. Samples were cooled with tap water and 5 ml *n*-butanol–alcohol was added, and the samples were vigorously shacked for 1 min and centrifuged. The *n*-butanol–alcohol phase, which contains the lipid peroxides, was used for malondialdehyde analysis with fluorospectrophotometer (F-2000, Hitachi Ltd. Tokyo, Japan) at excitation/emission of 515/553 nm. Freshly diluted tetrametoxypropane, which yields malondialdehide, was used as a standard and results are expressed as nanomoles of malondialdehide equivalents. Protein concentration was measured with BCA Protein Assay Reagent (Pierce, Florida, USA).

2.8. Statistical analysis

Data are expressed as mean±S.D. (the standard deviation) and analyzed using SPSS 10.0 statistical software (SPSS Inc., Chicago, IL, USA). The one-way analysis of variance (ANOVA) procedure followed by LSD's post hoc tests was used to determine the differences among groups.

3. Results

3.1. Effect of melatonin on calyculin A-induced decrease in cell viability

To detect the effect of calyculin A on cell viability, N2awt cells were exposed to calyculin A and cell viability was assessed by MTT reduction assay. Calyculin A treatment caused declined cell viability in a dose-dependent manner, and when the concentration of calyculin A was increased to 10 nM, most of the cells died away (Fig. 1A). Melatonin treatment has no effect on cell viability compared with control group. The calyculin A-induced decrease in cell viability was significantly attenuated by melatonin of 25 μM and 50 μM, but not of 100 μM (Fig. 1B). According to these results, calyculin A of 5 nM and melatonin of 25 μM and 50 μM were used for the rest of the study.

3.2. Effect of melatonin on calyculin A-induced tau hyperphosphorylation

To test whether calyculin A influence the phosphorylation of microtubule-associated protein tau and the effect of melatonin, Western blot was performed. The

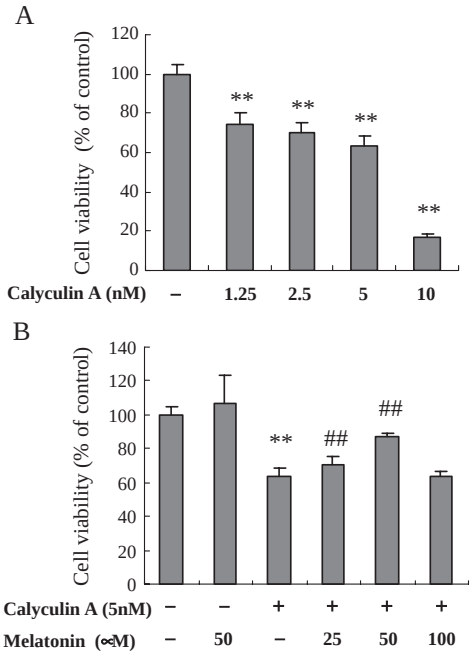

Fig. 1. Effect of calyculin A and melatonin on cell viability. (A) A dose-dependent cell death was induced by calyculin A treatment for 12 h **P<0.01 vs. untreated cells. (B) Melatonin (25 μM and 50 μM) partially restores MTT reduction induced by 5 nM calyculin A. Data are mean±S.D., of 10 observations. **P<0.01 vs. untreated cells, ##P<0.01 vs. calyculin A-treated cells.

results demonstrated that calyculin A treatment led to a decreased immunoreaction of tau to tau-1 (Fig. 2A) and an increased staining to PHF-1 (Fig. 2B), suggesting hyperphosphorylation of tau at Ser198/199/202 (tau-1) and Ser396/404 (PHF-1), respectively. Melatonin of 25 μM and 50 μM significantly attenuated calyculin A-induced tau hyperphosphorylation at these sites, and the most effective concentration of melatonin in antagonizing tau hyperphosphorylation was 50μM, especially at tau-1 epitope (Fig. 2A and B).

3.3. Effect of melatonin on calyculin A-induced inhibition of PP-2A and activation of GSK-3

To further elucidate the involvement of relevant phosphatase and kinase in calyculin A-induced tau hyperphosphorylation, we assayed the activity of PP-2A and GSK-3. It was shown that calyculin A (5 nM) significantly decreased the activity of PP-2A by 22% (Fig. 3A). To our surprise, we also found that treatment of the cells with calyculin A increased the GSK-3 activity up to twofold of the control level (Fig. 3B). Melatonin not only arrested calyculin A-induced PP-2A inhibition but also significantly increased the phosphatase activity to above the normal level (Fig. 3A). Additionally, melatonin at 25 μM suppresses

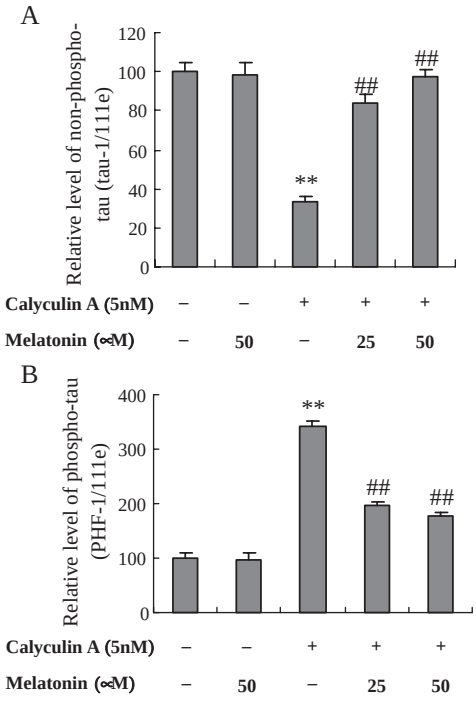

Fig. 2. Effect of melatonin on calyculin A-induced tau hyperphosphorylation. Calyculin A (5 nM) treatment for 12 h enhances tau phosphorylation at tau-1 and PHF-1 epitopes, and melatonin partially arrests calyculin A-induced tau hyperphosphorylation at tau-1 and PHF-1 sites. Quantitative analysis of tau-1 or PHF-1 sites phosphorylated tau is normalized by total tau (111e). The data were from 3 to 4 separate experiments. $^{**}P<0.01$ vs. untreated cells, $\#\#P<0.01$ vs. calyculin A-treated cells.

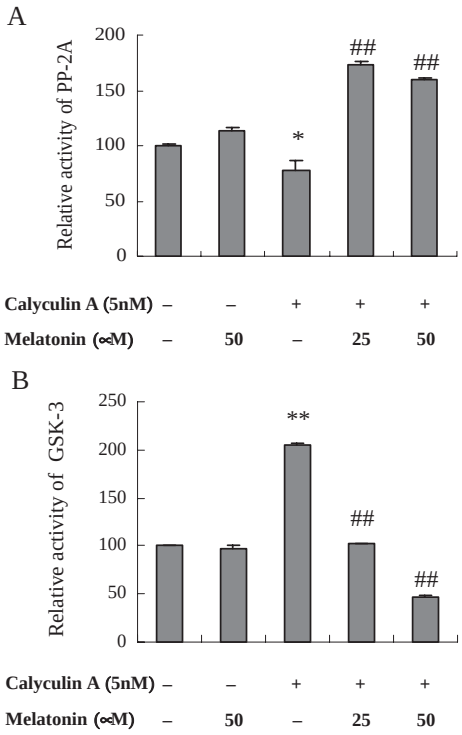

Fig. 3. Effect of calyculin A and melatonin on PP-2A and GSK-3 activities. Activities of PP-2A (A) and GSK-3 (B) in N2awt cells treated with 5 nM calyculin A or calyculin A plus melatonin for 12 h. PP-2A activity was assayed using [^{32}P]-labeled phosphorylase a as a substrate and was determined in the presence of inhibitor-I. Treatment of the cells with 5 nM of calyculin A decreases the activity of PP-2A by 22% (A) and stimulates the activity of GSK-3 up to twofold of the control level (B). Melatonin with concentrations of 25 μM, 50 μM restores GSK-3 and PP-2A activities. Each data point represents the mean±S.D. of 3 different experiments. $^{*}P<0.05$ and $^{**}P<0.01$ vs. untreated cells, $\#\#P<0.01$ vs. calyculin A-treated cells.

calyculin A-induced GSK-3 overactivation, and when the concentration was used to 50 μM, it inhibits the basal activity of GSK-3 (Fig. 3B). The PP-2A activity was slightly but not significantly elevated (Fig. 3A) and no obvious alteration in GSK-3 (Fig. 3B) was observed by the treatment of the cells with melatonin alone at 50 μM.

3.4. Effect of melatonin on calyculin A-induced oxidative stress

Oxidative stress maybe the upstream factor of GSK-3 activation (Gomez-Ramos et al., 2003). To explore the possible mechanism of the calyculin A-induced GSK-3 activation, we measured the level of malondialdehyde as indicator of lipid peroxidation and activity of superoxide dismutase. We found that incubation of N2awt cells with calyculin A resulted in oxidative stress, characterized by a significant increased level of malondialdehyde and a decreased activity of superoxide dismutase. When the cells were treated simultaneously with calyculin A and melatonin, the level of malondialdehyde obviously decreased (Fig. 4A), and the activity of superoxide dismutase significantly

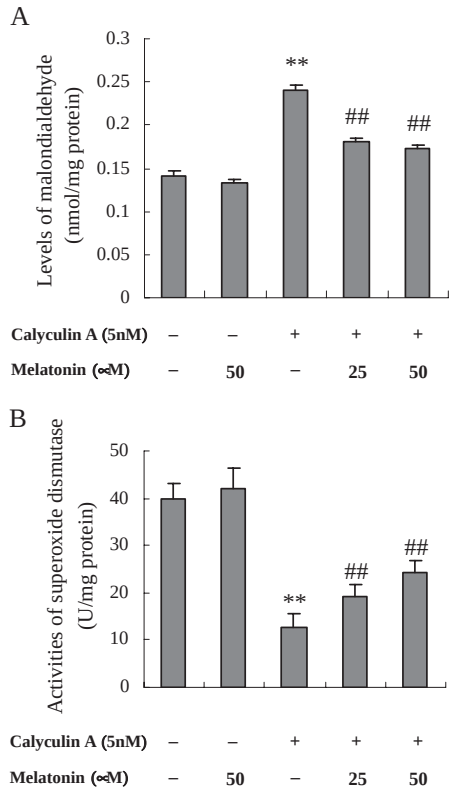

Fig. 4. Effect of melatonin on calyculin A-induced oxidative stress. The levels of malondialdehyde (A) and the activities of superoxide dismutase (B) of N2awt cells after 12 h exposure to calyculin A (5 nM) and melatonin. Melatonin with concentrations of 25 μM, 50 μM blocks calyculin A-induced elevation of malondialdehyde and rescues the activity of superoxide dismutase. The most significant protection was observed by 50 μM of Melatonin. $^{**}P<0.01$ vs. untreated cells, $\#\#P<0.01$ vs. calyculin A-treated cells.

increased compared with calyculin A group (Fig. 4B). No significant change was seen in the level malondialdehyde (Fig. 4A) and superoxide dismutase (Fig. 4B) by treatment of the cells with melatonin alone at 50 μM.

4. Discussion

It is well known that hyperphosphorylation of tau plays an important role in the formation of neurofibrillary tangles in Alzheimer disease. In vitro studies showed that PP-2A is the most effective phosphatase in dephosphorylating hyperphosphorylated tau isolated from brains of patients with Alzheimer disease (Wang et al., 1996). Calyculin A is an inhibitor of serine/threonine protein phosphatase of PP-2A and PP-1 classes, and it has little or no direct effect on other phosphatases or kinases (Kato et al., 1986; Ishihara et al., 1989). In vitro study has demonstrated that the activity of PP-2A is strongly yet incompletely inhibited by nanomolar of calyculin A (Favre et al., 1997). Recently, we have also reported that melatonin arrests tau and neurofilament hyperphosphorylation induced by calyculin A, wortmannin and isoproterenol (Li et al., 2004; Liu and Wang, 2002; Wang et al., 2004). However, the mechanism of the protection by melatonin is not understood. In the present study, we found that calyculin A treatment of N2awt resulted in not only inhibition of PP-2A but also significant activation of GSK-3 with a concomitant tau hyperphosphorylation at Ser198/199/202 and Ser396/404, and melatonin could attenuate tau hyperphosphorylation induced by calyculin A in N2awt cells. We have also demonstrated that melatonin effectively modulates the calyculin A-induced PP-2A inhibition and GSK-3 overactivation.

What might be the causative factors for the activated GSK-3 in this calyculin A-treated N2awt cell system? According to the previous report that calyculin A induces oxidative stress, and melatonin prevents oxidative damage (Acuna-Castroviejo et al., 1997; Benitez-King et al., 2003; Pappolla et al., 1997), we propose that oxidative stress manifested by the significantly increased malondialdehyde level and decreased superoxide dismutase activity induced by calyculin A may be one of the reasons responsible for GSK-3 activation, and the antioxidative effect of melatonin may contribute to its modulation of GSK-3 activity. This is supported by the results that melatonin simultaneously attenuates the GSK-3 activation and oxidative stress induced by calyculin A. Moreover, we have also demonstrated that vitamin E, a recognized antioxidant, restores the calyculin A-induced imbalance in the activity of GSK-3 but not in PP-2A (data not shown). This is coincidently supported by a recent finding that acrolein, a product of lipid peroxidation, activates GSK-3 (Gomez-Ramos et al., 2003). We have noted that melatonin restores calyculin A-induced inhibition of PP-2A and activation of GSK-3 to the levels beyond the basal activities (see Fig. 3), but it does not affect the activity of these particular enzymes under physiological condition. This suggests that melatonin may only exert influence to cells under stress. The underlying mechanism for this phenomenon needs further investigation.

According to these data, we believe that melatonin may play an important role in maintaining the physiological activity of PP-2A as well as in the regulation of GSK-3, and the decreased level of melatonin seen in Alzheimer disease patients may be critical in the development of their brain neurofibrillary degeneration. Taken together, we propose that the decreased melatonin and as a consequence, oxidative stress may be upstream pathological events, which ultimately results in activation of GSK-3 and inhibition of PP-2A, and thus hyperphosphorylation of tau in Alzheimer disease brain.

In summary, we have found in the present study (i) that calyculin A induces not only inhibition of PP-2A but also significant activation of GSK-3 in N2awt cells, and concurrently tau hyperphosphorylation at both PHF-1 and tau-1 epitopes; (ii) that melatonin modulates the activity of PP-2A and GSK-3, and thus maintains normal phosphorylation of tau; (iii) that oxidative stress induced by calyculin A might be the upstream effector of increased GSK-3

activity, and melatonin efficiently relieves calyculin A-induced effects.

Acknowledgments

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