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EFFECT OF MELATONIN ON NEURONAL DIFFERENTIATION REQUIRES CBP/P300-MEDIATED ACETYLATION OF HISTONE H3 LYSINE 14

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Abstract—The transition from multipotent neural stem cells (NSCs) to terminally differentiated neurons is a multistep process, and the transition is finely regulated by transcription factors with basic helix-loop-helix (bHLH) motifs. Melatonin is an endogenous neurohormone with profound neurotrophic and neuroprotective effects both during the embryonic developmental stage and adulthood. The effects of melatonin on the differentiation of NSCs have been reported, and these effects may be responsible for its neuroprotective properties. However, the mechanisms underlying the effects of melatonin are not well understood. It is unclear whether melatonin affects the expression of bHLH factors at the onset of neuronal differentiation, and the molecular mechanisms involved still need to be further explored. Using mouse NSCs, we identified a novel role for melatonin in the epigenetic regulation of bHLH factors during neuronal differentiation. Our data showed that melatonin promoted neuronal differentiation by specifically increasing the acetylation of histone H3 lysine14 (H3K14). Increased H3K14 acetylation altered the chromatin state of the promoters of bHLH factors *Neurogenin1* and *NeuroD1* and activated their transcription; then, Neurogenin1 and NeuroD1 initiated and sustained the commitment to neuronal fates. As we know, CBP/p300 is an important class of histone acetyltransferases that acetylate histone H3K14, we found that melatonin activated the histone acetyltransferase activity of CREB-binding protein (CBP)/p300 via ERK signaling pathways. For the first time, we systematically showed the molecular mechanism of action of melatonin, which suggested that melatonin functions as a regulator of the acetylation-dependent gene expression network.

Key words: melatonin, neural stem cells, neuronal differentiation, histone acetylation, histone H3 lysine 14, CREB-binding protein/p300.

INTRODUCTION

Neural stem cells (NSCs) are a subset of precursor cells that possess the ability to self-renew and differentiate into neuronal and glial cells (McKay, 1997; Temple, 2001). The progression from proliferation to neuronal commitment is a multistep process; in response to the dynamic interplay between extracellular cues and intracellular signals, multipotent NSCs exit the cell cycle and ultimately give rise to neurons with distinct cell identities (Morrison, 2001; Sauvageot and Stiles, 2002). With the successful development of NSC-based therapies for nervous system disorders, the molecular mechanisms that orchestrate this precise temporally and spatially regulated process have been the focus of intense research in recent years (Lindvall and Kokaia, 2006; Lederer and Santama, 2008; Ernst, 2016). Knowledge about these mechanisms may be useful for the development of appropriate therapeutic strategies for neurological diseases by enabling the behavior of transplanted or endogenous progenitors to be controlled.

Melatonin, an indoleamine mainly synthesized and secreted by the pineal gland in a circadian manner, is widely distributed in the brain (Pandi-Perumal et al., 2008; Hardeland et al., 2012). Melatonin has profound neurotrophic and neuroprotective effects during both the embryonic developmental stage and adulthood (Pappolla et al., 1998; Sarlak et al., 2013; Liu et al., 2015b; Mack et al., 2016; Sagrillo-Fagundes et al., 2016; Ramos et al., 2017). Emerging evidence suggests that melatonin might mediate such protective actions by influencing the proliferation and differentiation of NSCs (Chu et al., 2016; Yu et al., 2016). Kong and colleagues showed that melatonin significantly facilitates the differentiation of rat midbrain NSCs into dopaminergic neurons.

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Abbreviations: ac, acetylation; bHLH, basic helix-loop-helix; CBP, CREB-binding protein; CNS, central nervous system; CREB,, cAMP-response element binding protein; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; GPCRs, G protein-coupled receptors; H3K14, histone H3 lysine14; HAT, histone acetyltransferase; HDACs, histone deacetylases; MAP2, microtubule-associated protein 2; Ngn, Neurogenin; NSCs, neural stem cells; PHH3, phosphohistone-H3; Tuj1, neuron-specific class III beta-tubulin.

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The mRNA and protein levels of tyrosine hydroxylase, a marker of dopaminergic neurons, were higher in melatonin-treated cells than in untreated control cells (Kong et al., 2008). Our previous study demonstrated that melatonin reversed impairments in the differentiation of NSCs induced by hypoxia and increased the percentage of microtubule-associated protein 2 (MAP2; a neuronal marker)-positive cells (Fu et al., 2011). However, the mechanisms underlying the beneficial effects of melatonin are not fully understood.

The transition from multipotency NSCs to postmitotic neurons is regulated to a large degree by transcription factors with basic helix-loop-helix (bHLH) motifs (Lee, 1997; Ross et al., 2003). There are two types of bHLH genes, the repressor type (e.g., *Hes* genes) and the activator type (e.g., *Mash1*, *Math*, *Neurogenin*, and *NeuroD* genes). The activator bHLH factors promote the determination of neuronal fate and regulate neuronal subtype specification. They dimerize with E proteins (E12, E47, etc.) and bind specific DNA sequences in neuronal-specific genes. The sequences containing the core hexanucleotide motif CANNTG, are termed E boxes. Transactivation is mediated by the interaction of bHLH dimers with coactivators, which recruit a large transcription activator complex. (Ross et al., 2003; Kageyama et al., 2005; Seo et al., 2007). Among these bHLH factors, Neurogenin (Ngn) and NeuroD are key regulators of neuronal differentiation. Ngn acts at the top of the regulatory cascade to initiate neuronal differentiation, and NeuroD is a key Ngn-regulated transcriptional node (Ma et al., 1996; Seo et al., 2007; Pataskar et al., 2016). Although we have previously reported that melatonin increased the expression of bHLH factors in NSCs after hypoxia treatment (Fu et al., 2011), it is unclear whether melatonin regulates NSCs differentiation through Ngn1 and Neruod1. It is necessary to explore how melatonin affects the expression of key bHLH factors in the differentiation of NSCs and what molecular mechanisms are involved in this regulatory process. It will be helpful to understand the neuroprotective effect of melatonin in depth in order to effectively apply it in clinical treatment.

Here, we found that melatonin enhanced the histone acetyltransferase (HAT) activity of CREB-binding protein (CBP) and p300 via the ERK signaling pathway and increased histone H3 lysine14(H3K14) acetylation levels at the promoters of *Ngn1* and *NeuroD1*. The alterations promoted the expression of these two genes and eventually led to an increase in neuronal differentiation. On the basis of our analysis, we suggest a possible mechanism for melatonin action.

EXPERIMENTAL PROCEDURES

Primary NSC culture

NSCs were cultured according to a previously described method with minor modifications (Fu et al., 2011; Chen et al., 2016). Briefly, the cells were isolated from the brain of Kun Ming mouse at embryonic day 12.5. Tissues were finely minced and digested mechanically. After centrifugation, cells were resuspended and counted. Cell were seeded at 2×10^5 cells/mL in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) medium supplemented with 2% B27 (Gibco, Caithersburg, MD, USA), 20 ng/mL basic fibroblast growth factor (bFGF; R&D, Minneapolis, MN, USA), 20 ng/mL epidermal growth factor (EGF; Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell were seeded onto 25-cm^2 T-flasks and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After 5 days of incubation, the primary neurospheres were harvested by centrifugation and dissociated into single cells using trypsin and EDTA (Gibco, Caithersburg, MD, USA), and the single cells were re-plated in serum-free medium and cultured for 3–5 days (passage 1 neurospheres). For differentiation studies, the passage 1 neurospheres were dissociated and transferred into differentiation medium containing 2% fetal bovine serum (FBS) without the growth factors and cultured for 1–7 days. Animal care and treatment complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the experimental protocol was authorized by the Ethics Committee on Animal Experiments of the medical school of Shandong university (No. 201402020).

The melatonin (Sigma–Aldrich, St Louis, MO, USA) was dissolved in ethanol at a concentration of 20 mM and then diluted with culture medium to a final concentration of 100 nM (Fu et al., 2011). To study the effects of melatonin on neuronal differentiation, melatonin was added to the cell cultures for 1, 3, 5, and 7 days. The melatonin receptor antagonists luzindole (Tocris Bioscience) and 4-phenyl-2-propionamidotetralin (4-P-PDOT) (Sigma–Aldrich, St Louis, MO, USA) were added

Table 1. Primers used in quantitative real-time PCR analysis

Gene	Forward primer	Reverse primer
P21	5′-GAGAACGGTGGAACTTTGACTTC-3′	5′-GAGGAAGTACTGGGCCTCTTG-3′
P27	5′-CGACTTTCAGAATCATAAGCCCC-3′	5′-TTGCCTGAGACCCAATTAAAGG-3′
MAP2	5′-GAATAAGCAAGAGCCCAGAG-3′	5′-GTCCGTCGTGCTGAAGAG-3′
GFAP	5′-CTGGCTTCAAGGAGACACG-3′	5′-TCCGCCTGGTAGACATCA-3′
Ngn1	5′-CTGCGCTTCGCCTACAACT-3′	5′-TGAAGCCGAGGGACTACTGG -3′
NeuroD1	5′-TGCTACTCCAAGACCCAG-3′	5′- AAGAAAGTCCGAGGGTTGA-3′
Hes1	5′-AAGCCTATCATGGAGAAGAGG-3′	5′-GTTGATCTGGGTCATGCAGT-3′
Hes5	5′-GATGCTCAGTCCCAAGGAGA-3′	5′-CGTGGAAGTGGTAAAGCAG-3′
Ngn1(ChIP)	5′-CATTGTTGCGCGCCGTA-3′	5′-GCGATCAGATCAGCTCCT-3′
NeuroD1(ChIP)	5′-GTCCGCGGAGTCTCTAACTG-3′	5′-GAACCACGTGACCTGCCTAT-3′