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Potential effects and molecular mechanisms of melatonin on the dopaminergic neuronal differentiation of human amniotic fluid mesenchymal stem cells

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ABSTRACT

Melatonin, a highly lipophilic molecule secreted by the pineal gland in the brain, plays a role in various biological functions. Previous studies reported that melatonin exerts its effect on mesenchymal stem cell (MSC) survival and differentiation into osteogenic- and adipogenic-lineage. However, the effect of melatonin in neurogenic differentiation in amniotic fluid (AF)-MSCs remains to be explored, thus we investigated the potential role of melatonin on dopaminergic neuron differentiation in AF-MSCs. The results showed that various concentrations of melatonin did not affect cell viability and proliferative effects of AF-MSCs. Increases in the levels of neuronal protein marker (βIII-tubulin) and dopaminergic neuronal markers (tyrosine hydroxylase, TH and NURR1), but decrease in the level of glial fibrillary acidic protein (GFAP), were observed in melatonin-treated AF-MSCs. Melatonin induced alteration in differential expression patterns of mesenchymal stem cell antigens by reducing CD29, CD45, CD73, CD90 and CD105, but no changing CD34 expressing cells. AF-MSCs were sequentially induced in neurobasal medium containing standard inducing cocktails (ST: bFGF, SHH, FGF8, BDNF), 1 μM melatonin, or a combination of ST and melatonin. The levels of TUJ1, TH, MAP2, NURR1 and dopamine transporter (DAT) were significantly increased in all treated groups when compared with control-untreated cells. Pretreated AF-MSCs with non-selective MT1/MT2 receptors antagonist, luzindole and selective MT2 receptor antagonist, 4-P-PDOT diminished melatonin-induced increase in dopaminergic neuronal markers and phosphorylated ERK but did not diminish increase in phosphorylated CaMKII by melatonin. Pretreatment with mitogen-activated protein kinase (MEK) inhibitor, PD98059 and CaMKII inhibitor, KN-93 were able to abolish increase in the levels of dopaminergic markers in melatonin-treated AF-MSCs. These findings suggest that melatonin promotes dopaminergic neuronal differentiation of AF-MSCs possibly via the induction in ERK and CaMKII pathways through melatonin receptor-dependent and -independent mechanisms, respectively.

1. Introduction

Amniotic fluid derived stem cells have been studied as an alternative source for neuronal differentiation due to their high potential for multi-lineage differentiation and proliferation. Their pluripotential properties make these cells useful for therapeutic purposes (De Coppi et al., 2007; Prusa et al., 2004; Phermthai et al., 2010; Antonucci et al., 2012). Amniotic fluid stem cells express mesenchymal stem cells (MSCs) surface antigen markers including CD29, CD44, CD73 CD90, CD105 and lack on expression of CD34 and CD45. They also express pluripotency markers such as SSEA-4 and OCT-4 as well as stem cell marker CD133 (Phermthai et al., 2010). Amniotic fluid mesenchymal stem cells (AF-MSCs) have broad multi-potential properties that are able to differentiate into multiple cell types such as adipocyte, chondrocyte, osteocyte, hepatocyte, endothelial, and neuronal cells (De Coppi et al., 2007; Joo et al., 2012).

Melatonin is an endogenous neurosecretory hormone from the pineal gland in the brain (Reiter et al., 2002) and extrapineal tissues such as gastrointestinal tract, retina and lens, skin, immune and hematopoietic cells, some reproductive organs, and endocrine glands

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(Ramirez-Rodriguez et al., 2014). It has various functions in a number of physiological processes, such as circadian rhythms, sleep, inhibition of tumor growth, immune responses, and reproduction (Esquifino et al., 2004; Srinivasan et al., 2005, 2009; Luchetti et al., 2014). Melatonin possesses properties of antioxidant (Zhang et al., 2014), anti-inflammation (Song et al., 2015) and anti-apoptosis to prevent cell damage (Chetsawang et al., 2007; Parameyong et al., 2015; Suwanjang et al., 2016), and promotes cell survival and differentiation in rat embryonic cells and neural stem cells subjected to hypoxia in vitro (Ramirez-Rodriguez et al., 2009; Sotthibundhu et al., 2010; Fu et al., 2011; Tocharus et al., 2014). Recently, Shu et al. (2016) reported that melatonin promotes neurosphere formation, cell viability, and neuronal differentiation of induced pluripotent stem cell-derived neural stem cells by up-regulated gene and protein expression of nestin and MAP2. Melatonin has a modulatory effect on both proliferation and neural differentiation of neural stem cells (NSCs) (Moriya et al., 2007). In addition, melatonin stimulates rat ventral midbrain NSCs to differentiate into dopaminergic neurons (Kong et al., 2008). Melatonin was previously proven to increase the expression of neuronal markers such as NeuroD1, nestin, βIII-tubulin and MAP2 in NSCs from the subventricular zone and induced pluripotent cells (Sotthibundhu et al., 2010; Shu et al., 2016). Melatonin effects on osteogenic, chondrogenic, and adipogenic differentiation have been described in MSCs (Luchetti et al., 2014).

Recent report showed that melatonin involves in NSC proliferation and differentiation processes through melatonin receptors (Sotthibundhu et al., 2010). Melatonin also exerts a therapeutic effect in neurodegenerative diseases such as Parkinson's disease (Mayo et al., 2005; Srinivasan et al., 2011) and Alzheimer's disease (Wang, 2009), via stimulation of endogenous NSCs as well as increased expression of BDNF and GDNF (Niles et al., 2004; Lee et al., 2014; Kaiser et al., 2014). The potential ability of melatonin as the growth factor for synergizing NSC proliferation and differentiation have been proposed. Although the involvement of melatonin in embryonic neural stem cells and MSC differentiation has been studied, its effects and mechanism on neuronal differentiation in AF-MSCs is not elucidated. Therefore, in this study, the potential effect of melatonin as the neurogenic inducing factor to induce dopaminergic neuron differentiation was investigated in AF-MSCs.

2. Materials and methods

2.1. Amniotic fluid mesenchymal stem cell culture

Six individual amniotic fluid samples were obtained from 16 to 20 week pregnant woman undergoing amniocentesis for routine chromosomal analysis. The isolation protocol was approved by the Ethics Committee of Siriraj Hospital, Mahidol University, Thailand. Mesenchymal stem cells were isolated and derived from amniotic fluid. Briefly, the cells were immediately isolated and cultured with Chang medium to generate starter cells. Colony formation from a starter cell was continued by culturing in mixed medium defined by Phermthai et al. (2010). The cells underwent clonal expansion and were sub-cultured with α-MEM supplement with 10% FBS and 1% penicillin/streptomycin (expansion medium or EM) in a humidified atmosphere of 95% and 5% CO_2 at 37 °C. At passage 8, the cells were characterized as mesenchymal stem cells. The isolated cells from six individual samples were used in the next experiment.

2.2. Flow cytometric analysis of mesenchymal stem cell (MSC) markers

In order to characterize the MSC markers, amniotic fluid stem (AFS) cells (8th passage) were harvested and stained with FITC- or PE-conjugated antibodies against CD29, CD44, CD90, CD105, CD133, CD34, CD45, CD73, SSEA-4, and OCT4 (1:20). The cells were fixed with 1% paraformaldehyde. The cell analysis was performed using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA).

2.3. Cytotoxic effect of melatonin in AF-MSCs

The viability of cells was determined by the tetrazolium salt MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. AF-MSCs were plated into 96-well culture plates at 5000 cells/cm^2 per well. After 24 h, the culture media were replaced with low serum medium (LSM: αMEM with 1% FBS) to avoid the induction of cell differentiation by trophic factors in the serum, and then different concentrations of melatonin were added to the media. At 9 days after melatonin treatment, 5 mg/mL MTT (Sigma) was added to each well and incubated at 37 °C for 4 h. The medium was gently aspirated, following which 200 µl DMSO was added to each well to dissolve the formazan crystals. The optical density of each sample was then measured in a multi-well spectrophotometer at 570 nm.

2.4. Dose-dependent effect of melatonin on proliferation of AF-MSCs

The proliferative effect of melatonin at different concentrations was also evaluated in AF-MSCs by measurement of cellular DNA contents. The cells were plated on 6-well plates in LSM and treated with 0.05, 0.1 and 1 µM melatonin for 9 days. DNA was extracted with Genomic DNA mini kit (Geneaid, USA) according to the manufacturer's instructions. DNA concentrations were measured by absorbance at 280 nm by NanoDrop 1000 spectrophotometer (NanoDrop, Technologies).

Thymidine analog incorporates into proliferating cells was determined using ELISA for BrdU incorporation (Abcam, USA). AF-MSCs were seeded on 96- well plates and treated with melatonin. The assay was performed according to the manufacturer's instructions. Briefly, 2 h before the end of melatonin treatment, cells were incubated with 10 µM BrdU labeling reagent. Cells were then fixed and DNA was partial denatured in fix solution. After 30 min, fix solution was removed, anti-BrdU (monoclonal antibody from mouse conjugated with peroxidase) was added and incubated for 60 min. The TMB solution was added and the absorbance was measured at 450 nm by a micro-plate reader. The results were presented as a percentage of BrdU incorporation compared to control-untreated cells.

2.5. Dose-dependent effect of melatonin on neuronal markers

AF-MSCs were seeded onto matrigel coated plates at density < 3000 cells/cm^2. Twenty-four hours after cell seeding, the culture medium was replaced with LSM. Different concentrations of melatonin (0.05, 0.1 and 1 µM) were added into culture medium and incubated for 2–9 days. Control cells were treated with vehicle. Western blot was used to analyze neuronal markers.

2.6. Effect of melatonin on stem cells markers

AF-MSCs were cultured in EM, LSM and LSM plus 1 µM melatonin for 9 days and then the cells were harvested and stained with MSC markers, including CD29, CD73, CD90, CD105 and hematopoietic stem cell markers, CD34 and CD45, respectively. The staining cells were analyzed using a FACSCalibur flow cytometer.

2.7. Dopaminergic neuronal induction of AF-MSCs with melatonin and standard induction factors

The ability of melatonin was investigated and compared with standard protocol in dopaminergic neuronal induction. AF-MSCs 6^{th}-8th passages were trypsinized with 0.25% trypsine/EDTA. The cells were seeded on matrigel coated plates at low density (< 3000 cells/cm^2) in EM. Twenty-four hours later, the culture media was replaced with neurobasal medium/expansion medium (1:1) containing 2% B27. The cells were induced with 1 µM melatonin (M), standard cocktails

(250 ng/ml SHH, 100 ng/ml FGF8, 20 ng/ml bFGF and 50 ng/ml BDNF), and 1 μM melatonin combined with standard cocktails. In the control group, the cells were treated with vehicle. A half-volume neurobasal medium with 2% B27 was replaced at day 5 and 9. For standard cocktail treated or/and 1 μM melatonin cells, the cells were sequential induction with 50 ng/ml BDNF at day 9 and incubated until day 12.

2.8. Blockage of melatonin receptor and ERK and CaMKII pathways in melatonin-induced differentiation in AF-MSCs cells

To investigate the involvement of melatonin receptor in neuronal differentiation, MAP2 expression and an activation of phosphorylation of ERK1/2 and CaMKII were determined in response to melatonin treatment and luzindole, a non-selective MT1/MT2 antagonist and 4-P-PDOT, a specific MT2 antagonist pre-treatment. Involvement of melatonin receptor in the mechanism by which melatonin induces neuronal differentiation was approached by pre-treatment with 10 μM luzindole, 10 μM 4P-PDOT, 10 μM PD98059 and 1 μM KN-93 in AF-MSCs cells for 2 h, followed by 1 μM melatonin treatment and incubated for 12 days. Some cells were treated with 10 μM luzindole, 10 μM 4P-PDOT, 10 μM PD98059 and 1 μM KN-93 for 2 h. Western blot analysis was performed to evaluate the expression of phospho-ERK1/2 (pERK1/2) and phospho-CaMKII (pCaMKII), TH, NURR1 and DAT in the cells.

2.9. Western blot analysis

The cells were lysed by adding lysis buffer (150 mM NaCl, 50 mM Tris-base, 1 mM phenyl methanesulfonyl fluoride, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 1% protease inhibitor) into the culture plate after removing culture medium. The cells were scraped off the plate with a pipette tip and transferred to a microcentrifuge tube on ice. The cell suspension was centrifuged at 14,000 g for 5 min at 4 °C. The supernatant was collected for protein determination and kept at −20 °C. The extracted protein concentration was determined using Bradford reagent. Protein samples were electrophoretically separated on 10% SDS-PAGE. The protein bands were electrophoretically transferred to a PVDF membrane. The PVDF membranes were blocked with 5% skim milk or 5% bovine serum albumin and incubated with primary antibodies: mouse anti-βIII-tubulin monoclonal antibody (1:1000; Abcam); mouse anti-TH monoclonal antibody (1:1000; Millipore); mouse anti-GFAP antibody (1:1000; Sigma); mouse anti-TUJ1 monoclonal antibody (1:1000; Abcam); rabbit anti-MAP2 monoclonal antibody (1:1000, Cell Signaling); rabbit anti-Nuclear receptor related 1 protein (NURR1) polyclonal antibody (1:1000; Millipore); mouse anti-DAT monoclonal antibody (1:1000, Abcam); rabbit monoclonal antibody against MT1A (1:1000; Santa Cruz); mouse monoclonal antibody against phospho-ERK1/2 (1:1,000, Santa Cruz); and rabbit monoclonal antibody against phospho-CaMKII (1:1000, Cell Signaling). The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000). The protein bands were detected with Amersham ECL Prime Western Blotting Detection Reagent using a luminescence image analyzer with an electronically cooled charge-coupled device camera (Azure c600, Azure Biosystem, Dublin, CA, USA). Actin was used as the internal positive control to check the consistency of protein sample loading. The density of protein bands was analyzed by ImageJ program (NIH). The density of all protein bands were normalized to the matched density of actin values.

2.10. Immunocytochemical analysis

The cells were seeded on sterile glass coverslips and then the cells were treated melatonin. In some experiments, luzindole was added to the medium prior to incubation with melatonin. After incubation, the medium was removed, and then cells were washed with ice-cold PBS. The cells were then fixed with 4% paraformaldehyde for 30 min at 4 °C and washed with PBS three times for 5 min each. Next, the cells were permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature and were rinsed with PBS three times and then blocked with 1% normal goat serum in PBS containing 3% bovine serum albumin (BSA). The cells were incubated with the primary antibodies (TH 1:200 and MAP2; 1:200) overnight at 4 °C and were incubated with Alexa Fluor® 488- and Alexa - Fluor® 546-conjugate secondary antibodies (1:300) for 1 h at room temperature (RT). For nuclear staining, Hoescht 33,258 (1 mg/ml) for 5 μl in 250 μl PBS was added to the cells and incubated for 15 min at RT. The cells were washed three times with PBS and were mounted with antifade reagent in glycerol buffer (Vector Laboratories, Burlingame, CA, USA) and visualized under a confocal microscope.

2.11. Statistical analysis

Results were expressed as the means ± S.E.M, each from 3 to 4 independent experiments. Statistical difference was analyzed by one-way analysis of variance (ANOVA) followed with Tukey's multiple comparison test using the scientific statistical software GraphPad Prism version 6 (GraphPad Software). Probability (*P*) values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. AF-MSCs surface antigen markers

Flow cytometry analysis demonstrated high numbers of positive staining cells for the mesenchymal surface antigen markers; CD29, CD44, CD73, CD90 and CD105 in amniotic fluid stem cells. Moreover, these cells exhibited positive staining for stem cell markers CD133, SSEA4 and OCT4 and hematopoietic stem cell surface antigen markers CD34 and CD45 (Table 1).

3.2. Cytotoxic and proliferative effects of melatonin on AF-MSCs

The various concentration of melatonin were assessed for cytotoxic and proliferative effects on AF-MSCs by MTT assay and measurement of cellular DNA content and BrdU incorporation, respectively. Melatonin treatment at 0.05–1 μM for 9 days did not change the cell viability when compared to control-untreated cells (0 μM) (Fig. 1a). Cellular DNA contents were not changed in melatonin-treated cells (0.05 μM = 104 ± 8.3%, 0.1 μM = 99 ± 13.5% and 1 μM = 111 ± 5.7% of the control values, respectively) when compared to control-untreated cells (Fig. 1b). Moreover, the levels of BrdU incorporation were not changed in melatonin-treated cells (0.05 μM = 113.9 ± 3.9%, 0.1 μM = 99.21 ± 4.3% and 1 μM = 115.3 ± 4.6% of the

Table 1
The flow cytometry analysis for the expression of surface antigen markers of mesenchymal stem cells (CD44, CD73, CD90, CD105 and CD29), hematopoietic stem cells (CD34 and CD45), and stemness markers (SSEA4, OCT4 and CD133) in AF-MSCs. The data show the percentage of positive staining cells as mean ± S.E.M of 6 independent experiments.

Markers (% of positive staining)	Percentage of positive staining (Mean ± SEM)	Phermthai et al., 2010
CD34	3.57 ± 3.53	5.21
CD45	4.30 ± 3.49	3 28
CD44	97.73 ± 4.23	99.65
CD73	99.52 ± 0 58	99.74
CD90	64.54 ± 27.23	98.59
CD105	99.73 ± 0.22	99.88
CD29	99.61 ± 0.15	9984
CD133	95.59 ± 4.66	99.10
SSEA4	53.18 ± 31.48	91.41
OCT4	19.67 ± 13.40	34.18

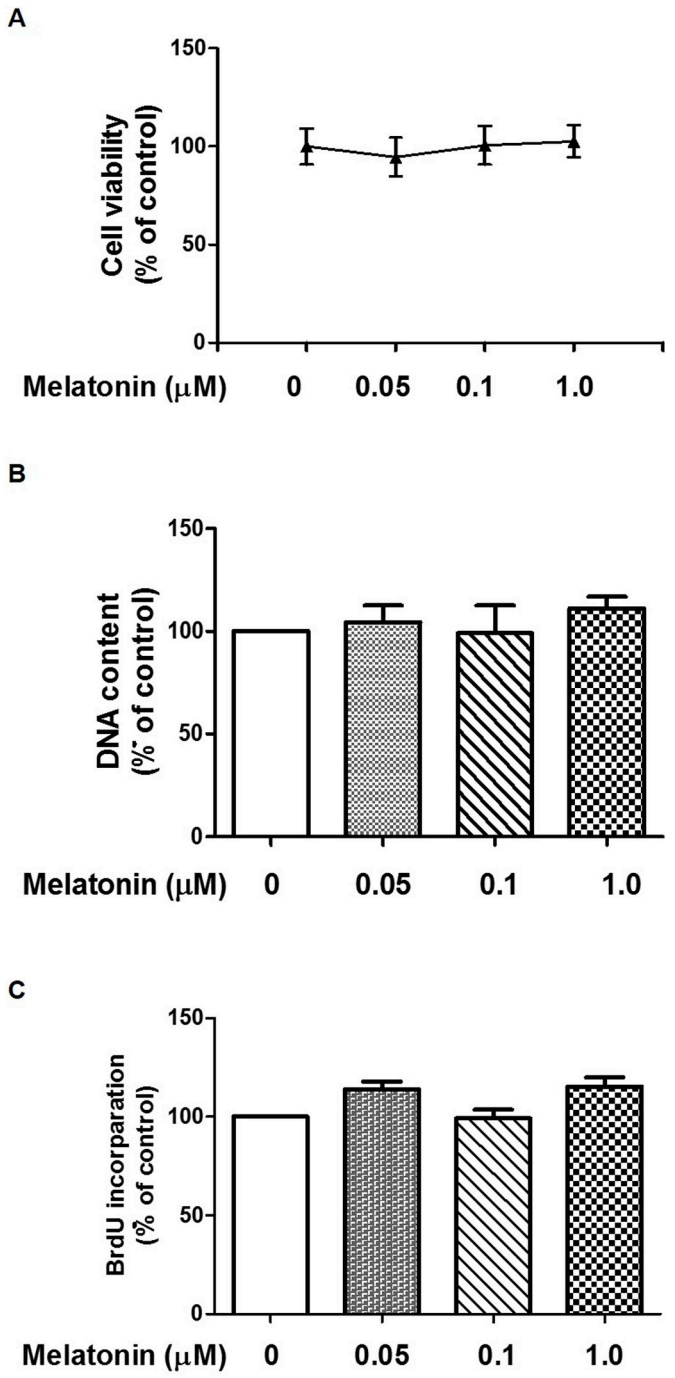


Fig. 1. Cytotoxic and proliferative effects of melatonin on AF-MSCs. Cells were treated with melatonin at 0.05, 0.1 and 1 μM for 9 days. (a) Cytotoxic effect was assessed by MTT assay. (b and c) Proliferative effect was determined by measurement of cellular DNA content and BrdU incorporation, respectively. Data are expressed as mean ± S.E.M of 4 independent experiments.

control values, respectively) when compared to control untreated cells (Fig. 1c). These results indicated that melatonin at 0.05–1 μM did not exert cytotoxic and proliferative effect on AF-MSCs.

3.3. Dose-dependent effect of melatonin on neural makers in AF-MSCs

To examine the effect of melatonin concentration on dopaminergic neuron marker protein expression, various concentration of melatonin (0.05, 0.1 and 1 μM) were used to treat AF-MSCs cultured with LSM for 2 days. Western blot analysis was performed to determine the levels of dopaminergic neuron markers (TH and NURR1). Melatonin at 0.1 μM and 1 μM significantly increased the levels of TH (259 ± 21.8% and 333 ± 48.5% of the control-untreated cell values, respectively) and NURR1 (457 ± 21.8% and 567 ± 32.3% of the control values, respectively) (Fig. 2a and b, respectively). Melatonin at 0.05 μM did not change the levels of TH and NURR1 in AF-MSCs when compared to control-untreated cells (Fig. 2a and b, respectively). Therefore, melatonin at 0.1 μM and 1 μM were used to investigate the effects of melatonin on the expression of protein markers of neuron (βIII-tubulin) and glial (GFAP) cells in AF-MSCs (Fig. 2c, d and 2e). Melatonin at 0.1 μM and 1 μM significantly increased the levels of βIII-tubulin (127 ± 3.0% and 141. ± 8.6% of the control values, respectively) (Fig. 2c and d) but significantly decreased the levels of GFAP (62 ± 9.6% and 66.8 ± 8.7% of the control values, respectively) (Fig. 2c and e) when compared to control-treated cells.

3.4. Effect of melatonin on surface antigen markers of AF-MSCs

The results from induction in neuronal markers of melatonin at 0.05, 0.1 and 1 μM in AF-MSCs demonstrated dose-dependent effects (Fig. 2). Thus, 1 μM melatonin was used to investigate the differentiative effect and differential expression pattern of the cluster density of MSC surface antigen markers (CD29, CD73, CD90 and CD105) and hematopoietic stem cell markers (CD34 and CD45) in AF-MSCs. AF-MSCs were divided into 3 groups: cells cultured with EM, LSM containing vehicle, and LSM containing 1 μM melatonin. After melatonin treatment for 9 days, the expression of CD29, CD45, CD73, CD90 and CD105 was significantly decreased in AF-MSCs when compared to cells cultured in EM and LSM with vehicle, respectively. The expression of CD29 was significantly decreased in cells cultured in LSM with vehicle when compared to cells cultured in EM. The expression of CD34 tended to increase in melatonin-treated cells and cells cultured in LSM with vehicle when compared to cells cultured in EM (Fig. 3).

3.5. Effect of melatonin on dopaminergic neuron differentiation

The effects of melatonin treatment on dopaminergic neuron differentiation in AF-MSCs were examined and compared with vehicle-, standard cocktails- and standard cocktails plus melatonin-treated cells. Following dopaminergic neuronal induction for 12 days, AF-MSC-induced cells in all treated groups exhibited cell bodies with long processes and neural-like morphology. Moreover, the characteristic of neuronal-like network morphology with cytoplasmic connection between cells was observed in all treated groups (Fig. 4a). The expression of MT1-melatonin receptor proteins was also detected in all AF-MSCs-treated groups by immunoblotting (Fig. 4b).

Dopaminergic neuronal markers in AF-MSCs-inducing cells were determined after induction with vehicle, standard cocktails, melatonin and standard cocktails plus melatonin for 12 days. The levels of dopaminergic neuronal markers, including TUJ1, MAP2, TH, NURR1, and DAT were determined using western blot analysis. The results showed that the levels of dopaminergic neuronal markers were significantly increased in melatonin-induced (M) cells (TUJ1 = 201.0 ± 29.1%; MAP2 = 134.5 ± 7.3%; TH = 165.5 ± 16.0%; NURR1 = 219.3 ± 10.4% and DAT = 163.8 ± 7.2%) when compared to control vehicle-treated (V) cells (Figs. 5 and 6, respectively). Standard cocktail-induced (ST) cells expressed significantly increased the levels of dopaminergic neuronal markers (TUJ1 = 173.7 ± 8.9%; MAP2 = 145.5 ± 13.4%; TH = 148.1 ± 7.4%; NURR1 = 245.7 ± 44%; and DAT = 180.6 ± 3.9) when compared to control vehicle-treated (V) cells (Figs. 5 and 6, respectively). In addition, ST plus M-induced cells also expressed significantly increased levels of dopaminergic neuronal markers (ST + M; TUJ1 = 184.2 ± 18.2%; MAP2 = 136.6 ± 0.3%; TH = 146.7 ± 10.7%; NURR1 = 275.7 ± 31.5%; and DAT = 284.6 ± 47.8%) when compared to control vehicle-treated (V) cells (Figs. 5 and 6, respectively). Moreover, the levels of DAT were significantly increased in

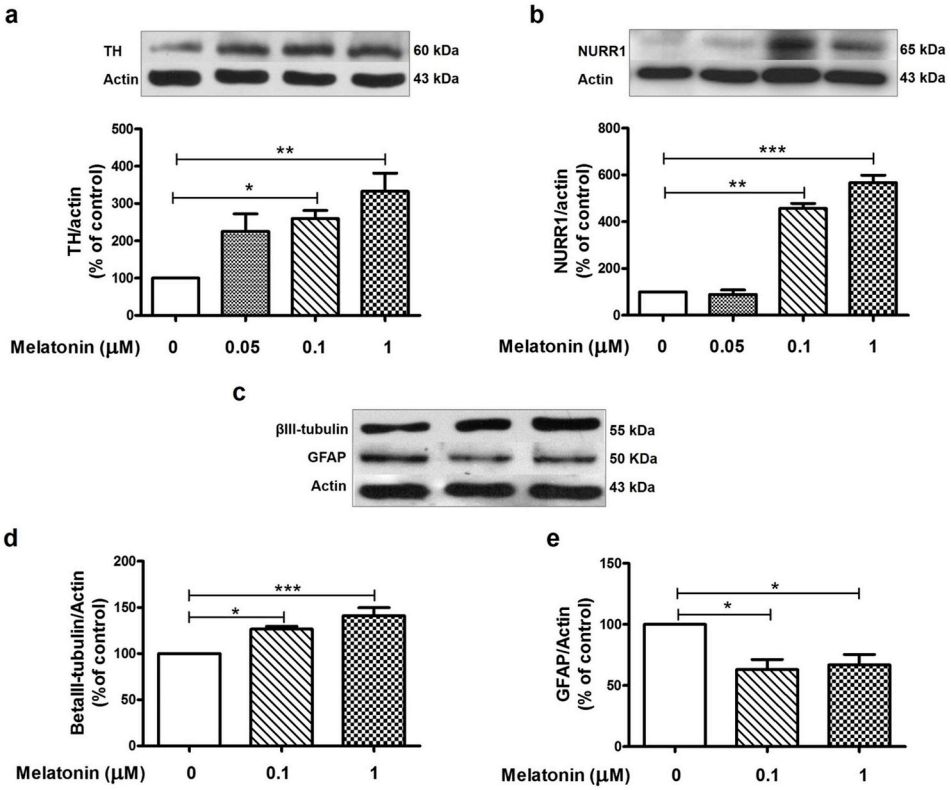


Fig. 2. The effect of melatonin on (a) TH, (b) NURR1 and (c) βIII-tubulin and GFAP protein expression in AF-MSCs. AF-MSCs were treated with melatonin for 2 days. The protein levels were determined by western blot analysis. The data are expressed as mean ± S.E.M of 3–4 independent experiments. ANOVA followed by Tukey's multiple comparison test was performed. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ versus control groups (0 μM). TH = Tyrosine hydroxylase; NURR1 = Nuclear receptor related 1 protein, GFAP = Glia fibrillary acidic protein.

ST + M-treated cells when compared to M-treated cells (Fig. 6c). This result shows that melatonin has a potential to induce dopaminergic neuronal marker expression in AF-MSCs.

3.6. Involvement of melatonin receptor in melatonin regulated AF-MSCs differentiation

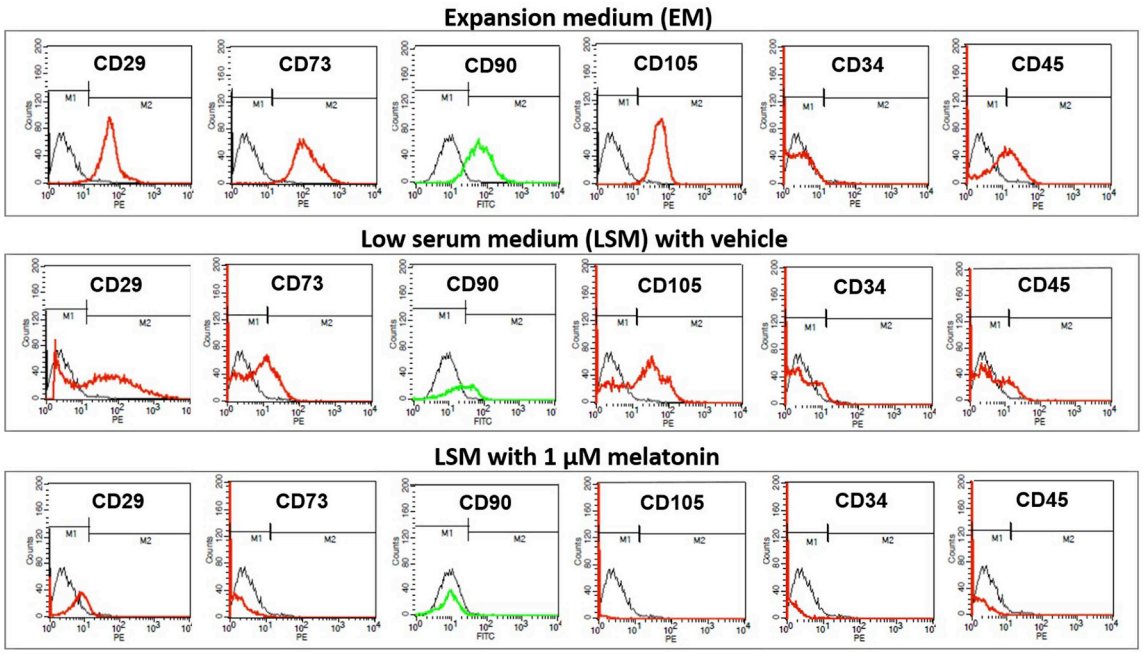
Involvement of melatonin receptor in mechanisms by which melatonin regulates neuronal differentiation was investigated by treatment of AF-MSCs cells with 10 μM luzindole, a MT1/2 melatonin receptor antagonist or 10 μM 4-P-PDOT, a selective MT2 receptor antagonist, prior to treating cells with 1 μM melatonin. Some cells were treated with luzindole or 4-P-PDOT. The dopaminergic neuronal markers (TH, DAT and NURR1) and phosphorylation of extracellular signal-regulated protein kinase 1 and 2 (pERK1/2) and calcium/calmodulin-dependent protein kinase II (pCaMKII) levels were determined in response to melatonin and luzindole and 4-P-PDOT treatment using Western blot analysis. The results showed that melatonin significantly increased the levels of dopaminergic neuronal markers; TH (147.0 ± 9.6%), NURR1 (143.6 ± 13.0%) and DAT (130.6 ± 4.2%) when compare to control-vehicle-treated cells, whereas pretreatment with luzindole and 4P-PDOT significantly decreased the levels of TH (82.5 ± 12.8% and 91.1 ± 12.9%), NURR1 (94.0 ± 3.8% and 70.2 ± 6.7%), and DAT (93.0 ± 8.1% and 88.5 ± 4.1%) when compared to melatonin-treated cells, respectively (Fig. 7a, b and c). Luzindole and 4P-PDOT treatment did not cause any changes in dopaminergic neuronal markers levels in AF-MSCs cells (Fig. 7a, b and c). Melatonin significantly increased the levels of phosphorylated ERK (173.2 ± 8.6%) and phosphorylated CaMKII (161.3 ± 13.5%) when compared to vehicle-treated cells (Fig. 8a and b, respectively). Pretreatment with luzindole and 4P-PDOT were able to inhibit the increase of phosphorylation of ERK (100.2 ± 8.6% and 87.3 ± 2.4%, respectively) in melatonin-treated cells. However, pretreatment with luzindole and 4P-PDOT were unable to inhibit the increase of phosphorylated CaMKII (147.5 ± 10.8% and 156.7 ± 13.7%, respectively) in melatonin-treated cells. Luzindole and 4P-PDOT treatment did not cause any changes in phosphorylation of ERK and CaMKII levels in AF-MSCs cells (Fig. 8a and b, respectively).

Furthermore, the cellular mechanism of melatonin-induced dopaminergic neuronal differentiation mediate through ERK or CaMKII pathways were investigated in AF-MSCs. Melatonin significantly increased the levels of TH (140.9 ± 3.3%), NURR1 (131.8 ± 7.7%), and DAT (136.8 ± 9.4%) when compared to vehicle-treated cells (Fig. 9a, b and c, respectively). Pretreatment with 10 μM PD58098 and 1 μM KN-93 significantly decreased the levels of TH (91.0 ± 20.3% and 116.8 ± 2.7%, respectively) and NURR1 (71.2 ± 8.8% and 111.1 ± 3.1%, respectively) when compared to melatonin-treated cells (Fig. 9a and b). Pretreatment with PD58098 and KN-93 significantly decreased and tended to decrease the levels of DAT (78.7 ± 4.3% and 114.8 ± 15.8%) when compared to melatonin-treated cells, respectively (Fig. 9c). PD58098 and KN-93 treatment did not cause any changes in TH, NURR1 and DAT levels in AF-MSCs cells (Fig. 9a, b and c, respectively).

The melatonin induced increase in the amount of TH and MAP2 protein expression in AF-MSCs was demonstrated using immunofluorescence staining. The green and red colors indicate the TH and the MAP2-positive immunostaining, respectively. The blue color indicate nuclear staining. The control-vehicle-treated cells exhibited less TH and MAP2 positive immunofluorescence staining cells. The exposure to melatonin resulted in increase in TH and MAP2 positive immunofluorescence staining cells as compared with the control cells. Melatonin induced increase in TH and MAP2 positive immunofluorescence staining cells disappeared with the luzindole pretreatment (Fig. 10).

4. Discussion

Amniotic fluid mesenchymal stem cells (AF-MSCs) are a heterogeneous population and have pluripotency properties which involve developmental and regeneration processes. Use of MSCs as therapeutic tools in tissue engineering and regenerative medicine is increasing



MSC markers (Percentage of positive cell)	EM (Mean ± S.E.M)	LSM with vehicle (Mean ± S.E.M)	LSM with 1 μM melatonin (Mean ± S.E.M)
CD29	99.03 ± 0.53	65.17 ± 2.33***	9.89 ± 4.41***,###
CD73	99.72 ± 0.06	60.72 ± 36.49	3.83 ± 3.57***,#
CD90	68.26 ± 23.27	63.85 ± 26.64	37.41 ± 18.94*,#
CD105	99.83 ± 0.06	79.76 ± 11.46	4.10 ± 3.12***,#
CD34	0.55 ± 0.28	19.28 ± 10.24	20.80 ± 10.24
CD45	14.01 ± 13.46	10.89 ± 1.75	0.63 ± 0.44*,#

Fig. 3. Flow cytometry analysis for the effect of melatonin on the differential expression pattern of membrane surface antigen markers of mesenchymal stem cells (MSC); CD29, CD73, CD90, CD105, and hematopoietic stem cells; CD34, CD45 in AF-MSCs. AF-MSCs were divided into 3 groups and cultured for 9 days in expansion medium (EM), low serum medium (LSM) with vehicle, and 1 μM melatonin, respectively. The histogram represents the number of cells counted and the fluorescence intensity of staining cells. The percentages of positive staining cells are shown as mean ± S.E.M of 3 independent experiments. $^{*}P < 0.05$, and $^{***}P < 0.001$ versus AF-MSCs cultured in expansion medium (EM), and $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ versus AF-MSCs cultured in low serum medium (LSM) with vehicle.

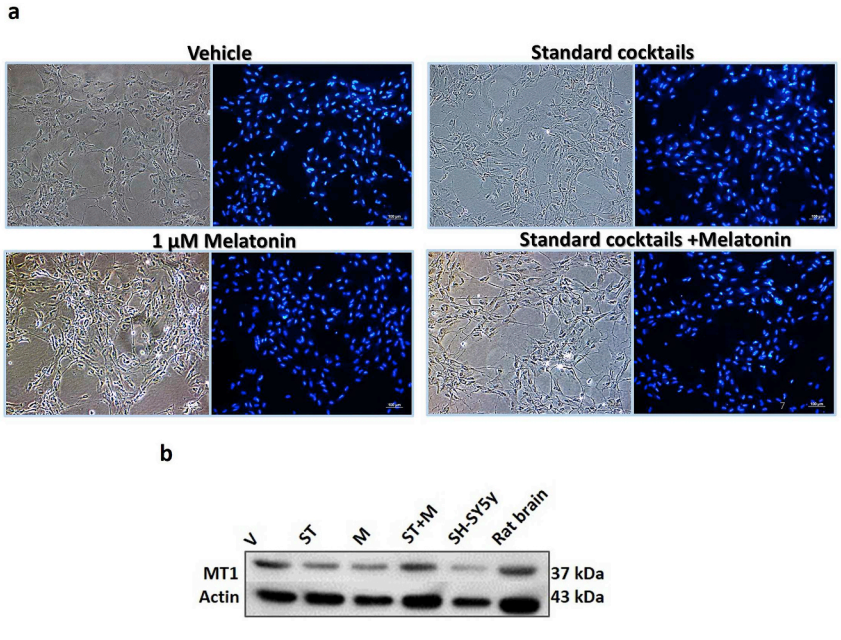


Fig. 4. (a) Phase contrast imaging of AF-MSCs morphology after inducing with standard cocktails (ST), melatonin (M) and ST combine with melatonin. (b) Melatonin receptor (MT1) proteins were detected in AF-MSCs-induced cells. Dopaminergic cell lines, SH-SY5Y cells and rat brain were used as internal standard control for MT1 melatonin receptor expression in dopaminergic cells and brain, respectively.

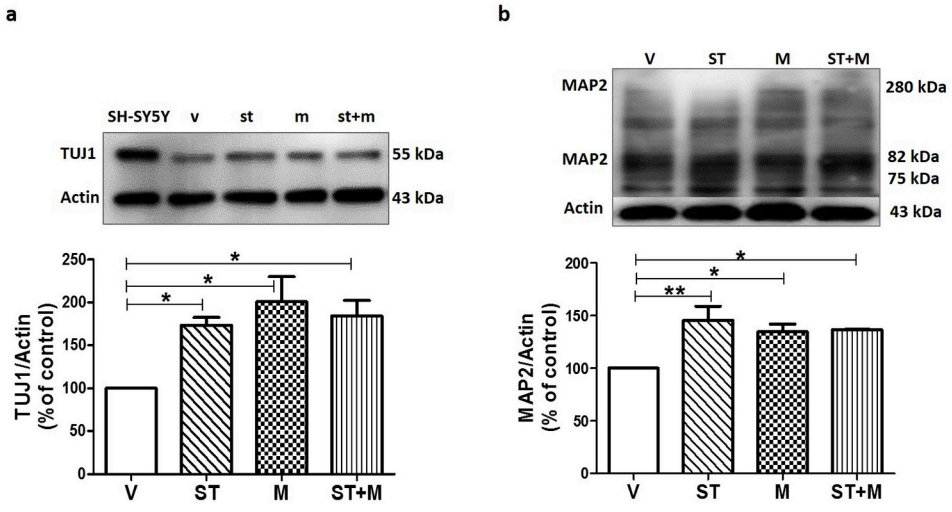


Fig. 5. The effect of melatonin and standard inducing factors on expression in neurons protein markers in AF-MSCs. AF-MSCs passage 6th-8th were cultured and sequentially induced by standard cocktails (ST; 50 ng/ml bFGF, 100 ng/ml FGF8, 250 ng/ml SHH and 50 ng/ml BDNF), 1 μM melatonin (M) and combination of ST and melatonin (ST + M) for 12 days. The density of TUJ1 (a) and MAP2 (b) protein bands was quantified using ImageJ software and normalized with actin. SH-SY5Y cells were used as internal standard control for dopaminergic cells. ANOVA was performed for statistical analysis and values are expressed as mean ± S.E.M. of 3–4 independent experiments. The significant difference represents as $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle-treated cells.

(Phermthai et al., 2010; Antonucci et al., 2012; Joo et al., 2012). In the present study, it was shown that six individual amniotic fluid-isolated (AF) cells expressed high levels of mesenchymal markers (CD29, CD44, CD73, CD90 and CD105), stemness markers (CD133, SSEA4) and OCT4. In contrast, AF cells expressed low levels of hematopoietic stem cell markers (CD34 and CD45). These results might indicate that AF cells exhibited characteristics of mesenchymal stem cells (AF-MSCs) (Prusa et al., 2004; Phermthai et al., 2010).

Recently, Thangnipon et al. (2016) reported that AF-MSCs are induced to differentiate into cholinergic neurons by BMP-9 and *N*-benzylcinamide. However, the potential role of melatonin in inducing neuronal differentiation in MSCs has not been reported. Several studies have shown that melatonin is a neuroprotective agent that induces neurogenesis in embryos (Moriya et al., 2007; Kong et al., 2008; Sotthibundhu et al., 2010). Melatonin combined with physical exercise potentiates adult rat hippocampus neurogenesis by enhancing cell survival (Liu et al., 2013), and administration of melatonin before and during sleep deprivation increases the number of neural precursor cells in adult rats (López-Armas et al., 2016). It has been reported that melatonin can induce neuronal differentiation in PC12 cells and induced pluripotent stem cells (Shu et al., 2016; Liu et al., 2013). In this study, we firstly demonstrate that melatonin has the capacity to induce AF-MSC differentiation into dopaminergic neurons. Our results showed that melatonin did not affect cell viability and proliferation of AF-MSCs. Melatonin treatment led to increases in levels of βIII-tubulin, MAP2, TH and NURR1, which possibly indicates that melatonin promotes dopaminergic neuron differentiation in AF-MSCs. Moreover, melatonin suppressed astrocyte differentiation by decreasing the expression of

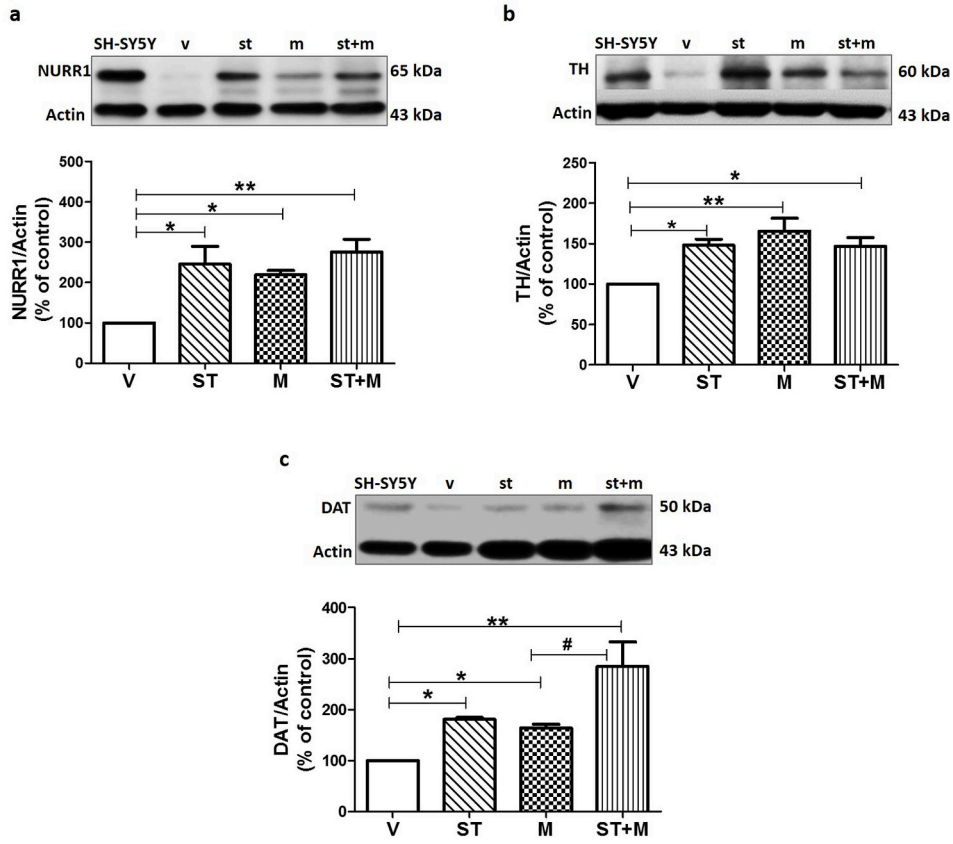


Fig. 6. The effect of melatonin and standard inducing factors on expression in dopaminergic neurons protein markers in AF-MSCs. AF-MSCs passage 6th-8th were cultured and sequentially induced by standard cocktails (ST; 50 ng/ml bFGF, 100 ng/ml FGF8, 250 ng/ml SHH and 50 ng/ml BDNF), 1 μM melatonin (M) and combination of ST and melatonin (ST + M) for 12 days. The density of NURR1 (a), TH (b) and DAT (c) protein bands was quantified using ImageJ software and normalized with actin. SH-SY5Y cells were used as internal standard control for dopaminergic cells. ANOVA was performed for statistical analysis and values are expressed as mean ± S.E.M. of 3–4 independent experiments. The significant difference represents as $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle-treated cells and $^{\#}P < 0.05$ versus melatonin-treated cells.

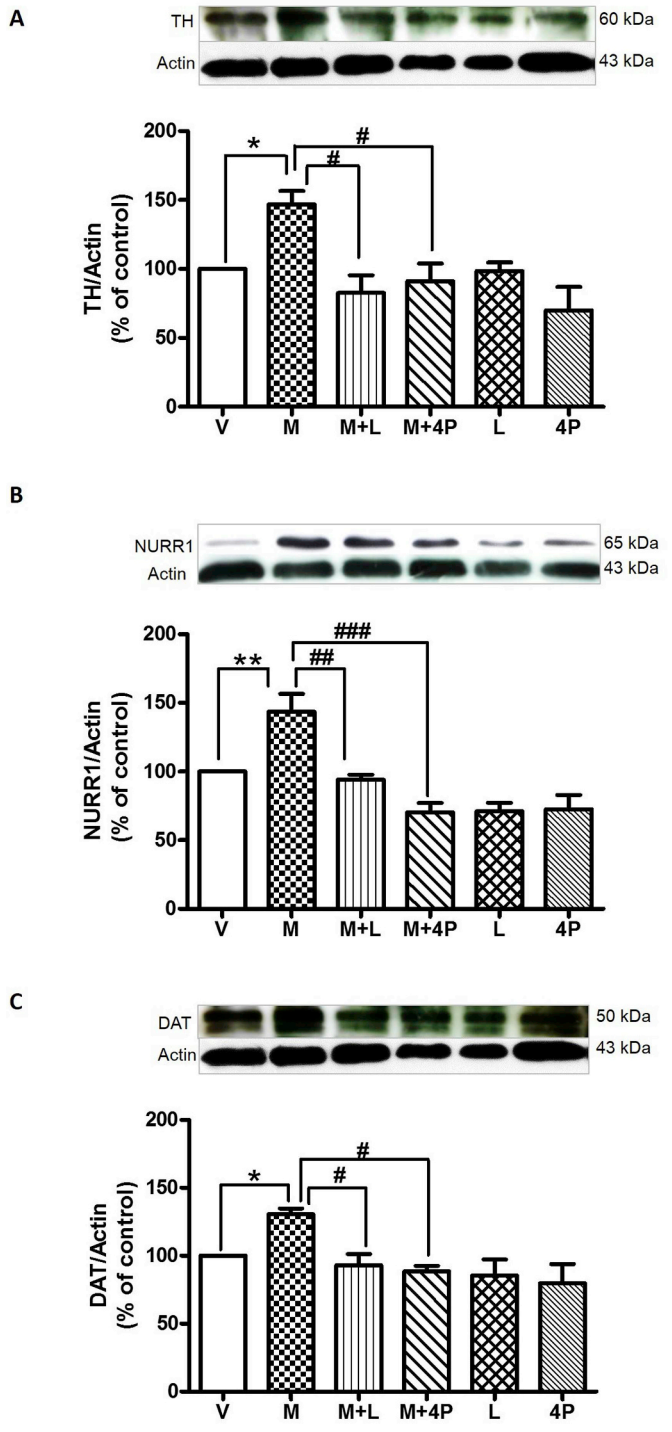


Fig. 7. The effect of antagonist of melatonin receptors, luzindole and 4-P-PDOT on the melatonin-induced increase in neuronal differentiation of AF-MSCs. AF-MSCs were cultured in the presence or absence of 10 μM luzindole or 10 μM 4-P-PDOT pretreatment for 2 h and then cells were treated with 1 μM melatonin for 12 days. Some cells were treated with 10 μM luzindole or 10 μM 4-P-PDOT for 2 h. The levels of dopaminergic neuronal markers (TH, NURR1 and DAT) were shown in density of protein bands which were quantified using ImageJ software. The TH (a), NURR1 (b) and DAT (c) levels were normalized with actin. The results are expressed as mean ± S.E.M of 3 independent experiments. An ANOVA with Turkey's multiple comparison test was performed to analyze the significant different. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle-treated cells, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus melatonin-treated cells. M = melatonin, L = luzindole, 4P = 4-P-PDOT.

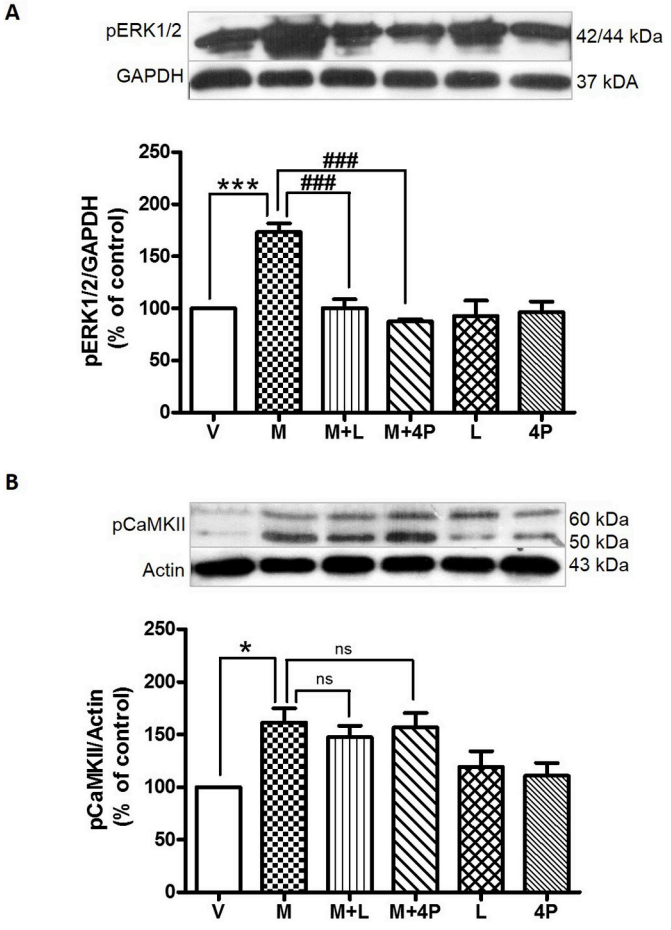


Fig. 8. The effect of antagonist of melatonin receptors, luzindole and 4-P-PDOT on the melatonin-induced phosphorylation in ERK and CaMKII in AF-MSCs. AF-MSCs were cultured in the presence or absence of 10 μM luzindole or 10 μM 4-P-PDOT pretreatment for 2 h and then cells were treated with 1 μM melatonin for 12 days. Some cells were treated with 10 μM luzindole or 10 μM 4-P-PDOT for 2 h. The levels of ERK1/2 and CaMKII phosphorylation were shown in density of protein bands which were quantified using ImageJ software. The pERK (a) and pCaMKII (b) levels were normalized with GAPDH or actin, respectively. The results are expressed as mean ± S.E.M of 3 independent experiments. An ANOVA with Turkey's multiple comparison test was performed to analyze the significant different. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus vehicle-treated cells, and $^{\#\#\#}P < 0.001$ versus melatonin-treated cells. ns = non-significant different. M = melatonin, L = luzindole, 4P = 4-P-PDOT.

GFAP. These results are consistent with previous studies in neural stem cells (Ramirez-Rodriguez et al., 2009; Moriya et al., 2007; Kong et al., 2008). Whether, they are interaction among neuron, astrocyte and microglia in intact and injured brain (Joe et al., 2018). In this study, the role of melatonin on microglia differentiation was not determined in AF-MSCs. Melatonin may be important role for neural differentiation of mesenchymal stem cells. As we know, ROS and the oxidative defense signaling interfere with MSC and pluripotent stem cell differentiation pathways. Melatonin could regulate oxidant defense between undifferentiated stem cells and their differentiated progeny indicates the important role of ROS in the regulation of stem cell fate (Sart et al., 2014). During differentiation, stem cells need high energy demand of differentiation process. Therefore, mitochondria generate ATP through oxidative phosphorylation and also release multiple signaling molecules, including ROS and calcium. High level of ROS could induce microglia or astrocyte differentiation substitute neuronal differentiation. Melatonin may play a role in control microenvironment of neuronal

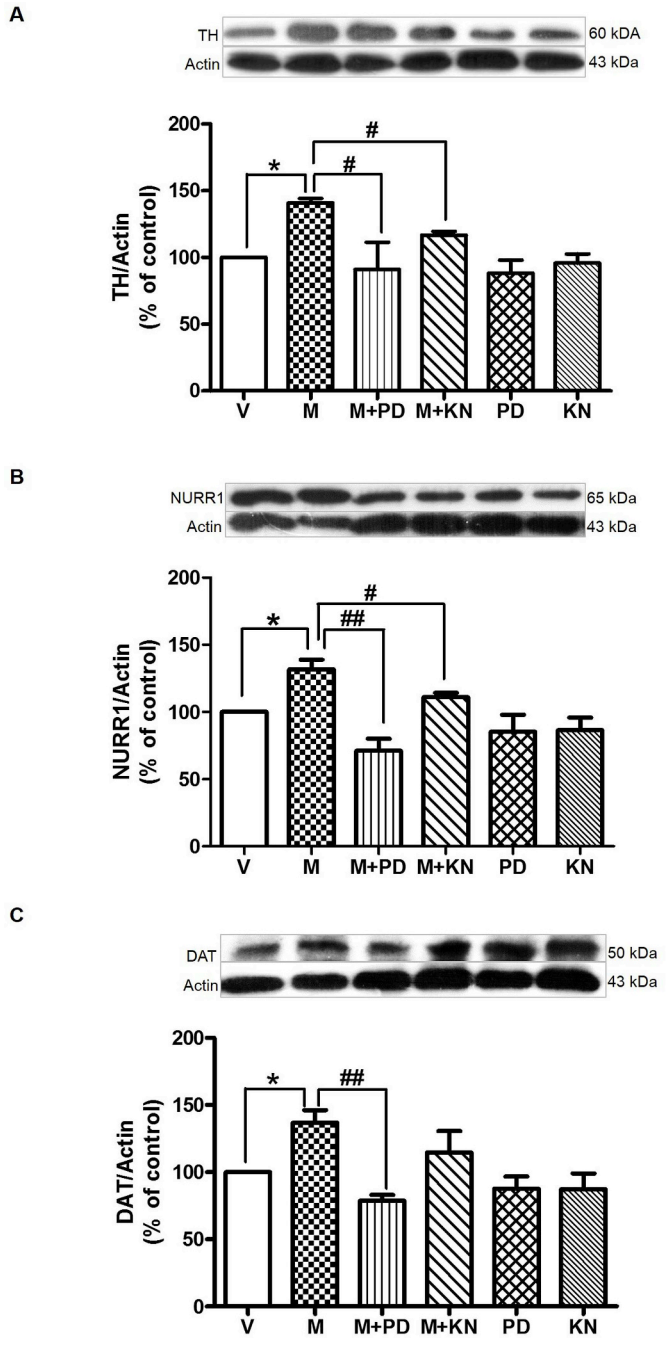


Fig. 9. The effect of inhibition of ERK and CaMKII pathways (PD98059 and KN-93, respectively) on the melatonin-induced increase in neuronal differentiation of AF-MSCs. AF-MSCs were cultured in the presence or absence of 10 μM PD98059 and 1 μM KN-93 pretreatment for 2 h and then cells were treated with 1 μM melatonin for 12 days. Some cells were treated with 10 μM PD98059 and 1 μM KN-93 for 2 h. The levels of dopaminergic neuronal markers (TH, NURR1 and DAT) were shown in density of protein bands which were quantified using ImageJ software. The TH (a), NURR1 (b) and DAT (c) levels were normalized with actin. The results are expressed as mean ± S.E.M of 3 independent experiments. An ANOVA with Turkey's multiple comparison test was performed to analyze the significant different. $^{*}P < 0.05$ versus vehicle-treated cells, and $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus melatonin-treated cells. M = melatonin, PD = PD98059, KN = KN-93.

differentiation during oxidative state in differentiation process.

The rate limiting enzymes for dopamine synthesis, tyrosine hydroxylase (TH) and its transcription factor NURR1 have been used as markers for determining the differentiation of bone marrow mesenchymal stromal cells (BM-MSC) and amniotic epithelial cells (Trzaska et al., 2007; Datta et al., 2011; Niknejad et al., 2012; Pfeiffer and McLaughlin, 2010). NURR1 is known as a transcriptional factor to induce TH expression in dopaminergic neurons (Kim et al., 2013). NURR1 is expressed by over 95% of dopaminergic neurons in the substantia nigra (SN) and ventral tegmental area (VTA) (Backman et al., 1999). In the mouse, NURR1 appears at embryonic day 10.5 (Saucedo-Cardenas et al., 1998). Various studies implicate NURR1 in the regulation of dopamine neurotransmission. Dopamine synthesizing enzymes TH and AADC are both expressed under NURR1 regulation (Hermanson et al., 2003; Smits et al., 2003; Saucedo-Cardenas et al., 1998).

Concomitant melatonin-induced differentiation of AF-MSCs resulted in a decrease in positive staining of MSC markers such as CD29, CD73, CD90, and CD105 and hematopoietic stem cell marker, CD45. The reduction of these antigen markers may have been due to the induction of cell differentiation after melatonin treatment. Recent studies have reported that melatonin can enhance chondrogenic differentiation (Gao et al., 2014) and osteogenic differentiation and inhibit adipogenic differentiation in BM-MSCs (López-Armas et al., 2016; Zhang et al., 2013). Calvo-Guirado et al. (2015) reported that melatonin enhances human adult mesenchymal stem cell (MSC) differentiation into osteoblasts, in comparison with MSCs cultured with dexamethasone, and decreased the expression of CD73, CD105 and CD90. Ali et al. (2015) also reported that CD29, CD73, CD90, and CD105 genes and proteins were down-regulated during chondrogenic differentiation whereas CD29 was up-regulated and CD73, CD90, and CD105 were down-regulated during osteogenic differentiation in Wharton's jelly mesenchymal stem cells. MSC migration is controlled by CD73 and CD29 which in turn are regulated by mechanical stimulation of cells (Ode et al., 2011). Reduction of CD29 (integrin β) is associated with the decreasing migratory capacity of AF-MSCs during differentiation. CD90 has been observed to be down-regulated during differentiation in BM-MSCs (Ode et al., 2011; Hu et al., 2013; Lee et al., 2009). The differentiated adult MSCs were reported to reduce stem cell properties; thus, they were able to differentiate into the chondrogenic, osteogenic, and adipogenic lineages (Barbero et al., 2006). Human MSCs, once they have differentiated into multiple lineages, could not regain their stem cell capacity. Therefore, the typical human MSC markers such as CD44, CD90, and CD105 were lost during differentiation (Moraes et al., 2016). It is interesting that MSC phenotypes change after differentiation of amniotic fluid stem cells. These findings might indicate the pivotal role of melatonin to induce AF-MSCs to differentiate into neuronal cells. Down-regulation of CD29, CD73, CD90, and CD105 during neuronal differentiation is associated with terminal differentiation, capability of migration and loss the properties of MSCs.

We also investigated the efficiency of melatonin on induction of dopaminergic neuron differentiation in AF-MSCs and its underlying mechanisms. The standard protocol to induce dopaminergic differentiation as previously done in BM-MSCs, WJ-MSCs, and AECs (Trzaska et al., 2007; Datta et al., 2011; Niknejad et al., 2012) was applied to induce AF-MSCs into dopaminergic neurons. The standard induction cocktails (ST) contained FGF8, SHH, bFGF, and BDNF, and induced BM-derived MSCs in vitro to become dopamine neurons in 12 days (Trzaska et al., 2007). Therefore, we compared the potential role of ST on induction in neuronal differentiation with melatonin and ST plus melatonin. The expression of MT1 melatonin receptor were shown to present in AF-MSCs and induced cells. Our results showed that melatonin increased the expression levels of dopaminergic neuronal marker proteins (TUJ1, MAP2, TH, NURR1, and DAT) in AF-MSCs as the same result has been observed in ST- and ST plus melatonin-treated cells. The effect of melatonin on neuronal differentiation is consistent with previous studies. Previous reports showed that melatonin increased expression of βIII-tubulin, NURR1, and MAP2 but decreased GFAP expression in mouse NSCs (Sharma et al., 2008; Kong et al., 2008; Sotthibundhu et al., 2010; Fu et al., 2011), PC12 cells, P19 cells (Chen et al., 2014),

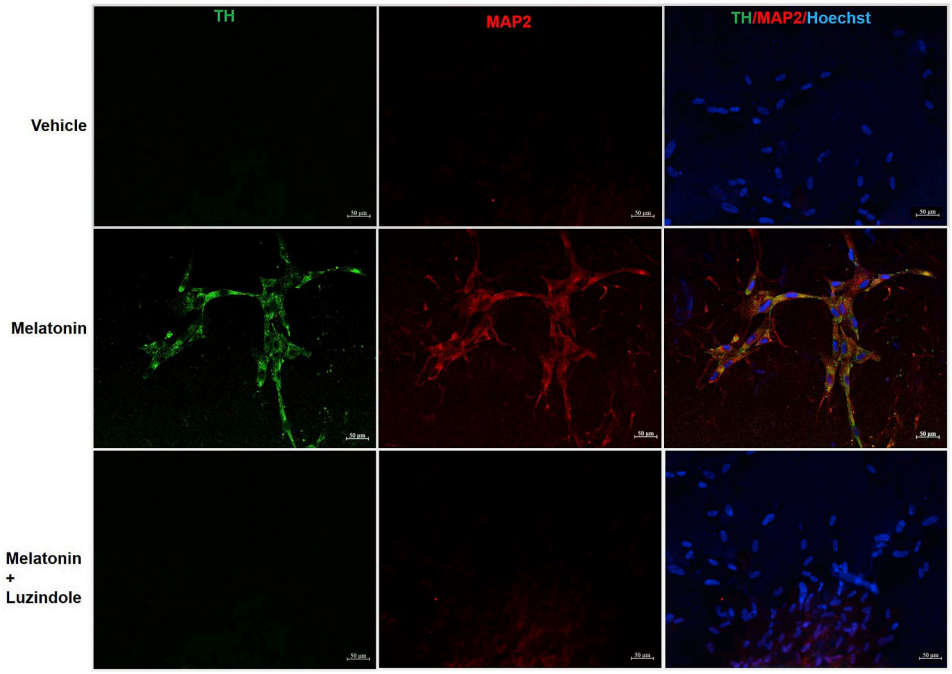


Fig. 10. Immunofluorescence images showing the TH and MAP2 in melatonin- and luzindole-treated AF-MSCs. AF-MSCs were cultured in the presence or absence of 10 μM luzindole pre-treatment for 2 h and then cells were treated with 1 μM melatonin for 12 days. Control cells were incubated with vehicle in culture medium for 12 days. The blue color of Hoechst staining indicates the nucleus. The green and red colors indicate the TH- and MAP2-positive immunofluorescence staining. Co-localization of immunofluorescence staining are shown in merged images.

AECs (Kaneko et al., 2011), and induced pluripotent stem cells (iPSCs) (Shu et al., 2016).

Several factors have been identified and used to determine the fate of midbrain dopaminergic neurons in the embryonic brain, including TH, NURR1, SHH/FGF8, WNT, EN1/2, PITX3, and LMX1b/TGFα/β. SHH and FGF8 are secreted, signaling proteins that function as early determinants to specify the identity of early proliferating dopaminergic progenitors (Ye et al., 1998). SHH is released by the floor plate in the ventral midline and plays the critical role in the specification of different populations by controlling various transcription regulators (Placzek and Briscoe, 2005; Ho and Scott, 2002). FGF8 is derived from the midbrain-hindbrain border, also called organizer (Rhinn and Brand, 2001) which regulates growth and differentiation of the dopaminergic fate of the progenitors, thus generating DA neurons with midbrain characteristics, including expression of TH, LMX1a/b, FOXA2, NURR1, and EN1 as well as typical electrophysiological properties (Xi et al., 2012). The neural floor plate exists throughout the entire length of the neural tube, but the midbrain-hindbrain organizer is a centralized organization playing a key role in controlling the size and relative location of mesencephalic dopaminergic neurons (Prakash and Wurst, 2006). Finally, expression of factors involved in dopamine storage or reuptake such as the dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2) has been shown to be regulated by NURR1 (Hermanson et al., 2003; Smits et al., 2003; Sacchetti et al., 2001, 2006). NURR1-mediated regulation of DAT and VMAT2 indicates that besides its role in embryonic stages, NURR1 also has functioned as the survival factor in dopaminergic neurons in the adult.

The present study explored an involvement of melatonin receptor in neuronal differentiation of AF-MSCs. Our result showed that melatonin activated phosphorylation of ERK1/2 and CaMKII and increased dopaminergic neuronal markers (TH, NURR1 and DAT) levels in melatonin-treated AF-MSCs. Blocking melatonin receptor with luzindole (a non-selective MT1/MT2 antagonist) and 4-P-PDOT (a 1 selective MT2 receptor antagonist) abolished the increased phosphorylation of ERK1/2 and dopaminergic neuronal markers levels. However, the levels of ERK 1/2 and CaMKII phosphorylation, and dopaminergic neuronal markers did not change in luzindole- and 4-P-PDOT treated AF-MSCs. Moreover, the inhibition in ERK 1/2 and CaMKII pathways by MEK inhibitor (PD58098) and CaMKII inhibitor (KN-93) were able to block the effect of melatonin-induced increase in dopaminergic neuronal markers levels. The results suggest that possibly mechanisms of neuronal differentiation mediate through melatonin receptor-dependent pathways. Our result is consistent with the study in mouse NSCs (Fu et al., 2011), P19 cells (Chen et al., 2014), and PC12 cells (Liu et al., 2016) which demonstrated that ERK1/2 was activated, via phosphorylation, in response to melatonin (Fu et al., 2011; Chen et al., 2014). Recent studies showed that ERK1/2 was activated by phosphorylation in response to melatonin in neuronal cell cultures and NSCs of the adult mouse subventricular zone (SVZ) (Roy and Belsham, 2002; Sotthibundhu et al., 2010; Tocharus et al., 2014). The MAPK-ERK pathway regulates the promotion of cell growth, differentiation, and survival (Pearson et al., 2001; Sotthibundhu et al., 2010; Tocharus et al., 2014). Accumulated evidence showed that melatonin plays an important role in NSCs, including its survival, proliferation, and differentiation, which are modulated by many factors, including MAPK/ERK signaling pathway, histone acetylation, neurotrophic factors, transcription factors, and apoptotic genes (Cohen-Armon et al., 2007; Lee et al., 2006; Chu et al., 2016). Liu et al. (2013) revealed that melatonin increases neurite outgrowth of PC12 cells and luzindole and PD98059 (MEK inhibitor) attenuated these increases. Recently, Bai et al. (2016) revealed that melatonin increased proliferation of N-iPS cells via increased phosphorylation of intracellular ERK1/2 pathway in M-N-iPS cells via MT1 receptor.

Furthermore, the potential role of melatonin activation on CaMKII signaling pathway was examined in AF-MSCs. We found that melatonin could activate phosphorylation of CaMKII, but this effect was not inhibited by luzindole and 4-P-PDOT. It is possible that melatonin receptor-independent pathway may partly involve in the neuronal differentiation process. Recent study in organotypic cultures from adult rat hippocampus demonstrated that melatonin induced autophosphorylation of CaMKII and PKC, as well as phosphorylation of ERK1/2 and dendritic formation. The stimulation of dendritogenesis by melatonin is abolished by CaMKII inhibitor (KN-26) while luzindole partially blocked this effect of melatonin (Dominguez-Alonso et al., 2012). CaMKII is a Ca^{2+} activated enzyme that is highly abundant in the brain, enriched at synapses, and is the main protein of the postsynaptic density (PSD). CaMKII is central to the regulation of glutamatergic synapses. Several lines of evidence indicate that CaMKII can be detected under Ca^{2+} elevation and initiates the biochemical cascade that potentiates synaptic transmission (Lisman et al., 2012)w. Thus, CaMKII

may be a marker of functional neurons. In this study, the increase in the levels of phosphorylation of CaMKII in differentiated cells may indicate the capacity of induced cells to develop the function or differentiate into dopaminergic neurons, which associated with the increase in MAP2 and DAT expression and neuronal like-network morphology in AF-MSCs-induced cells.

5. Conclusion

Based on these findings, we conclude that melatonin has the ability of inducing AF-MSCs to differentiate into neurons, especially dopaminergic neurons, indicated by increased the expression of neuronal dopaminergic phenotype markers. In contrast, melatonin suppresses the differentiation of astrocytes as indicated by decreased expression of the glia marker. Melatonin may exert its role to regulate neuronal differentiation by activated ERK signaling pathway via melatonin receptors (receptor-dependent pathway) and CaMKII pathway possibly through intracellular calcium regulation (receptor-independent pathway). On basis knowledge of this study may lead to development of AF-MSCs into neuron cells as a therapeutic tool for neurodegenerative diseases.

Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuint.2018.12.012.

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