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Melatonin influences the proliferative and differentiative activity of neural stem cells

Abstract: Though melatonin has a wide variety of biological functions, its effects on the neural stem cells (NSCs) is still unknown. In this study, we examined the effects of melatonin at either physiological (0.01–10 nM) or pharmacological concentrations (1–100 μM) on the proliferation and neural and astroglial differentiation of NSCs derived from the mouse embryo striatum using an in vitro culture system. We found that melatonin at pharmacological concentrations, but not at physiological concentrations, suppressed epidermal growth factor (EGF)-stimulated NSC proliferation (increment of viable cells, DNA synthesis and neurosphere formation) in a concentration-dependent manner. Furthermore, treatment with melatonin at a pharmacological concentration during the proliferation period facilitated 1% FBS-induced neural differentiation of NSCs without affecting the astroglial differentiation. In contrast, the treatment with melatonin at pharmacological concentrations during the differentiation period decreased the neural differentiation of the NSCs. As with melatonin, MCI-186, an antioxidant, suppressed EGF-stimulated NSC proliferation and facilitated the subsequent neural differentiation of NSCs. These results suggest that melatonin exerts potent modulatory effects on NSC functions including the suppression of the proliferation and facilitation of neuronal differentiation, likely via its antioxidant activity. As neurogenesis is thought to play an important role in ameliorating the deficit in neurodegenerative diseases, melatonin might be beneficially used for the treatment diseases such as cerebral infarction.

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Introduction

Recent extensive efforts have revealed that three cell types in the brain (neurons, astrocytes and oligodendrocytes) originate from the immature precursor cells referred to as neural stem cells (NSCs) [1, 2]. The NSCs possess self-renewal and multipotential abilities and are located not only in the developing mammalian brain but also in the adult brain, especially abundant in the anterior subventricular zone and subgranular zone of the hippocampal dentate gyrus [3]. Furthermore, the proliferative and differentiative activities of the NSCs are not stable, rather they are dynamically regulated by various humoral and adhesive factors under physiological and pathophysiological conditions [4]. Thus, the identification of the signaling molecules regulating NSC activity may contribute not only to the understanding of the neural ontogeny but also toward the development of new therapy against neural death.

Melatonin is a major indole released from the pineal gland, and it possesses a wide variety of potent neurobiological actions including control of seasonal reproduction [5] as well as the regulation of circadian rhythms and body temperature via high affinity receptors on the cell membrane or presumptively via low affinity receptors in the nucleus [5–8]. In addition to these physiological functions, melatonin also protects neurons from ischemia-reperfusion injury [9–11] probably because of its radical scavenging actions [12–14]. These observations have documented the ability of melatonin to prevent neural cell death. The effects of melatonin on neurogenesis from the NSCs in the central nervous system, however, are unknown. Using clonal neural cell lines, a few studies demonstrated that this indole influences cell growth and differentiation of the neural blastoma [15] and PC12 cells [16–18].

In this study, we examined the effects of melatonin at either physiological (0.01–10 nM) and pharmacological concentrations (1–100 μM) on the proliferation and differentiation of the NSCs derived from mouse embryo striatum using in vitro culture systems. In these in vitro experiments of NSCs, it is possible to discriminate the proliferation and differentiation processes and also delineate the direct actions of extrinsic factors [2]. In addition, we also investigated whether the effects of melatonin on the NSC activity depended on the drug exposure timing i.e. during the proliferation or differentiation periods. Here, we showed that the melatonin at pharmacological concentrations (1–100 μM) had potent modulatory effects on the

proliferation and neural differentiation of the NSCs in an exposure timing-dependent manner.

Material and methods

Cell culture

The NSCs were isolated and propagated using a neurosphere method developed by Reynolds and Weiss [19] and Reynolds et al. [20] with minor modifications [21]. Fetal ICR (Slc : ICR) mice at embryonic day 15.5 (where embryonic day 0 = a midnight of the day of overnight mating) were isolated from their mothers under deep anesthesia using ether and placed in an ice cold 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 nutrient (DMEM/F-12; Gibco-BRL, Gaithersburg, MD, USA). The ganglionic eminence (the developing striatum) tissues were carefully micro-dissected using a stereomicroscope and triturated with a 1 mL plastic pipette to obtain a single cell suspension in N-2 medium (DMEM/F-12 supplemented with 0.34% glucose, 23 μg/mL insulin, 92 μg/mL transferrin, 55 μM putrescine, 27.5 nM sodium selenite, 20 nM progesterone, 50 U/mL penicillin–streptomycin). The viable dissociated cells at a density of 25×10^4 cells in 5 mL of N-2 medium containing 20 ng/mL EGF (Invitrogen Corp., Carlsbad, CA, USA) were seeded into uncoated T25 culture flasks (Becton-Dickinson, Franklin Lakes, NJ, USA) and were maintained in a humidified incubator at 37°C and 95% atmospheric air/5% CO_2. Cultures were fed with 1.25 mL of fresh medium every second day and were incubated for 7–8 days to form a sufficient number of neurospheres. All animal housing and surgical procedures were in accordance with the guidelines of the Japanese Physiological Society and were approved by the Institutional Animal Care and Use Committee of Graduate School of Biomedical Sciences Nagasaki University.

Experimental procedure

Melatonin was purchased from Sigma (St. Louis, MO, USA) and dissolved in ethanol. MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5-one) was kindly gifted by Mitsubishi Pharma (Osaka, Japan) and dissolved in water. An equivalent volume of ethanol (final: 0.01%) or water was added to the control wells and all melatonin-containing wells. The effects of melatonin on the proliferative activity of the NSCs were evaluated by three methods; (i) indirect measurement of the number of viable cells by WST-8 assay; (ii) BrdU incorporation assay; and (iii) the counting the number of neurospheres. The single cell suspensions from primary neurospheres were prepared by a centrifugation (300 g, 5 min) followed by a mechanical dissociation. They were seeded in a nontreatment 96-well plate (Becton-Dickinson) in the absence or presence of various concentrations of melatonin (0.01 nM to 100 μM) and the number of viable cells, their BrdU incorporation and the number of secondary neurospheres were measured on an appropriate day (Fig. 1A,B). The numbers of wells per a group were four (WST-8 assay) and 40 or 12 (the counting the number of neurospheres). The single cell suspensions from primary neurospheres were re-expanded by EGF (20 ng/mL) in T25 culture flask in the absence or presence of melatonin (1–50 μM). The resultant secondary neurospheres were mechanically dissociated and seeded in nontreatment 96-well plate without melatonin and the number of viable cells and ternary neurospheres were counted on an appropriate day (Fig. 1C).

The effects of melatonin on the differentiation of the NSCs were evaluated by the fluorescence-based immunocytochemistry and ELISA. In addition, we examined the effects of melatonin when applied during the proliferation and differentiation periods as a previous report described that a facilitatory action of hypoxia on NSC differentiation depended on the timing of exposure [22]. Briefly, the single cell suspensions from primary neurospheres were re-expanded by EGF (20 ng/mL) in T25 culture flask in the absence or presence of melatonin (1–25 μM). The resultant secondary neurospheres were mechanically dissociated and differentiated for 7 days in the absence or presence of melatonin (1–100 μM) by an incubation in 1% FBS-containing N-2 medium without EGF in Lab-Tek® Chamber Slide^{TM} (Nunc Inc., Naperville, IL, USA) precoated with 0.005% poly-L-ornithine (Sigma) for immunocytochemistry (Fig. 2A) or tissue culture treated 96-well plate (Becton-Dickinson) precoated with 0.0025% poly-L-ornithine for enzyme-linked immunosorbent assay (ELISA) (Fig. 2B). The numbers of wells per a group were 4 (immunocytochemistry) and 4–5 (ELISA).

WST-8 assay

The indirect counting of viable cells was carried out by WST-8 assay using a Cell Counting Kit-SF (Tesque Nacalai, Kyoto, Japan). Briefly, the single cell suspension of EGF-expanded NSCs were seeded in nontreatment 96-well plate (Becton-Dickinson) at a cell density of 5000 cells per well. After 1, 4 and 7 day incubation periods in the absence or presence of melatonin, 5 μL of the Cell Counting Kit (Tesque Nacalai) solution was added to each well and incubated for an additional 5 hr at 37°C. The absorbance at 450 nm with a reference wavelength of 630 nm was measured by a microplate reader (Thermo Labsystems, Vantaa, Finland) and the net absorbance subtracting the values of cell-free wells was calculated. Our preliminary studies showed that these experimental procedures produced a good linear relationship between the net absorbance and the cell density (0–15 000 cells per well) (data not shown).

BrdU incorporation assay

The BrdU incorporation assay was performed using a Cell Proliferation ELISA BrdU kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the single cell suspension of EGF-expanded NSCs was seeded in nontreatment 96-well plate (Becton-Dickinson) at a cell density of 5000 cells per well. After a 24-hr incubation period, cells were labeled with a 10 μM BrdU for 8 hr. After centrifugation at 300 g for 10 min, the labeling medium was removed and cells were dried and the cellular DNA was denatured by adding FixDenat solution (Roche) for 30 min at room temperature. After removal of the FixDenat

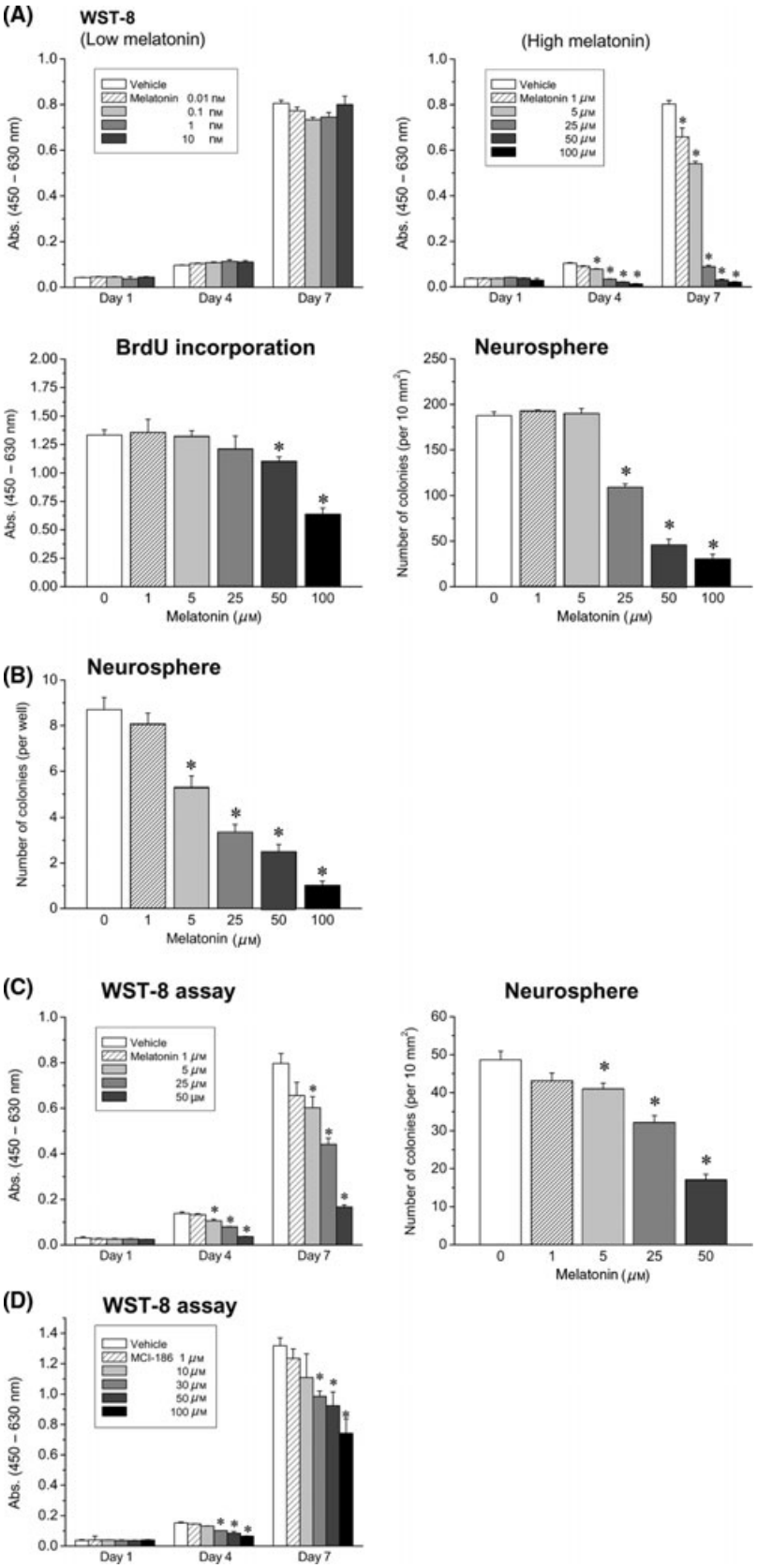


Fig. 1. Effects of low or high concentration of melatonin on the proliferative activity of the cultured neural stem cells (NSCs). The single NSCs from the primary neurospheres were subcultured on 96-well plate at the high-cell density (5000 cells/well) (A) or the low density (100 cells/well) (B) in the EGF-containing medium in the absence or presence of low (0.01–10 nM) or high (1–100 μM) concentrations of melatonin. The increment in the number of viable cells was assessed by WST-8 assay on day 1, 4 and 7 and the DNA synthesis was evaluated by BrdU incorporation assay 1 day after the subculture (day 1). The number of neurospheres on day 7 was also measured by a computer-assisted image analysis. The averages and S.E.M. of the data from four (A) or 40 (B) wells are shown. (C) Inhibitory effect of pretreatment with melatonin on the re-proliferative activity of the NSCs in the EGF-containing medium without melatonin. The dissociated NSCs from the primary neurosphere were re-expanded with EGF in the absence or presence of melatonin for 5 days and the resultant secondary neurospheres were mechanically dissociated and re-subcultured on 96-well plate at the high cell density (5000 cells/well) in the EGF-containing medium without melatonin. The increment in the number of viable cells and the number of neurospheres were examined as described above. The averages and S.E.M. of the data from four (WST-8 assay) or 12 (Neurosphere) wells are shown. (D) Inhibitory effect of an antioxidant, MCI-186, on the proliferative activity of the NSCs. The single NSCs from the primary neurospheres were subcultured on 96-well plate at the high cell density (5000 cells/well) in the EGF-containing medium in the absence or presence of MCI-186. The increment in the number of viable cells was assessed by WST-8 assay on day 1, 4 and 7. The averages and S.E.M. of the data from four wells are shown. Asterisks show the significant difference from the vehicle control ($P < 0.05$; one-way ANOVA followed by Dunnett's test).

solution (Roche), anti-BrdU antibody conjugated with peroxidase solution was added onto each well and incubated for 90 min at room temperature. After three washes with phosphate buffered saline (PBS), TMB solution was added to each well and the cells were incubated for 60 min at room temperature. Immediately after stopping the total

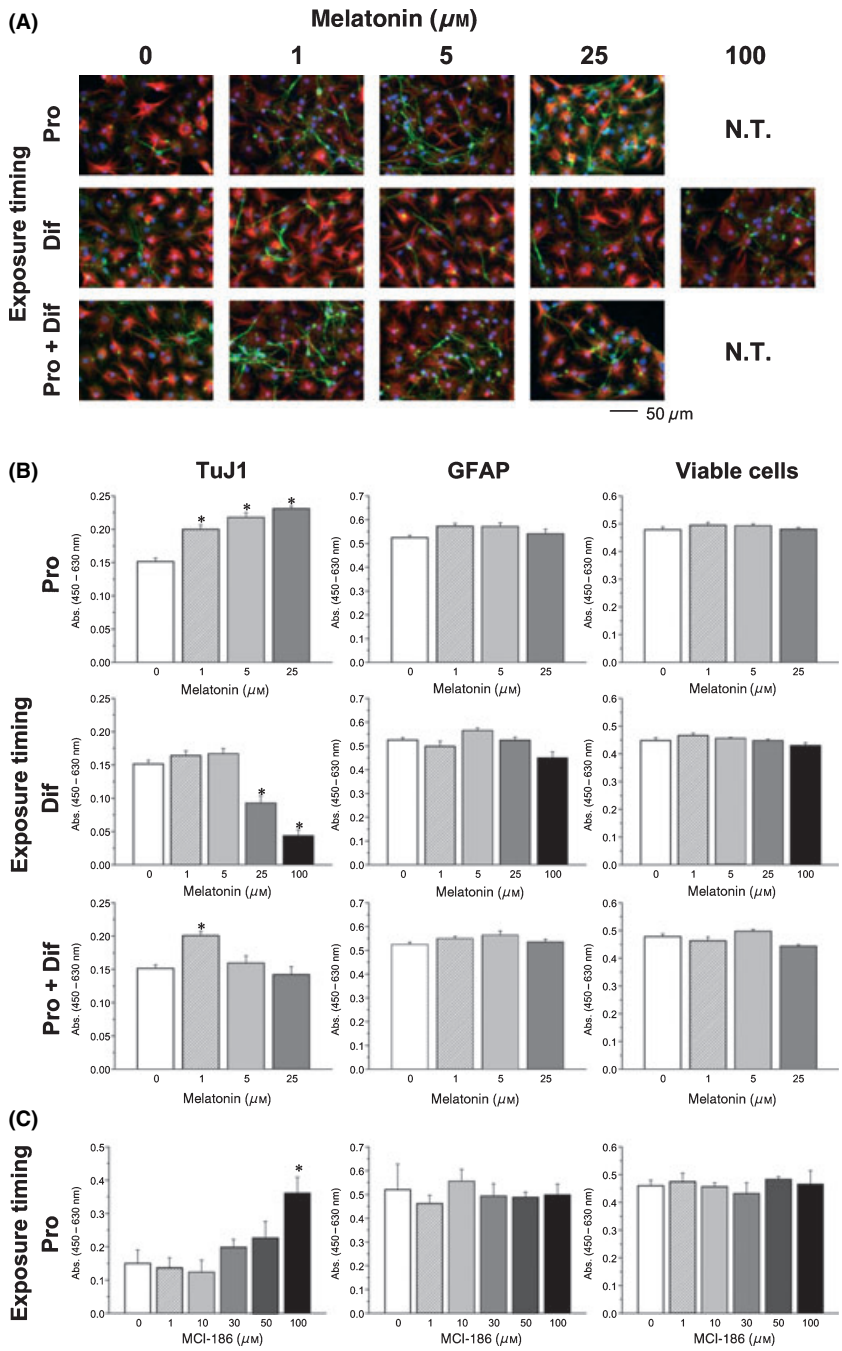


Fig. 2. Modulation on the neural differentiation of the neural stem cells (NSCs) by melatonin as a function of exposure-timing. The primary neurospheres produced in the EGF-containing medium were dissociated and differentiated in the Lab-Tek® Chamber Slide^{TM} or 96-well plate by an incubation with 1% FBS-containing medium for 7 days in the absence or presence of melatonin (Dif). Otherwise, the dissociated NSCs from the primary neurosphere were re-expanded with EGF in the absence or presence of melatonin or MCI-186 for 5 days and the resultant secondary neurospheres were mechanically dissociated and differentiated as described above in the absence (Pro) or presence of melatonin or MCI-186 (Pro + Dif). The differentiations were evaluated by the fluorescence-based immunocytochemistry on the Lab-Tek® Chamber Slide^{TM} using a mouse monoclonal antibody against TuJ1, a premature neural marker, and a rabbit polyclonal antibody against GFAP, an astroglial marker (A) or by enzyme-linked immunosorbent assay (ELISA) on the 96-well plate using same primary antibodies as in the immunocytochemistry (B, C). The photographs in (A) are the representative immunofluorescence images in four stained wells of Lab-Tek® Chamber Slide^{TM}. N.T. in (A) means 'not tested' because 100 μM of melatonin yielded much less neurosphere even in the EGF-containing medium (see Fig. 1A). The relative amount of viable cells of the differentiated NSCs was also measured by WST-8 assay. The averages and S.E.M. of the data from five (B) and four (C) wells are shown. Asterisks in (B) and (C) show the significant difference from the vehicle control ($P < 0.05$; one-way ANOVA followed by Dunnett's test). The control value of 'Pro + Dif' group was re-plotted from that of 'Pro'.

reaction by adding 1N H_2SO_4 solution, the absorbance at 450 nm (reference wavelength 630 nm) was measured by a microplate reader (Thermo Labsystems) and the net absorbance subtracting the value of cell-free wells was calculated. Our preliminary studies showed that these experimental procedures produced a good linear relationship between the net absorbance and the cell density (0–15 000 cells per well) (data not shown).

Neurosphere counting

The single cell suspension of EGF-expanded NSCs were seeded in nontreatment 96-well plate (Becton-Dickinson) at a cell density of 100 or 5000 cells per well and was incubated for 7 days with EGF in the absence or presence of melatonin. The fixed area (10 mm^2) at the center of each well was converted into a digital image using a digital still camera (DSC-S70; Sony, Tokyo, Japan) and the number of neurospheres which diameter was over 60 μM was counted by Scion Image Beta 4.02 (Scion Corporation, Frederick, MD, USA).

Immunocytochemistry

The differentiated cells in Lab-Tek® Chamber Slide^{TM} (Nunc) were fixed in PBS containing 4% paraformaldehyde

for 20 min at room temperature and they were permeabilized by incubation with 100% methanol at −20°C for 10 min. After removal of methanol, cells were incubated overnight with anti-TuJ1 antibody (1:200; mouse monoclonal antibody, Sigma) and anti-GFAP antibody (1:80; rabbit polyclonal antibody, Sigma) in PBS containing 0.1% Triton X-100 and 3% normal goat serum at 4°C. After removal of the primary antibody solution, cells were washed by PBS three times and were incubated with two secondary antibodies (Alexa Fluoro® 488 goat anti-mouse IgG, 1:200 and Alexa Fluoro® 568 goat anti-rabbit IgG, 1:200, Molecular Probes Inc., Eugene, OR, USA) and 1.25 μg/mL of Hoechst 33258 (Sigma, St. Louis, MO, USA) for a nuclear counter staining for 4 hr at room temperature under light-shading conditions. After sequential washing with PBS and water, the slides were mounted with PermaFluor^{TM} Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA, USA) and their fluorescent images were viewed with a microscope (LSM10; Carl Zeiss, Dublin, CA, USA).

ELISA

Cells differentiated in tissue culture treated 96-well plate (Becton-Dickinson) were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and they were permeabilized by incubation with 100% methanol at −20°C for 10 min. After removal of methanol, cells were incubated with one of the following primary antibodies in PBS containing 0.1% Triton X-100 and 3% normal goat serum overnight at 4°C; anti-TuJ1 mouse monoclonal antibody (1:600, Sigma) and anti-GFAP rabbit polyclonal antibody (1:300, Sigma). After three washes with PBS, cells were incubated with appropriate secondary antibody (goat anti-mouse or anti-rabbit antibody conjugated with horseradish peroxidase; 1:7000) for 4 hr. After three washings with PBS, cultures were incubated TMB solution with H_2O_2 (Sumilon, Tokyo, Japan) for 60 min at room temperature. Immediately after stopping the reaction by 1N H_2SO_4 solution (Sumilon), the absorbance at 450 nm with a reference wavelength of 630 nm was measured by a microplate reader (Thermo Labsystems) and the net absorbance subtracting the values of cell-free wells was calculated.

Statistical analysis

Results are listed as the mean ± S.E.M. Statistical difference was analyzed by one-way ANOVA followed by Dunnett's test. P value < 0.05 was considered to be significant.

Results

We first examined the direct effect of melatonin on the proliferative activity of the cultured NSCs. As EGF is a well-known growth factor which stimulates proliferation of cultured NSCs in vitro [19, 20], we tested whether melatonin influences the EGF-induced proliferation of the cultured NSCs. The number of viable cells increased day-by-day in the presence of EGF (20 ng/mL) (Fig. 1A), although, in the absence of EGF, the number of viable cells did not increase even after the 7-day incubation period (data not shown), suggesting that the proliferation of the NSCs in our culture system was completely dependent on EGF. The population doubling time from day 1 to day 7 was calculated to be 32.2 hr. As shown in Fig. 1A, physiological concentrations of melatonin (0.01–10 nM) failed to affect the number of viable NSCs 1, 4 and 7 days after the melatonin treatment as assessed by WST-8 assay. Treatment with pharmacological concentrations (1–100 μM) of melatonin also did not affect the number of viable cells of the NSCs after 1-day incubation, although the treatment suppressed the increment in the number of viable cells elicited by EGF on days 4 and 7 in a concentration-dependent manner (Fig. 1A). Melatonin at pharmacological concentrations (1–100 μM) also significantly suppressed the amount of BrdU incorporation and the number of secondary neurospheres in the presence of EGF (20 ng/mL) in a concentration-dependent manner (Fig. 1A). Even when the NSCs were grown at a low-cell density (100 cells per well), melatonin at pharmacological concentrations (1–100 μM) also suppressed the number of secondary neurospheres (Fig. 1B), which excluded a possibility that melatonin inhibited the nonspecific aggregation of seeded single cells. Next, we examined whether primary neurosphere-derived NSCs to pharmacological concentrations (1–100 μM) of melatonin affected the abilities of secondary neurosphere-derived NSCs to proliferate and give rise to next ternary neurospheres. The secondary neurosphere-derived NSCs formed in the presence of melatonin exhibited much less activity to proliferate and to form neurospheres under EGF-containing medium without melatonin than that formed in the control medium (Fig. 1C). In similar to melatonin, MCI-186, an antioxidant [23], did not affect the number of viable cells of the NSCs after 1-day incubation, although the treatment suppressed the increment in the number of viable cells elicited by EGF on day 4 and 7 in a concentration-dependent manner (Fig. 1D).

Cultured NSCs are known to undergo differentiation into neurons and astrocytes under a differentiation-preferring condition, i.e. low concentration of FBS-containing medium lacking EGF [2]. We next examined whether melatonin exposure influenced the 1% FBS-induced NSC differentiation into TuJ1-immunoreactive neurons and GFAP-expressing astrocytes by immunocytochemistry and ELISA. As mentioned above, we changed the timing of melatonin exposure, the proliferation period or differentiation period, as a previous report described the importance of the timing of exposure on NSC differentiation [22]. The incubation of 1% FBS-containing medium lacking EGF for 7 days differentiated the NSCs into the TuJ1-expressing neurons dispersing among the large amount of GFAP-immunoreactive astrocytes (Fig. 2A,B). Physiological concentrations of melatonin (0.01–10 nM) during the proliferation period and differentiation period failed to affect the differentiation of the NSCs into either TuJ1-immunoreactive neurons or GFAP-immunoreactive astrocytes when examined by immunocytochemistrty (data not shown). On the other hand, NSCs which had been expanded in the presence of higher concentrations of melatonin (1–25 μM) differentiated into more abundant

neurons than those in control medium, when evaluated by immunocytochemistry and ELISA (Fig. 2A,B), while the exposure to melatonin during the proliferation period failed to affect the differentiation into GFAP-expressing astrocytes and the number of total viable cells under differentiation-preferring conditions (Fig. 2A,B). In contrast, melatonin (1–100 μM) decreased the neural differentiation of the NSCs when the exposure occurred during the differentiation period in a concentration-dependent manner. Melatonin, up to a concentration of 100 μM, however, did not affect the differentiation into GFAP-expressing astrocytes or the number of total viable cells under differentiation-preferring conditions (Fig. 2A,B). When melatonin was applied during both proliferation and differentiation periods at same concentration, it increased the differentiation into TuJ1-immunoreactive neurons at only 1 μM without affecting the number of GFAP-expressing astrocytes and the number of total viable cells (Fig. 2A,B). In similar to melatonin, NSCs which had been expanded in the presence of MCI-186 differentiated into more abundant neurons than those in control medium (Fig. 2C), while the exposure to MCI-186 during the proliferation period failed to affect the differentiation into GFAP-expressing astrocytes and the number of total viable cells under differentiation-preferring conditions (Fig. 2C).

Discussion

These results clearly show that melatonin modulates the proliferative and differentiative ability of the NSCs from fetal mouse brain on embryonic day 15.5 in a concentration- and exposure-timing-dependent manner. When pharmacological concentrations of melatonin (1–100 μM) were applied during the proliferation period, it diminished the EGF-induced proliferation, but increased neural differentiation without affecting astroglial differentiation. On the other hand, these concentrations (1–100 μM) of melatonin during the differentiation period diminished 1% FBS-induced neurogenesis from NSCs. In contrast, physiological concentrations of melatonin (0.01–10 nM) failed to affect the proliferation and differentiation of the NSCs whether the exposure occurred during the proliferation or differentiation period. These results suggest that melatonin at pharmacological concentrations, but not at physiological concentrations, has potent modulatory actions on the proliferation and the neural differentiation of NSCs. To our knowledge, this is the first report demonstrating that melatonin exerts modulatory effects on the NSCs primarily derived from the fetal mouse brain.

One of the most interesting actions of melatonin in the present study was that the NSCs, which had been propagated in melatonin-containing medium, exhibited a decrease in the proliferation and neural differentiation even when incubated without melatonin. These results suggest that melatonin exhibited the aftereffect on the function of the NSCs. The NSCs may be induced to decelerate proliferation and neural differentiation once they are pre-exposed to melatonin. This long-term effect of pharmacological concentrations of melatonin is also reported in its protective actions against PC12 cell death [16]. Although we could not explain why pharmacological concentrations of melatonin influence the fate of the NSCs in the long-term, it is possible that melatonin might not affect the cellular components regulating the cell cycle and differentiation process via cellular membrane receptors.

The blood concentration of melatonin is reported to be in the nano- or subnanomolar range [24]; these concentrations influenced neither the proliferation nor the differentiation of the NSCs in the present study. However, it should be noted that there are orders of magnitude higher concentrations of melatonin in the cerebrospinal fluid [25] and melatonin concentrations within cells are also likely greater than in blood [26]. Considering the levels of melatonin in blood as physiological concentrations, those in the cerebrospinal fluid or within cells would be considered pharmacological [26]. Thus, these pharmacological concentrations in the cerebrospinal fluid may influence the function of NSCs. In mammals, at least three high-affinity cell surface receptors and three nuclear receptors for melatonin have been identified [6, 7]. The cell surface receptors, which linked to the G proteins and could influence intracellular Ca^{2+} levels, adenylate cyclase activity and inositol phosphate turnover, have binding affinities for melatonin in the picomolar range [27]. Also, nuclear melatonin receptors belonging to the RZR/ROR family have the high binding affinity for melatonin in the nano or subnanomolar region [7]. These reports taken together with our present findings suggest that high affinity receptors do not play a critical role in regulating NSC activities. However, several studies have shown that melatonin at physiological concentrations affects the functions of the clonal neural cell lines. For example, melatonin inhibits cell proliferation and greater neurite outgrowth of neuroblastoma cell line [15]. Furthermore, the C17.2 neural stem cell line expresses MT1 melatonin cell surface receptor and melatonin in the picomolar range increases the expression of GDNF [28]. These actions of physiological concentrations of melatonin on cell lines seem to oppose to those on the NSCs in the present study, suggesting that the effect of melatonin on clonal neural cell lines is quite different from that on the NSCs primarily derived from the brain. Recently, it was reported that melatonin increases the proliferation of the neural stem or progenitor cells in the dentate gyrus of rat pups [29] and this facilitative action seems to be contrary to our result demonstrating that melatonin decreased the proliferation of neural stem cells. Although the reason for the difference in the effects of melatonin is unclear at present, the difference in the source of neural stem cells (the dentate gyrus and the striatum) or the experiment system (in vivo and in vitro) might produce the difference in melatonin's effects.

Melatonin has been shown to be a potent free radical scavenger capable of preventing oxidative stress in a number of biological systems [12–14, 30, 31]. It is well known that melatonin is highly lipid soluble and thus readily crosses the plasma membrane into the cell, where it can interact with a variety of reactive products [31]. The effective concentration of melatonin in the present study is within the range of concentrations at which it exhibits

radical scavenging actions [13]. To support the idea that melatonin modulates the functions of the NSCs via radical scavenging actions, we investigated the effects of MCI-186, which is known to be an antioxidant [23, 32], on the proliferation and the differentiation of the NSCs. As a result, we found that MCI-186 also suppressed EGF-stimulated NSC proliferation and facilitated the subsequent neural differentiation of NSCs. In similar to our results, MCI-186 was shown to inhibit the proliferation of rheumatoid synovial cells [33] and various tumor cells [34] via its antioxidant actions. Furthermore, the concentrations of MCI-186 inhibiting cell proliferation in these reports [33, 34] were comparative with those suppressing the proliferation of the NSCs in the present study. These reports taken together with our finding suggested that the suppressed proliferation and the modulated neural differentiation of the NSCs by melatonin as well as MCI-186 may be caused via its antioxidative actions. Likewise, the molecules that are formed when melatonin scavenges radicals are likewise potent scavengers [35, 36]. Furthermore, melatonin has important actions in oxidative defense by stimulating antioxidative enzymes [37]. Therefore, the modulatory actions of melatonin on NSCs observed in this study may be caused by melatonin metabolites, such as N^1-acetyl-N^2-formyl-5-methoxykynuramine [36] or N^1-acetyl-5-methoxykynuramine [35], or because of its ability to stimulate antioxidative enzymes [37]. Further studies examining gene expression involved in the proliferation and the neural differentiation of the NSCs as well as using other radical scavengers will be required to confirm this hypothesis.

Recent evidence demonstrates that global or focal ischemia enhances the proliferation and neural differentiation of endogenous NSCs not only in continually neurogenic regions, such as hippocampal dentate gyrus and anterior subventricular zone [38–43], but also in sites where neurogenesis normally does not occur such as in the hippocampal CA1 area [4]. This compensatory neurogenesis is thought to contribute to ameliorating neurological deficits because an intraventricular infusion of growth factors markedly augments ischemia-elicited neurogenesis and restores hippocampal electrophysiological impairment as well as the learning deficits [4]. Interestingly, it was reported that administration of melatonin at pharmacological doses reduces cerebral infarct volume induced by middle cerebral artery occlusion in rat [9–11] and this is primarily explained by melatonin's direct action of scavenging abundant free radical molecules generated in the injured neurons. Therefore, it would be important to examine the possibility that melatonin would facilitate ischemia-induced neural differentiation in vivo as we observed under in vitro culture system and ameliorate the ischemia-elicited behavioral deficits.

In summary, the present study provides evidence that melatonin at pharmacological concentrations has potent modulatory effects on the self-renewal and neural differentiation of the NSCs in an exposure-timing-dependent manner. These effects of melatonin on NSC functions might contribute the amelioration of ischemia-induced neuronal loss and behavioral deficits. Additional studies are required to uncover the mechanisms underlying melatonin's actions on NSCs.

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