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Melatonin Modulates Cell Survival of New Neurons in the Hippocampus of Adult Mice

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Regulation of adult hippocampal neurogenesis is influenced by circadian rhythm, affected by the manipulation of sleep, and is disturbed in animal models of affective disorders. These observations and the link between dysregulation of the circadian production of melatonin and neuropsychiatric disorders prompted us to investigate the potential role of melatonin in controlling adult hippocampal neurogenesis. *In vitro*, melatonin increased the number of new neurons derived from adult hippocampal neural precursor cells *in vitro* by promoting cell survival. This effect was partially dependent on the activation of melatonin receptors as it could be blocked by the application of receptor antagonist luzindole. There was no effect of melatonin on cell proliferation. Similarly, in the dentate gyrus of adult C57BL/6 mice *in vivo*, exogenous melatonin (8 mg/kg) also increased the survival of neuronal progenitor cells and post-mitotic immature neurons. Melatonin did not affect precursor cell proliferation *in vivo* and also did not influence neuronal and glial cell maturation. Moreover, melatonin showed antidepressant-like effects in the Porsolt forced swim test. These results indicate that melatonin through its receptor can modulate the survival of newborn neurons in the adult hippocampus, making it the first known exogenously applicable substance with such specificity *Neuropsychopharmacology* (2009) **34,** 2180–2191; doi:10.1038/npp.2009.46; published online 6 May 2009

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INTRODUCTION

Neurogenesis in the adult hippocampus appears to contribute to hippocampal function by allowing lifelong adaptation processes in the network of the mossy fiber system (Kempermann, 2008). Several theories have emerged that link failure of adult hippocampal neurogenesis to neuropsychiatric disorders, most notably depression and schizophrenia, but also dementia (Duman, 2004; Kempermann *et al*, 2008; Sahay and Hen, 2007). In this context the role of intrinsic modulators of adult neurogenesis became interesting, especially as these might provide novel targets to treat the diseases—as least as far as the contribution by adult neurogenesis is concerned. A novel class of antidepressants is based on the action of melatonin, and the first prototypic substance of this type, Agomelatine, has been tested with impressive clinical results (Eser *et al*, 2007). Part of the underlying rationale is that circadian rhythms are disrupted in depressed patients and that patients with depression suffer from chronically disturbed sleep patterns (Darcourt *et al*, 1992).

Melatonin, the main product of the pineal gland is cyclically synthesized in synchrony with the dark period of the circadian cycle (Reiter, 1991). Melatonin has pleiotropic neurobiological actions mediated through cell membrane receptors (Dubocovich, 2007; Dubocovich *et al*, 2005) and by intracellular signaling cascades (Benitez-King *et al*, 2001; Benitez-King *et al*, 1993; Soto-Vega *et al*, 2004). In addition, melatonin might scavenge free oxygen radicals and thereby act neuroprotective (Reiter, 1998a, 1998b).

A number of observations point into the direction that melatonin might be an interesting molecule in the context of adult neurogenesis as well. Melatonin modulated proliferative activity in the dentate gyrus in early postnatal rats (Kim *et al*, 2004) and influenced proliferation and differentiation of embryonic neural stem cells (Moriya *et al*, 2007). In addition, melatonin increased differentiation of rat midbrain neural stem cells (Kong *et al*, 2008).

Adult hippocampal neurogenesis is affected by circadian rhythms with slightly higher numbers of proliferating cells in the middle of the active period (Holmes *et al*, 2004). In several paradigms of sleep deprivation adult neurogenesis was impaired (Guzman-Marin *et al*, 2008; Guzman-Marin *et al*, 2005; Mueller *et al*, 2008). In none of these studies, however, were the effects of melatonin investigated. But

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several preclinical reports suggested that melatonin itself could be effective as an antidepressant (Overstreet *et al*, 1998; Papp *et al*, 2003). So far, every substance with antidepressant effects examined were also found to affect adult neurogenesis (Kempermann *et al*, 2008; Sahay and Hen, 2007).

We thus hypothesized that melatonin might be effective in regulating certain aspects of neuronal development in the adult hippocampus. We used isolated neural precursor cells from the adult hippocampus to measure the melatonin effects on specific cell populations. We performed most experiments in adult C57BL/6 mice that synthesize melatonin with plasma levels below that of other strains of mice. In those inbred mice high performance liquid chromatography analysis confirmed by radioimmunoassay has revealed a short-term peak of melatonin in the middle of the dark period (Conti & Maestroni, 1996; Vivien-Roels *et al*, 1998).

MATERIALS AND METHODS

Animals

C57BL/6 mice were obtained from Charles River (Sulzfeld, Germany). They were held in standard laboratory cages under 12-h light/12-h dark cycles. The light/dark cycle corresponded to the timing of lights on (Zeitgeber time 0; ZT0) at 0700 hours and to the timing of lights off (Zeitgeber time 12; ZT12) at 1900 hours, respectively. The animals had access to food and water *ad libitum*. A total of 80 female mice, eight weeks old at the beginning of the experiment, were used. All Institutional and legal regulations regarding animal ethics were followed for *in vivo* experiments and for isolating adult hippocampal neural precursor cells.

Isolation of Precursor Cells from Hippocampus of Adult Mice

Adult hippocampal neural precursor cells were isolated from the hippocampus of adult female mice as reported earlier (Babu *et al*, 2007). Briefly, animals were killed by cervical dislocation. Brains were removed from the skull and placed in cold artificial CSF (aCSF) containing 124 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM $NaHCO_3$, 10 mM D-glucose. The aCSF was constantly bubbled with 95% O_2/5% CO_2. Brains were sliced coronally using a vibratome and hippocampal coronal slices (300 μm) were collected to dissect out the dentate gyrus. Dentate gyri were dissociated by enzymatic digestion for 30 min and cell suspension separated by centrifugation using a Percoll gradient.

Proliferation, Differentiation, and Cell Survival Assays

Precursor cells were plated on laminin precoated coverslips or 96 multi-well plates and cultured with 20 ng/ml of human epidermal growth factor and 20 ng/ml of human fibroblast growth factor-2 (Fibroblast Growth Factor-2 ; both from PeproTech, Hamburg, Germany) in Neurobasal medium supplemented with B27 (Gibco, Germany), for 24 h.

To explore melatonin effects on precursor cell proliferation a dose–response analysis was performed with 10^{-8}, 10^{-6}, 10^{-4}, 10^{-2} mM of melatonin (Sigma-Aldrich, Munich, Germany) and incubation for 48 h. Twelve hours before the end of the experiment, proliferating cells were labeled with 10 μM of bromodeoxyuridine (BrdU; Sigma-Aldrich). BrdU-labeled cells were identified by immunocytochemistry and quantified using a Zeiss Axioplan2 epifluorescence microscope. Experiments were done in duplicates and repeated at least three times.

For differentiation studies, precursor cells were cultured in medium containing epidermal growth factor and FGF2 for 1 day and on the next day switched to a medium without growth factors in the presence of a different concentration of melatonin (10^{-8}, 10^{-6}, 10^{-4}, 10^{-2} mM) for 5 days. Simultaneously, precursor cells were prelabeled with 1 μM of BrdU during 24 h. Melatonin concentrations were replaced every second day. Phenotype of newborn cells (BrdU-labeled cells) was analyzed using specific markers for astrocytes and neurons. Co-labeled cells were quantified using a Leica TCS SP2 confocal microscope (Leica, Bensheim, Germany). All analyses were done in sequential scanning mode to avoid false-positive co-labeling. In some experiments cells were cultured with 10^{-4} mM of melatonin receptor antagonist luzindole (Ramirez-Rodriguez *et al*, 2003) (Tocris, Bristol, United Kingdom). Experiments were done in duplicates and repeated at least three times.

Survival of precursor cells was assessed using the WST-1 assay (Roche) or by quantifying cells that underwent differentiation after 5 days. WST-1 analysis from six wells per culture condition was performed three times. Precursor cells were prelabeled with 1 μM of BrdU to further quantify labeled and non-labeled cells as the total number of cells per coverslip (Babu *et al*, 2007). Twelve randomized fields per coverslip were counted using a Zeiss Axioplan2 epifluorescence microscope. Experiments were done by duplicate and repeated at least three times.

Immunocytochemistry

Cultures were fixed with 4% *p*-formaldehyde in 0.1 M phosphate buffer (PBS; pH 7.4) for 20 min. After PBS washing, cells were permeabilized with 0.5% Triton X-100 in PBS for 30 min. Non-specific sites were blocked with 5% donkey serum (Chemicon, Germany) containing 0.5% Triton X-100 (blocking buffer), free aldehyde groups were reduced with 1 M glycine for 20 min. Primary antibodies were diluted in blocking buffer and monolayer incubated overnight at 4°C. After washes with PBS, incubation with secondary antibodies was allowed for 2 h at room temperature. The primary antibodies were: monoclonal mouse anti-Nestin 1 : 400 (Becton-Dickinson, Heidelberg, Germany); monoclonal mouse anti-βIII-tubulin 1 : 1000 (Promega, Mannheim, Germany); rat anti-BrdU 1 : 500 (Biozol, Germany); rabbit anti-βIII-tubulin (TuJ1) 1 : 1000 (Covance, Münster, Germany); guinea-pig anti-GFAP 1 : 1000 (Advanced Immunochemistry). Secondary antibodies raised in donkey (Jackson Immunoresearch-Dianova, Hamburg, Germany) were used at 1 : 250 dilution.

Western blot

Precursor cells were lysed with RIPA buffer (150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 0.5% Triton X-100, 1 mM phenyl-methyl-sulphonyl-fluoride, 25 μg/ml leupeptin,

25 μg/ml aprotinin and 1 mM sodium ortho-vanadate in 50 mM Tris-HCl, pH 7.6) and homogenized with an ultrasonic homogenizer for 30 s. Cellular debris was removed by centrifugation at 14 000 r.p.m. Total protein content was quantified using Bradford reagent (Bio-Rad, Munich, Germany). Protein separation was performed by the Laemmli Method (Cleveland *et al*, 1977) and transferred to PVDF paper. Membranes were blocked with 5% skim milk in 0.5% Tween 20–TBS and incubated with the monoclonal mouse anti-βIII-tubulin antibody (1 : 2000). Blots were washed 3 times with Tween 20–TBS and incubated for 1 h in a 1 : 3000 dilution of phosphatase-conjugated donkey anti-mouse. Proteins were visualized with the enhanced chemiluminescence detection system. Autoradiograms were scanned and βIII-tubulin levels were estimated by densitometry with a GS-800 densitometer and PDQuest Advanced Software (Bio-Rad). Protein levels were normalized with regard to β-actin levels using a monoclonal mouse anti-β-actin antibody (1 : 5000; Sigma-Aldrich, Munich, Germany).

Determination of Melatonin Levels

Melatonin levels were determined from plasma samples that were obtained at 0000, 0500, and 0700 hours (ZT17, ZT22, and ZTO, respectively). C57Bl6 mice were decapitated after ketamine overdose injection. Five hundred microliters of blood was collected in a small-heparinized vial. Blood was kept on ice until centrifugation (1500 r.p.m. at 4°C) to allow for extraction of plasma. Tubes containing plasma were wrapped in aluminum foil to prevent light-induced degradation. Plasma was kept at −80°C until analysis. Melatonin levels were determined taking all the necessary care to prevent light degradation by using the melatonin enzyme immunoassay kit (IBL Hamburg, Germany).

Melatonin Treatments and Brdu-Labeling in Adult Mice *in Vivo*

Melatonin was freshly prepared every day and dissolved in minimum volume of pure ethanol plus saline solution (0.9%. NaCl) with the final working concentration of 8 mg/kg body weight. Tubes containing the melatonin solution were wrapped in aluminum foil to prevent light-induced degradation. The final volume of ethanol in the melatonin vehicle was less than 1%. The thymidine analog BrdU (Sigma) was administered intraperitoneally (i.p.) at a concentration of 50 mg/kg body weight. Mice received one single BrdU injection.

To study the acute and chronic effects of melatonin on cell proliferation in the hippocampus, adult mice were injected with melatonin (8 mg/kg, i.p.) or vehicle once daily for 1–7 days at 1900 hours. Ten hours after last injection, proliferating cells were labeled with BrdU. This time was chosen considering previous reports about the half-lives of melatonin (rapid half-life of 3.04–5.97 min, a slower half-life of 36–47.52 min and the slowest half-life of 24 h), and the bioavailability of melatonin up to 48 h after its administration (Anton-Tay *et al*, 1988; Vitte *et al*, 1988) as well as the melatonin levels in plasma (Supplementary Table 1). Mice were killed 2 h after the BrdU injection. The i.p. route of administration was chosen given that melatonin is absorbed through any route and that it crosses all morpho-physiological barriers (Reiter *et al*, 2007, Reiter, 2000 no. 3751).

Chronic effects of melatonin on cell survival and differentiation of newborn cells were studied in adult mice that first received a single injection of BrdU (50 mg/kg, i.p.) at 1700 hours. (ZT10). Two hours later (1900 hours; ZT12), vehicle or melatonin was injected (8 mg/kg, i.p.). Melatonin/vehicle treatments were applied daily for 14 consecutive days. Twelve hours after the last injection, mice were killed by an overdose of ketamine and perfused transcardially with 4% *p*-formaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and kept in the fixative for 24 h. After fixation, brains were transferred into 30% sucrose in phosphate buffer.

Tissue Preparation

Brains were cut into 40 μm coronal sections on a sliding microtome (Leica). The sections were stored at −4°C in cryoprotectant solution containing 25% ethylene glycol and 25% glycerin in 0.05 M phosphate buffer. Sections were stained following the free floating immunohistochemistry method and pretreated for BrdU-immunodetection by incubation in 2 N HCl for 30 min at 37°C followed by three washes in 0.1 M borate buffer (pH 8.5) for 10 min each.

Quantification of Brdu-Labeled Cells

The number of BrdU-labeled cells was determined in series of every 6th section from all animals. BrdU-labeling was visualized with the peroxidase method (Kempermann *et al*, 2003). Positive cells were counted exhaustively using a × 40 objective throughout the rostro–caudal extent of the granule cell layer. Counting was done as described earlier, with a modified optical dissector method. The cells appearing in the uppermost focal plane were disregarded to avoid over-sampling (Kempermann *et al*, 2003). The resulting numbers were multiplied by six to obtain the estimated total number of BrdU-labeled cells per granule cell layer.

Immunohistochemistry

Phenotypic analysis of newly formed cells in the dentate gyrus of adult mice was done in one-in-twelve series of sections from animals of vehicle and melatonin groups by immunofluorescent triple staining (Kempermann *et al*, 2003). Fifty BrdU-labeled cells within the granule cell layer were analyzed for coexpression of the different markers. From the percentages of every phenotype and to the total number of BrdU-labeled cells the total number per phenotype was calculated.

Newborn neurons were identified by the co-labeling of BrdU with NeuN, new astrocytes by BrdU and S100β. Intermediate stages of neuronal development were studied with antibodies against Doublecortin and calretinin. Analysis was done by confocal microscopy (Leica TCS SP2) in sequential scanning mode to avoid cross-bleeding between channels. Double-labeling was confirmed by three-dimensional reconstructions of *z*-series covering the entire nucleus (or cell) in question. Net neurogenesis was

calculated multiplying the total number of BrdU-labeled cells per the percentage of each phenotype.

The primary antibodies were: monoclonal mouse anti-NeuN (1 : 100; Chemicon); monoclonal mouse anti-calretinin (1 : 250; Swant, Bellinzona, Switzerland); rabbit anti-S100β (1 : 250; Swant, Bellinzona, Switzerland); goat anti-doublecortin (1 : 200; Santa-Cruz Biotech, Santa-Cruz, California, USA). Fluorophore-coupled secondary antibodies were: anti-rat TRITC, anti-mouse FITC, anti-rabbit Cy5, anti-goat FITC, anti-mouse Cy5. All secondary antibodies were raised in donkey and diluted 1 : 250 (Jackson Immunoresearch, Dianova). Sections were mounted in polyvinyl alcohol with diazabicyclo-octane as an anti-fading agent (DABCO).

Behavioral Testing in the Porsolt Forced Swim Test

The Porsolt swim test (also called 'test of behavioral despair') is assessing the animal's response to a strong stressor (Porsolt *et al*, 1977; Porsolt *et al*, 1977). Mice were treated with melatonin or vehicle during 14 days. Thirty-six hours after the last injection of melatonin or vehicle, mice were habituated to the testing room for 30 min before behavioral analysis. Behavioral testing was performed at the end of the dark phase of the light/dark cycle (ZT23). The testing area was dimly lit to reduce stress or anxiety. During the forced swim test the experimenter was blind to treatment. Mice were gently placed in a beaker (18 cm in diameter) filled to a depth of approximately 10 cm with water maintained at room temperature. Each mouse was tested in for 6 min; the first 2 min served as habituation period. The time spent immobile and the number of bouts of immobility were recorded during the last 4 min. After testing, each mouse was gently dried and placed in a preheated holding cage with normal bedding covered by an absorbent paper towel for 30 min. Mice were then returned to their home cages (Porsolt *et al*, 1978).

Statistical Analysis

Analysis was performed using SigmaStat 3.1 and Statview 5.0.1 software. Results are presented as mean ± SEM. Statistical analysis from the *in vitro* data was performed using one-way ANOVA for repeated measures, followed by the Tukey's *post hoc* test. Unpaired Student's *t*-test was also used to compare mean differences between two groups. For *in vivo* results, data were analyzed using one-way ANOVA followed by Fisher's *post hoc* test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Melatonin Increases Neuronal Differentiation of Adult Hippocampal Neural Precursor Cells Without Increasing Cell Proliferation

First, we analyzed whether melatonin might induce the differentiation of adult hippocampal neural precursor cells. Immunocytochemistry of precursor cells cultured under differentiation conditions (ie, growth factor withdrawal) in the presence of melatonin caused an increase in the density of cells positive for neuronal marker βIII-tubulin. These cells also showed characteristic neuronal morphologies with more than two processes establishing contacts between different cells (Figure 1a, panel a2). Quantitative analysis revealed that all concentrations of melatonin (10^{-8}, 10^{-6}, 10^{-4}, and 10^{-2} mM) increased the number of new neurons compared with vehicle treatment ($P<0.05$, Figure 1b). The effect of 10^{-6} mM melatonin, however, was greater ($\sim$70%) than that of the 10^{-8} and 10^{-2} mM concentrations ($\sim$40%; Figure 1b). Measuring βIII-tubulin levels by western blot corroborated the increase in neuronal differentiation in response to melatonin. The blots revealed an increase of βIII-tubulin levels in cells incubated with 10^{-6} mM melatonin. Densitometry confirmed this increase (Figure 1c). These data suggest that melatonin increases the formation of neurons from adult hippocampal neural precursor cells.

This increase in neurogenesis could be due to increased melatonin-induced neuronal differentiation from precursor cells or increased precursor cell proliferation. Cell proliferation as determined by quantification of dividing cells labeled with BrdU, revealed that melatonin alone or in the presence of growth factors did not change the percentage of precursor cells labeled with BrdU at any concentration tested ($P<0.49$, $P<0.083$; Supplementary Figure 1, panels b1 and b2). This result was confirmed with the WST-1 assay that also did not show significant changes between vehicle and melatonin-treated cultures (Supplementary Figure 1c). These data indicate that the increase in neuronal differentiation caused by melatonin was independent of changes on the level of precursor cell proliferation and was due to a direct neuronal differentiation stimulus on the precursor cells.

Melatonin Promotes Precursor Cell Survival *in Vitro*

As melatonin increased the number of new neurons without altering the proliferation rate of precursor cells, we investigated whether the effect was due to melatonin-affecting cell survival. We therefore quantified cells that underwent differentiation in the presence of vehicle or different concentrations of melatonin (10^{-8}, 10^{-6}, 10^{-4}, 10^{-2} mM) for 5 days. At 10^{-4} mM melatonin a significant increase in the survival of differentiated cells was found ($P<0.05$, Figure 2a), with the lower concentrations suggesting a similar—albeit statistically not significant—increase. This finding was confirmed with the WST-1 assay ($P<0.05$, Figure 2b). These results suggest that melatonin promotes the survival of precursor cells undergoing differentiation.

Melatonin Receptors are Expressed in Adult Hippocampal Neural Precursor Cells and Participate in Neuronal Differentiation Induced by Melatonin

Melatonin mediates some of its specific effects through activation of membrane receptors (Dubocovich *et al*, 2005). Therefore, we reasoned that if melatonin was able to regulate adult hippocampal neurogenesis, precursor cells capable of producing new neurons should contain melatonin receptors. Expression of transcripts of melatonin receptors was analyzed by RT-PCR in precursor cells cultured under proliferation conditions and after 36 h of

differentiation. We identified two bands of the predicted size corresponding to low- and high-affinity receptors (Mt1 = 397 bp; Mt2 = 297 bp) suggesting the presence of Mt1 and Mt2 transcripts in adult hippocampal neural precursor cells (Figure 2c). In addition, we investigated mRNA of melatonin receptors in tissue homogenate from the adult dentate gyrus and again found two products that corresponded to the two melatonin receptors (Figure 2c).

The presence of transcripts of melatonin receptors in precursor cells prompted us to investigate whether the observed effects were in fact due to the activation of functional melatonin receptors. To this end melatonin receptor activation was pharmacologically blocked with luzindole. Again, 10^{-6} and 10^{-4} mM concentrations of melatonin caused a relative 30% increase in the number of new neurons positive for βIII-tubulin and BrdU, compared with the vehicle control ($P<0.015$, Figure 2d), whereas 10^{-2} mM concentration did not modify neuronal differentiation. The increased proportion of neurons induced by melatonin was almost entirely blocked (~84%) when the precursor cells were differentiated in the presence of 10^{-4} mM luzindole (Figure 2d). The antagonist alone, however, did not induce changes in neuronal differentiation.

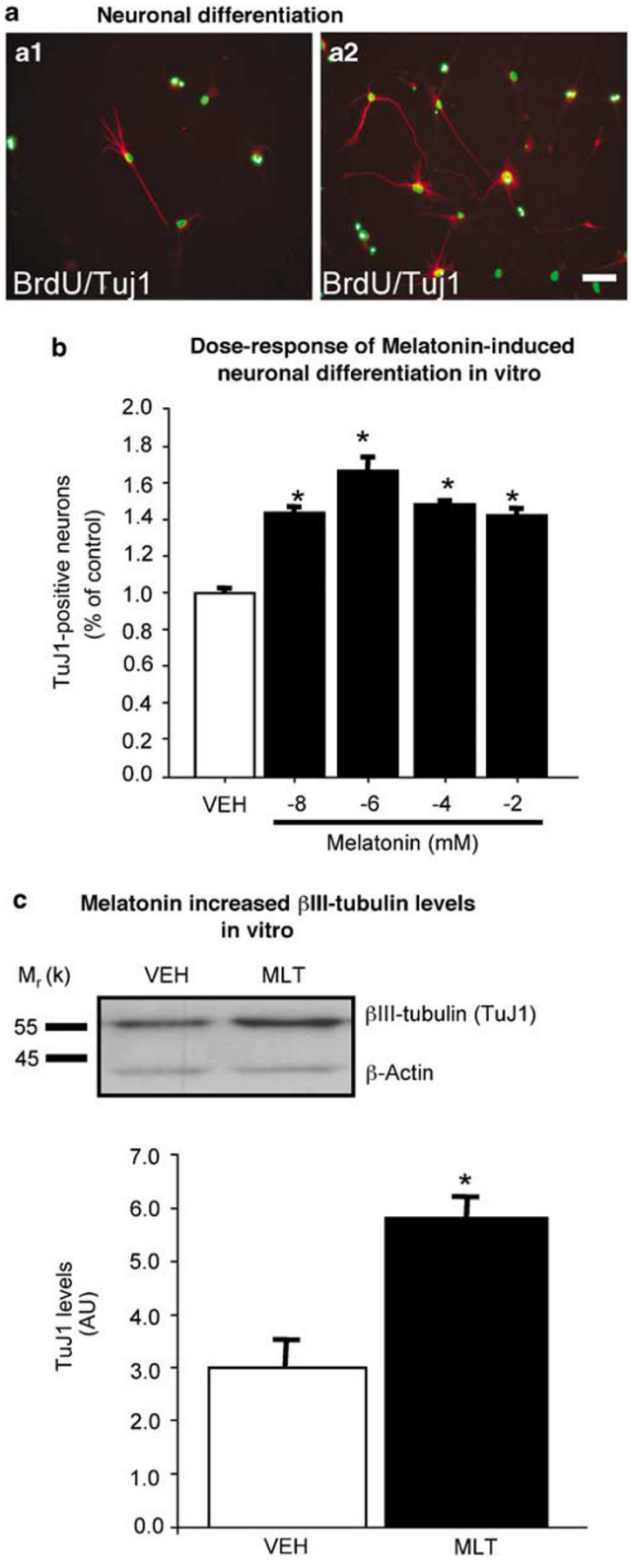

Figure 1 Melatonin induces neuronal differentiation of neural precursor cells *in vitro*. (a) New neurons were identified by βIII-tubulin/Tuj1 (red) and BrdU-labeling (green). Representative images of new neurons derived from adult hippocampal neural precursor cells that underwent differentiation in the presence of vehicle (a1) or melatonin (a2) for 5 days. Scale bar, 10 μm. (b) Histogram analysis of melatonin dose–response showed an increase in neuronal differentiation by melatonin. Results represent the mean + SEM of percentage of control. *$P<0.05$ by Tukey's *post hoc* test after one-way ANOVA. (c) Western blot analysis in adult hippocampal neural precursor cells lysates of βIII-tubulin/Tuj1 levels in cells incubated with VEH or 10^{-6} mM melatonin (MLT) showed an increase in βIII-tubulin/Tuj1 levels by melatonin. Protein levels were normalized using β-actin as a reference. Error bars represent SEM. *$P<0.05$ by unpaired Student's *t*-test. Experiments were done by duplicate and repeated at least three times.

Melatonin Increases Survival Of Newborn Neurons *in Vivo*

Precursor cells in culture allowed us to stringently study the direct effects of melatonin on proliferating cells in a controlled environment. We next performed *in vivo* studies to confirm our results in an intact system.

We first measured melatonin plasma levels in treated and untreated C57Bl6 mice (8 mg/kg melatonin). C57BL/6 mice showed a peak of melatonin in the middle of the dark period as reported earlier by others (Conti and Cattaneo, 2005; Vivien-Roels *et al*, 1998). The endogenous melatonin levels significantly decreased at the end of the dark period (0700 hours). However, in melatonin-treated mice, the plasma levels remained constantly higher compared with untreated mice (vehicle: 97.68 ± 9.84 pg/ml; melatonin: 145.68 ± 4.97 pg/ml; $P<0.012$; Supplementary Table 1). We next studied the acute and chronic effects of exogenous melatonin on hippocampal precursor cell proliferation in adult C57BL/6 mice *in vivo* (Figure 3a). Quantification of BrdU-labeled cells at 1 day after treatment revealed that acute treatment did not significantly change the absolute number of the proliferating cells in the dentate gyrus, as predicted from the *in vitro* results (vehicle: 778.50 ± 82.60; melatonin: 621.00 ± 52.22; $F_{1,6}=2.59$; $P<0.15$; Figure 3b). A similar result was found after 7 days of chronic melatonin administration (Vehicle: 709.50 ± 79.87; melatonin:

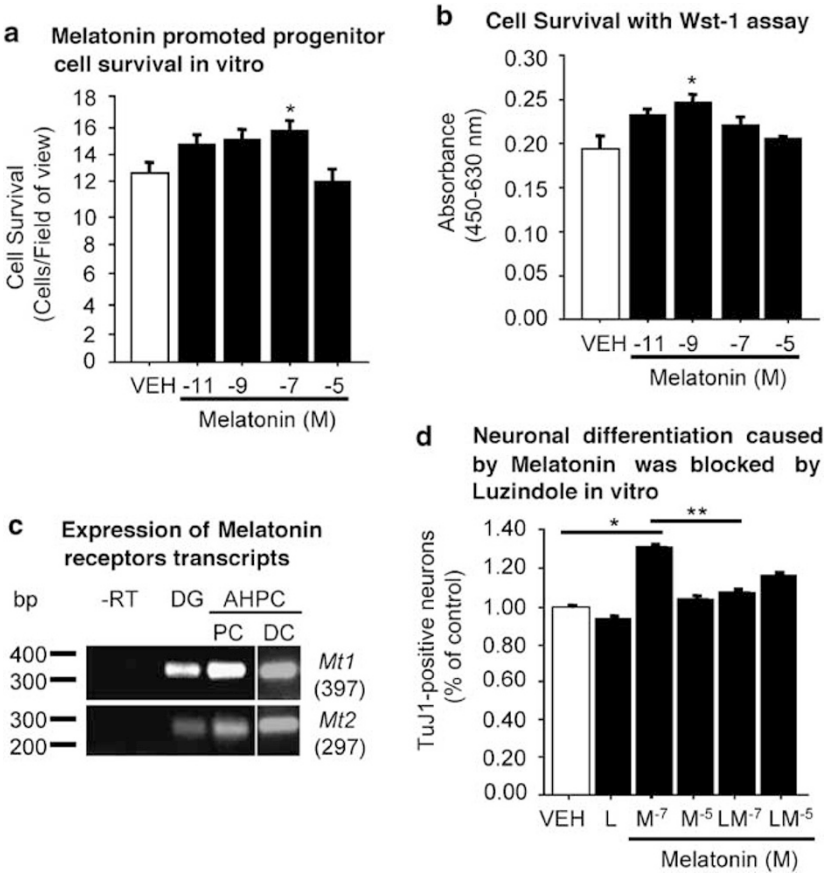

Figure 2 Melatonin modulates precursor cell survival *in vitro* and involves activation of melatonin receptors in the neuronal differentiation. (a) Survival index was determined in adult hippocampal neural precursor cells that underwent differentiation with either the vehicle (VEH) or melatonin (10^{-8}, 10^{-6}, 10^{-4}, 10^{-2} mM) for 5 days. Histogram revealed that 10^{-4} mM melatonin significantly increased cell survival. Experiments were done by duplicate and repeated at least three times. (b) Effect of melatonin on cell survival was corroborated with WST-1 showing that 10^{-6} mM melatonin significantly increased cell survival. WST-1 analysis from six wells per culture condition was performed three times. Error bars represent SEM. $^{*}P<0.05$ by Tukey's *post hoc* test after one-way ANOVA. (c) RT-PCR analysis confirms melatonin receptors expression (Mt1 and Mt2) in adult hippocampal neural precursor cells cultured under proliferation (PC) or differentiation conditions (DC) and in the dentate gyrus (DG; c). (d) Participation of melatonin receptors in neuronal differentiation caused by melatonin was studied in adult hippocampal neural precursor cells cultured with the vehicle (VEH), 10^{-4} mM luzindole (L) or 10^{-4} and 10^{-2} mM melatonin (M) in the presence or absence of luzindole (LM) for 5 days. New neurons identified by BrdU/Tuj1 co-labeling were quantified as described in methods. Inhibition of melatonin receptors by luzindole avoided neuronal differentiation caused by melatonin. Results represent the mean + SEM of percentage of control. $^{*}P<0.015$; $^{**}P<0.023$ by Tukey *post hoc* test after one-way ANOVA.

607.20 ± 53.47; $F_{1,7} = 1.22$; $P<0.30$; Figure 3b). These results indicate that after 1 or 7 days of treatment, melatonin does not modify cell proliferation in the dentate gyrus of adult mice.

In contrast, after 14 days of treatment melatonin increased the survival of newborn neurons in the dentate gyrus of adult mice (Figure 3c) as indicated by an increase in the number of BrdU-labeled cells compared with vehicle treatment (Figure 3d). The number of surviving cells in the dentate gyrus in melatonin-treated mice (8 mg/kg) was increased by 63% and significantly greater than that in vehicle-treated mice (vehicle: 320.40 ± 34.18; melatonin: 523.20 ± 71.66; $F_{1,8} = 6.52$; $P<0.034$; Figure 3e). These results indicate that melatonin increases survival of newly formed cells in the dentate gyrus of adult mice rather than stimulating cell proliferation.

Phenotypic analysis of BrdU-labeled cells after chronic melatonin treatment revealed that vehicle- and melatonin-treated mice exhibited a similar proportion of newborn mature neurons (Figure 4a). These results indicate that melatonin did not modulate or accelerate neuronal differentiation. Consequently, with increased survival and constant differentiation, calculation based on the absolute number of new neurons showed that melatonin significantly increased net neurogenesis in the dentate gyrus (vehicle: 265.08 ± 28.90; melatonin: 433.75 ± 55.80; $F_{1,8} = 7.20$; $P<0.02$; Figure 4b).

Analysis of Intermediate Stages of Neuronal Development

In the course of neuronal development new neurons first go through an intermediate phase, which is associated with the expression of doublecortin (DCX) and ranges from a progenitor cell to post-mitotic stage, followed by a transient post-mitotic phase characterized by the expression of the calcium-binding protein calretinin together with the first expression of persistent neuronal marker NeuN (Brandt *et al*, 2003; Kempermann *et al*, 2004; Plumpe *et al*, 2006). These intermediate DCX- and CR-associated stages of adult neurogenesis were analyzed by studying co-labeled cells for

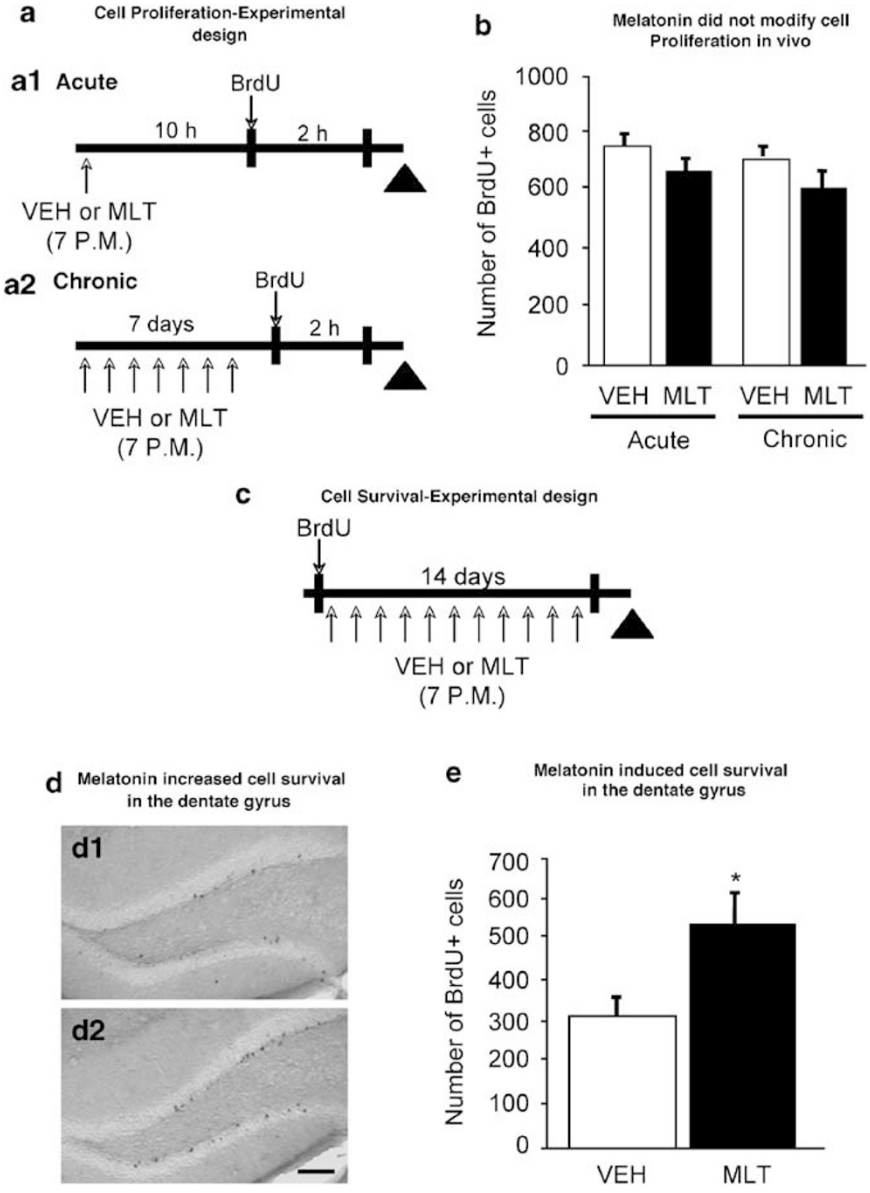

Figure 3 Melatonin increases survival of newborn neurons *in vivo*. Experimental design for proliferation study is shown in (a). Proliferation studies were conducted in mice treated with vehicle (VEH) or 8 mg/kg of melatonin (MLT) at 1900 hours for one (acute; panel a1) or 7 (chronic; panel a2) days followed by a single injection of BrdU (50 mg/kg). (b) Quantification of BrdU-labeled cells did not show significant changes in cell proliferation between vehicle (VEH) and melatonin (MLT) groups after acute or chronic treatment. Experimental design for cell survival study is shown in (c). Cell survival studies were performed in mice that received a single injection of BrdU (50 mg/kg) followed by chronic treatment with vehicle (VEH) or 8 mg/kg of melatonin (MLT) for 14 days (c). Representative images of BrdU-labeled cells are shown in (d): d1, VEH; d2, MLT. Scale bar 120 μm. (e) Quantification of BrdU-labeled cells indicated an increase in cell survival caused by melatonin (MLT) in comparison with vehicle (VEH). $n = 5$–6 mice per group. Error bars represent SEM. *$P < 0.034$ by Fisher's *post hoc* test after one-way ANOVA.

DCX- and CR- with BrdU (Figure 5a). Analysis of the absolute number of BrdU/DCX-labeled cells showed a significant increase after 14 days of treatment with 8 mg/kg of melatonin compared with vehicle-treated mice (vehicle: 45.88 ± 7.33; melatonin: 95.43 ± 18.24; $F_{1,8} = 6.35$; $P < 0.036$; Figure 5b). Furthermore, melatonin significantly increased the population of BrdU/DCX/CR-labeled cells (vehicle: 230.37 ± 13.86; melatonin: 342.80 ± 23.50; $F_{1,8} = 16.98$; $P < 0.003$; Figure 5b). The absolute numbers of cells with undetermined phenotype (BrdU only) and BrdU/CR-labeled cells did not show significant changes after chronic treatment (vehicle $= 11.72 \pm 6.15$; melatonin $= 35.68 \pm 9.54$; $P < 0.07$. BrdU/CR cells: vehicle $= 28.77 \pm 7.00$; melatonin $= 49.20 \pm 11.96$; $P < 0.18$; Figure 5b). These results indicate that melatonin increases the numbers of intermediate neuronal progenitor cells and immature postmitotic neurons in the dentate gyrus of adult mice after 14 days of chronic treatment.

Melatonin Decreases the Immobility Time in the Porsolt Forced Swim Test

Considering that a dysregulation of the circadian production of melatonin has been associated to neuropsychiatric

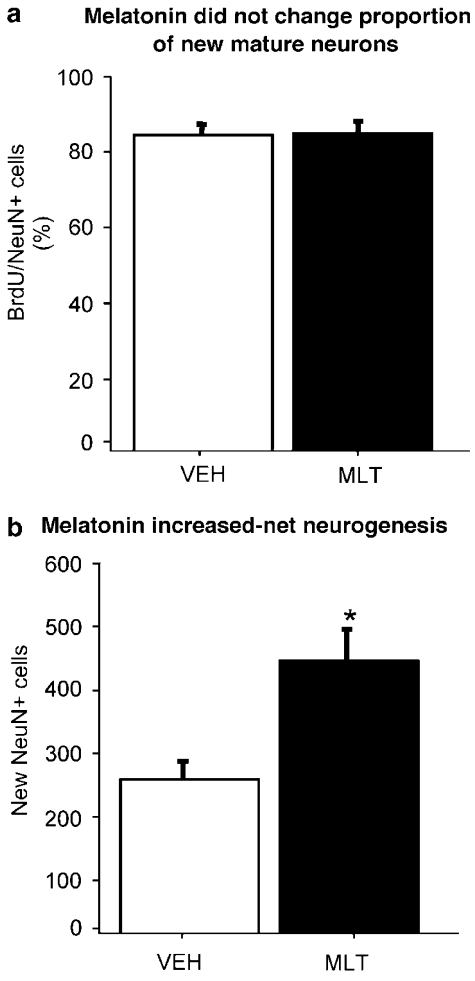

Figure 4 Melatonin modifies net neurogenesis in the adult mice. (a) Proportion of mature cells identified by BrdU- and NeuN-labeling after 14 days treatment revealed that melatonin did not modulate or accelerate neuronal differentiation in the adult brain. Net neurogenesis was calculated as described in methods. (b) Absolute numbers indicated that melatonin increased net neurogenesis in the adult brain. Error bars represent SEM. *$P<0.02$ by Fisher's *post hoc* test after one-way ANOVA.

disorders (Pacchierotti *et al*, 2001) in which regulation of adult neurogenesis is disturbed, specifically in models of affective disorders, and that melatonin induced an increase of cell survival of new neurons in the adult mice, we analyzed the possible antidepressant like effect of exogenous melatonin in the behavioral despair model, the Porsolt forced swim test (Porsolt *et al*, 1977; Porsolt *et al*, 1977). This test does not address 'depression' but is sensitive to the action of antidepressant drugs. Thirty-six hours after the last injection of melatonin or vehicle of a total of 14 daily injections, mice were habituated to the testing room for 30 min before behavioral analysis.

We found that melatonin significantly decreased the duration of immobility (vehicle: 199.59 ± 5.46 seconds; melatonin: 93.19 ± 20.46 seconds; $F_{1,8} = 25.22$; $P<0.001$; Supplementary Figure 2) and the number of bouts of immobility compared with the vehicle-treated mice (vehicle: 6.73 ± 0.12; melatonin: 3.73 ± 0.44; $F_{1,8} = 42.85$; $P<0.001$; Supplementary Figure 2). Thus, these data suggest that exogenous melatonin exerted an antidepressant-like effect in the adult C57BL/6 mice.

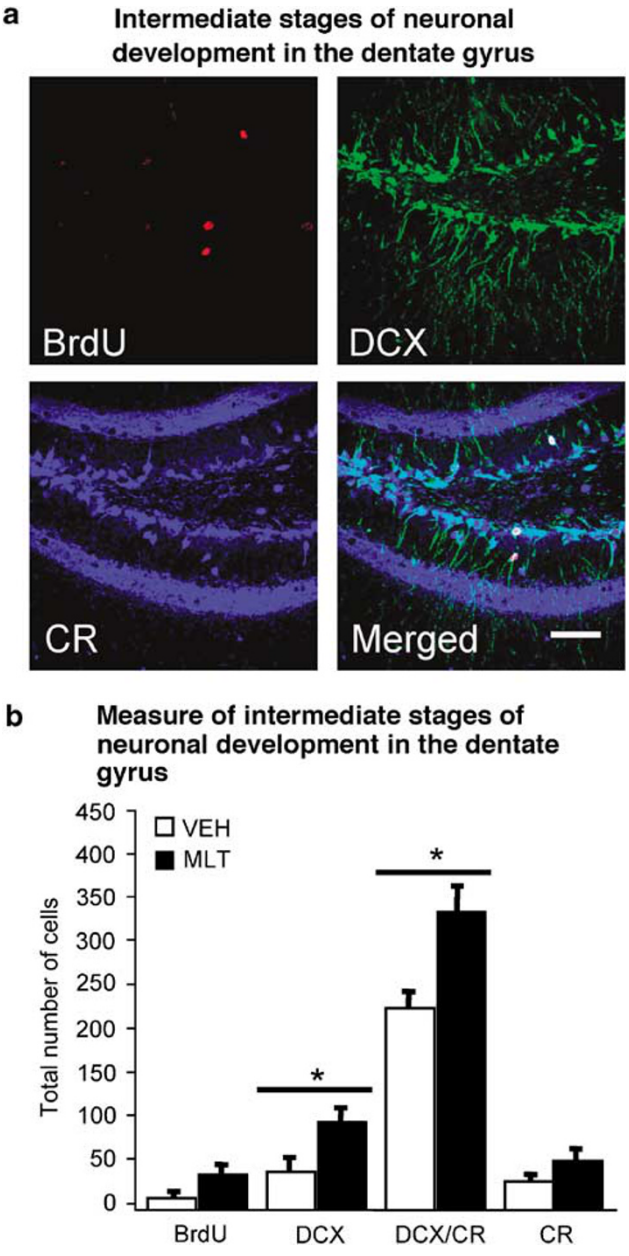

Figure 5 Analysis of intermediate stages of neuronal development. (a) Neurogenesis was assessed by counting BrdU- (red), doublecortin- (DCX; green), calretinin- (CR; blue) labeled cells by triple immunofluorescent staining in the granule cell layer after chronic treatment with either the vehicle (VEH) or 8 mg/kg melatonin (MLT) for 14 days. Scale bar 100 μm. Granular cell triple-labeled for BrdU/DCX/CR appears in purple in the merged image. (b) Total cell numbers show a significant increase in BrdU/DCX- and BrdU/DCX/CR-labeled cells in melatonin-treated mice related to vehicle. $n = 5$–6 mice per group. Error bars represent SEM. *$P<0.05$ by Fisher's *post hoc* test after one-way ANOVA related to the VEH.

DISCUSSION

We show here that melatonin modulates cell survival of new neurons derived from adult hippocampal neural precursor cells *in vitro* and *in vivo*. However, the highest effect of melatonin on cell survival was observed in the intact *in vivo* system, which might be due to the fact that in the hippocampus melatonin might affect the hippocampal

microenvironment rather than only isolated precursor cells as in the *in vitro* studies. We confirmed that melatonin effects might be partly mediated through melatonin receptors activation on the precursor cells. Moreover, exogenous melatonin administration decreased the duration of immobility of adult C57BL/6 mice through its antidepressant-like effect, thus adding melatonin to the list of factors with antidepressant-like effects and at the same time effects on adult hippocampal neurogenesis (Eisch *et al*, 2008).

Melatonin Promotes Neuronal Differentiation of Precursor Cells *in Vitro*

Melatonin has been previously found by others to enhance neurogenesis *in vitro* but no data on adult neural precursor cells from the hippocampus were available (Kong *et al*, 2008; Moriya *et al*, 2007). The published findings were difficult to extrapolate to adult neurogenesis because embryonic and adult neural stem cells have distinct properties, the details of which are poorly understood. For example, embryonic stem cells are committed to form neurons, whereas adult neural stem cells predominantly generate astrocytes (Palmer *et al*, 1997; Qian *et al*, 2000). A previous study had reported that melatonin signaling cans regulate proliferation and neuronal differentiation of embryonic neural stem cells across a wide range of concentrations including pharmacological doses (10^{-3}–10^{-1} mM) (Moriya *et al*, 2007). In our study we observed a bell shape response in neuronal differentiation (10^{-8}–10^{-2} mM) without changes in proliferation. The most effective dose to induce neuronal differentiation corresponded to the nocturnal plasma- and cerebrospinal fluid-concentrations of melatonin (10^{-6}–10^{-4} mM). In addition, we observed that in melatonin-treated cell cultures, new neurons established contacts with surrounding cells and formed a network. Recently, another *in vitro* study has reported that 10^{-6} mM melatonin modulated rat midbrain neural stem cells viability (Kong *et al*, 2008). Our data suggest that 10^{-6}–10^{-4} mM of melatonin caused a 28 and 26% increase in the number of surviving progenitor cells undergoing differentiation—possibly through an antiapoptotic effect (Reiter, 1998b). Additional experiments revealed that neuronal differentiation induced by melatonin was partially modulated through membrane receptors activation, here tested by receptor blockage with luzindole, which caused a roughly 84% decrease in neuronal differentiation. These data suggest that melatonin exerts its effects across a wide range of concentrations depending on the system studied. Pharmacological and physiological concentrations of melatonin are an issue, as serum melatonin levels do not correlate with levels in other fluids or cells (Reiter *et al*, 2005). Melatonin levels in the blood are normally low, however tissue levels of melatonin can be considerably higher. Nocturnal melatonin concentrations in plasma and cerebrospinal fluid reach 10^{-6} and 10^{-4} mM, respectively (Skinner & Malpaux, 1999). Autocrine and paracrine effects of melatonin occurred at 10^{-2} mM. Melatonin concentrations differ in different body fluids, different cells and cell types, and in different subcellular compartments. In our study the effects of melatonin on neuronal differentiation and cell survival of adult neural precursor cells occurred in physiological ranges, if we consider melatonin levels in plasma. Some effects of melatonin occur within nanomolar concentrations involving the membrane receptor activation. Our data indicate that the antagonist could not fully block the effects of melatonin on neuronal differentiation. This observation suggests the involvement of other additional mechanisms. Modulation of cytoskeleton structure and integrity might be contributing to melatonin effects on neuronal differentiation (Bellon *et al*, 2007; Benitez-King, 2006). Melatonin also promoted neurite outgrowth in murine neuroblastoma cells (Bellon *et al*, 2007; Benitez-King *et al*, 1990). Neurite formation is important for differentiation and consequently for establishing contacts among cells. It has also been reported that the establishment of synaptic connectivity promotes the integration of the new neurons into the hippocampus (Ge *et al*, 2007). In addition to the effects at physiological concentrations, pharmacological concentrations of melatonin seem to be affecting the regulation of neurogenesis by an independent membrane receptor activity. Melatonin might influence adult neurogenesis by different mechanisms that require different concentrations. Thus, melatonin affects neurogenesis at both physiological and pharmacological concentrations.

Melatonin Increases Precursor Cell Survival

Adult hippocampal neurogenesis reportedly was affected by sleep deprivation and by circadian rhythms, which are also disrupted in many affective neuropsychiatric disorders (Holmes *et al*, 2002; Mueller *et al*, 2008). In addition, in clinical populations dysregulation in the circadian release of melatonin has been correlated with depressive states (Pacchierotti *et al*, 2001). In our study, we increased nocturnal melatonin levels in adult female C57BL/6 mice.

We also found that neither acute nor chronic administration of melatonin increased cell proliferation in the dentate gyrus of adult mice. These results were in some contrast to another study, in which melatonin reportedly increased cell proliferation in the dentate gyrus of maternally separated young rats and in non-separated rats after 7 days treatment (Kim *et al*, 2004). Besides the species differences that study also used a cumulative BrdU-labeling protocol, blurring the distinction between proliferation and survival effects. The data seem consistent with a scenario, in which the measured increase in fact represents a survival effect on the cells marked early during the labeling period.

Along similar lines, it has been reported that melatonin might modulate reactive neurogenesis in the ischemic striatum of adult mice (Kilic *et al*, 2008). However, that finding was based on the BrdU and doublecortin-labeled cells only, which will not be sufficient to prove neurogenesis because cell proliferation of doublecortin-positive cells reflects only the initial stages of neurogenic process and has not been validated outside the 'canonical' neurogenic regions. Nevertheless, melatonin might have modulatory effects on several types of cell genesis in the adult brain.

Melatonin Induces Net Hippocampal Neurogenesis in Adult Mice

Phenotypic analysis of the BrdU-labeled cell population in the dentate gyrus at 2 weeks after labeling revealed that

melatonin did not affect differentiation into neurons or glia after 14 days of treatment. However, with increased survival and constant differentiation, melatonin increased net adult hippocampal neurogenesis.

Analysis of intermediate stages of neuronal development revealed that melatonin increased the number of BrdU/DCX- and BrdU/DCX/CR-cell populations in the dentate gyrus. As described earlier, during the phase of DCX expression cells are still able to divide and undergo morphological changes associated with migration (Plumpe *et al*, 2006). Calretinin expression is linked to early post-mitotic neuronal development (Brandt *et al*, 2003). In addition, the number of calretinin-labeled cells reflects changes in the number of newborn neurons, so that to some degree, calretinin expression can be used as a surrogate measure of adult neurogenesis. Our data indicate that melatonin induced survival of newly generated neurons by affecting intermediate DCX- and CR-expressing stages of adult neurogenesis. Given the directionality of development this implies that the main melatonin effect is likely to occur on precursor cells.

Although our study showed that exogenous melatonin modulates adult neurogenesis by affecting cell survival and also exerts antidepressant-like effects, long-term studies will be necessary to elucidate how melatonin affects adult neurogenesis over prolonged periods of treatment.

Furthermore, the effects of exogenous melatonin on adult neurogenesis might also be partly resulting from melatonin's antioxidant and antiapoptotic capacities (Reiter, 1998b). Preservation of cytoskeletal organization by melatonin might be important for migration, differentiation, survival, and maturation of newborn neurons in the adult brain (Benitez-King, 2006).

Behavioral Effects of Melatonin Treatment

Preclinical studies have suggested an antidepressant-like effect of melatonin in the chronic mild stress and in the forced swimming test, both models used to test the effectiveness of antidepressants, albeit of limited validity as depression models *per se* (Overstreet *et al*, 1998).

Essentially all pharmacological and non-pharmacological antidepressants increased adult neurogenesis (Thomas and Peterson, 2008). However, there are many questions around the 'neurogenesis theory' of major depression (Jacobs, 2002; Kempermann & Kronenberg, 2003; Sahay & Hen, 2007) but the coincidence remains suggestive.

Banasr *et al* (2006) have shown that Agomelatine, a novel antidepressant drug that acts as both serotonin receptor subtype 2C antagonist and melatonin agonist, increased cell proliferation and cell survival in the dentate gyrus of rats through 5HT2C receptor inhibition and melatonin receptor activation (Millan *et al*, 2005). This result points into the same direction of our findings, by which exogenous melatonin modulated cell survival in the dentate gyrus of the adult brain, although the details of the interaction with serotonergic signaling have to be sorted out.

In some neuropsychiatric disorders and in advanced age circadian rhythms are altered and levels of endogenous melatonin fall markedly (Asayama *et al*, 2003; Brusco *et al*, 2000; Liu *et al*, 1999; Tan *et al*, 2002). Our study might support the idea that the decrease of melatonin in aging might play a certain role in the decrease of neurogenesis in aged subjects and in neuropsychiatric disorders in which adult neurogenesis might be impaired and in which increasing adult neurogenesis might be thus beneficial (Kempermann, 2008). Under this assumption a compound like melatonin is of particular interest because it represents an endogenous modulatory factor, presumably able to restore disturbed physiological balances. Although such ideas are suggestive, they remain to be tested. Nevertheless, our present data indicate that the role of melatonin in the context of adult neurogenesis and potential medical relevance is worth pursuing.

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CONFLICT OF INTEREST

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