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Melatonin ameliorates dexamethasone-induced inhibitory effects on the proliferation of cultured progenitor cells obtained from adult rat hippocampus

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ABSTRACT

Glucocorticoids, hormones that are released in response to stress, induce neuronal cell damage. The hippocampus is a primary target of glucocorticoids in the brain, the effects of which include the suppression of cell proliferation and diminished neurogenesis in the dentate gyrus. Our previous study found that melatonin, synthesized primarily in the pineal, pretreatment prevented the negative effects of dexamethasone, the glucocorticoid receptor agonist, on behavior and neurogenesis in rat hippocampus. In the present study, we attempted to investigate the interrelationship between melatonin and dexamethasone on the underlying mechanism of neural stem cell proliferation. Addition of dexamethasone to hippocampal progenitor cells from eight-week old rats resulted in a decrease in the number of neurospheres; pretreatment with melatonin precluded these effects. The immunocytochemical analyses indicated a reduction of Ki67 and nestin-positive cells in the dexamethasone-treated group, which was minimized by melatonin pretreatment. A reduction of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation and G1-S phase cell cycle regulators cyclin E and CDK2 in dexamethasone-treated progenitor cells were prevented by pretreatment of melatonin. Moreover, luzindole, a melatonin receptor antagonist blocked the positive effect of melatonin whereas RU48, the glucocorticoid receptor antagonist blocked the negative effect of dexamethasone on the number of neurospheres. Moreover, we also found that dexamethasone increased the glucocorticoid receptor protein but decreased the level of MT1 melatonin receptor, whereas melatonin increased the level of MT1 melatonin receptor but decreased the glucocorticoid receptor protein. These suggest the crosstalk and cross regulation between the melatonin receptor and the glucocorticoid receptor on hippocampal progenitor cell proliferation.

1. Introduction

Stress is a significant threat to homeostasis that must be re-established by various physiological and behavioral adaptive responses [1]. Stress activates the hypothalamo-pituitary-adrenal axis that triggers the release of corticortropin-releasing hormone from the hypothalamus. This cascade of signals stimulates the release of glucocorticoids from the adrenal cortex. The release of glucocorticoids can then act on neurons that display the appropriate receptor in the brain. The glucocorticoid receptors (GR) are highly observed in various brain areas, including the dentate gyrus of the hippocampus [2]. The hippocampus – one of the important target of glucocorticoids – is a brain region noted for its plasticity in response to stressful events [3]. Recent studies suggest that a variety of stressors cause the suppression of various stages of neurogenesis; most of them involving the inhibition of cell proliferation [4]. Several studies have shown that treatment with antidepressants increases the number of cells born in the

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hippocampus – the majority of which differentiate into neurons [5,6]. This outcome could feasibly improve scores in behavioral assays, such as the forced swimming [7] and tail suspension tests [8].

Melatonin is a hormone that is primarily released from the pineal gland. Different groups of investigators found alteration of melatonin levels in animal models of mood disorders and depression [9,10]. The recent studies suggested that melatonin, *via* its receptor, can modulate the survival of newborn neurons in the adult hippocampus [11,12]. Moreover, melatonin can prevent glucocorticoid-mediated inhibition of cell proliferation in rats [13] and mouse hippocampal HT22 neuronal cell line [14]. Our previous study found that pretreatment with melatonin prevented both cognitive impairment caused by dexamethasone and dexamethasone-induced reduction in brain-derived neurotrophic factor (BDNF), synaptophysin, glutamate receptor subunit NR2A/B and Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) in adult mouse hippocampus [15]. Moreover, our recent work *in vivo* found that pretreatment with melatonin prevented dexamethasone-induced depressive-like behavior [16]. Whereas many studies focus on the influence of melatonin and stress on cell proliferation, little is known regarding the mechanism underlying these effects.

In this study, we used an *in vitro* model to study the direct effects of melatonin and dexamethasone, a GR agonist, on the proliferation of adult hippocampal progenitor cells. We also established whether the antagonistic effect of melatonin on glucocorticoid-induced alteration of hippocampal progenitor cell proliferation is mediated *via* membrane-bound melatonin receptors or independently regulated by other pathways.

2. Materials and methods

2.1. Reagents and chemicals

DEX-21-phospho disodium salt, melatonin and luzindole were purchased from Sigma–Aldrich Chemical Company (St. Loius, MO). Mifepristone (RU486) was purchased from EMD chemicals, Inc. (San Diego, CA). A mouse anti-β-actin monoclonal antibody was purchased from Merk Millipore (Darmstadt, Germany). Goat polyclonal antisera against doublecortin (DCX), mouse antisera against the extracellular signal-regulated kinase 1 and 2 (ERK1/2) and P-ERK1/2, mouse monoclonal antibody against β-III tubulin, rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP), rabbit polyclonal antibody against phospho-c-myc antibody, rabbit polyclonal antibody against cyclin E antibody, rabbit polyclonal antibody against CDK2 antibody, mouse monoclonal antibody against GR, goat polyclonal antibody against MEL-1A-R and goat polyclonal antibody against MEL-1B-R were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal antibody against Ki67 was purchased from abcam (Cambridge, UK). Mouse monoclonal antibody against nestin was purchased from Millipore (Bradford, MA). Neurobasal medium, B27, fetal bovine serum (FBS) and trypsin were purchased from GIBCO-BRL (Life Technologies, Carlsbad, CA). Basic fibroblast growth factor (bFGF) was purchased from Roche (Mannheim, Germany), and epidermal growth factor (EGF) was purchased from BD Biosciences (Franklin Lakes, NJ).

2.2. Animals

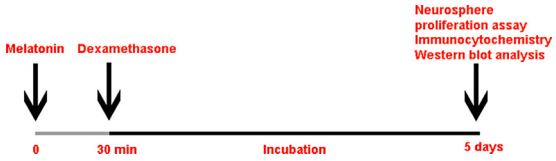
Eight-week old male Wistar rats were obtained from the National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand. The animals were housed under a 12:12-h light/dark cycle, and the room temperature was maintained at $24 \pm 1\,^{\circ}C$; water was supplied ad libitum. The experimental protocol was approved by the Animal Ethics Committee in accordance with the guide for the care and use of laboratory animals prepared by Mahidol University.

2.3. Preparation of NSC from hippocampus of adult rats

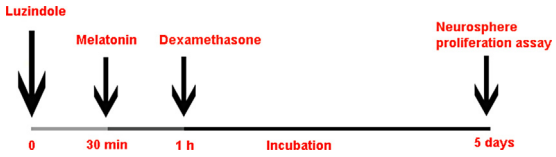
Adult hippocampal neural precursor cells were isolated from the hippocampus of 8-week old male rats as previously described [17]. Briefly, animals were killed by decapitation. Brains were removed from the skull and placed in cold artificial CSF. Hippocampal tissue was dissociated in 0.05% trypsin for 7 min. After trypsin neutralization, cells were spun down at 800 rpm for 5 min. Supernatant was removed and cells were suspended in 500 μl culture media (neurobasal medium containing 20 ng/mL bFGF, 20 ng/mL EGF, 2% B27, 2 mM Glutamax™ supplement and 1% penicillin–streptomycin mixtures). The hippocampal cells were gently triturated to yield a single-cell suspension and passed through a 40 μm cell strainer into a sterile 50 ml tube. Cells were plated in 4-well plates and incubated at 37 °C in 5% CO_2 for five days. To investigate the effects of melatonin and dexamethasone, melatonin or dexamethasone was added into the culture medium. To determine the protective effects of melatonin, cells were pretreated with 1 μM melatonin for 30 min prior to 1 μM dexamethasone treatment. Cells were plated at a density of 0.5×10^5 cells/ml for protein extraction and 2500 cells/ml for the neurosphere proliferation assay.

2.4. Neurosphere proliferation assay

The neurospheres were dissociated with 0.05% trypsin for 5 min. The single cells were plated at a density of 2500 cells in a 24-well plate in serum-free medium containing neurobasal medium, B27, 20 ng/ml EGF and 20 ng/ml bFGF. At day three, clusters of cells started to form small neurospheres floating in the medium. The size of neuropsheres continued to grow larger at day five. Various concentrations of melatonin and/or dexamethasone were added into the culture medium. A number of neurospheres (diameter > 80 μm) were counted directly under the microscope after five days of incubation.



To study the role of the melatonin receptor, the melatonin receptor antagonist luzindole was added to the culture medium 30 min prior to the addition of melatonin. The number of neurospheres (diameter > 80 μm) were counted directly under microscope after five days of incubation.



To investigate the role of the glucocorticoid receptor, the glucocorticoid receptor antagonist mefiprinstone (RU486) was added to culture medium 30 min prior to the addition of