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Cultured rat cortical astrocytes synthesize melatonin: absence of a diurnal rhythm

Abstract: Melatonin not only plays a major role in the regulation of circadian rhythms, but is also involved in antioxidative defense and immunomodulation. Circulating melatonin levels are derived primarily from the pineal gland while other sources of melatonin have also been reported. Here, we show for the first time that astrocytes from the rat cortex and glioma C6 cell line synthesize melatonin in vitro. In addition, we show the presence of serotonin, the precursor of melatonin and the two key enzymes in the pathway of melatonin synthesis, i.e. *N*-acetyltransferase and hydroxyndole-*O*-methyltransferase in the cultured rat cortical astrocytes. Release of melatonin into the culture medium showed no diurnal changes. These point to astrocytes as a local source of melatonin in the rat brain. Its exact physiological function remains a topic for future studies.

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Introduction

It is generally accepted that circulating melatonin is primarily synthesized by the pineal gland and plays an important role in many physiological and pathological functions, including the regulation of circadian rhythms [1, 2], antioxidative protection [3–6], improvement of learning and memory [7], and immunostimulation [8]. Melatonin is synthesized from tryptophan (Trp) by a series of catalyzing enzymes. First Trp is taken up from the circulation and is both hydroxylated and decarboxylated to produce serotonin catalyzed by both tryptophan hydroxylase and aromatic L-amino acid decarboxylase. Then serotonin is catalyzed by the *N*-acetyltransferase (NAT) to *N*-acetyl-5- hydroxytryptamine, which is in turn catalytically converted by hydroxyndole-*O*-methyltransferase (HIOMT) to melatonin. Following its synthesis, pineal melatonin is passively secreted into the circulation [9]. Although there is debate about whether NAT is the rate-limiting enzyme of melatonin synthesis, many researches considered that NAT is necessary for the production of melatonin [10].

Recently, our group reported that there is a dramatic decrease in the cerebrospinal fluid (CSF) melatonin levels starting very early in the process of Alzheimer's disease (AD) and a further reduction in CSF melatonin levels during the progression of AD neuropathology [11, 12]. In addition, it has been reported that MT1 melatonin receptor expression was decreased in aging and AD patients and pineal clock gene oscillation was disturbed in AD [13, 14]. The deceased production of melatonin in the pineal gland is considered to be the main reason to the reduced CSF melatonin levels are measured in aging and AD. It is proposed in the present study that a decreased local production of melatonin may also contribute to the lower level of CSF melatonin in aging and AD [15], although there are other sites of melatonin production. Such alternative sources are the retina [16], ovary [17, 18], testis [19], Harderian gland [20], bone marrow [21, 22], gastrointestinal tract [23], bile [24], skin [25], platelets [26] and lymphocytes [27]. Furthermore, it was reported that melatonin receptors exist in retina [28, 29], ovary [30], lymphocytes [31], skin [32], platelets [33] and astrocytes [34]. Such a wide distribution suggests a local effect of melatonin in these tissues and cells [35]. Although cultured cortical astrocytes have been demonstrated to express clock genes and can thus function as circadian oscillators independent of interactions with neurons [36], it is not known whether this property is associated with endogenous melatonin production. As astrocytes represent 70% cells in the brain and play many important functions in the central nervous system, it was of interest to investigate whether cortical astrocytes can synthesize melatonin, and if so, whether this would occur with an endogenous diurnal rhythm.

Materials and methods

Animals and reagents

Neonatal (1-day-old Sprague–Dawley, SD) rats from the Experimental Animal Center of Anhui province were used in this study. The parental rats were housed at 20–23°C under a 12:12 hr light/dark cycle (light on at 07:00 hr; light off at 19:00 hr). Animal housing, care and application of experimental procedures were in accordance with all relevant local guidelines and legislation to minimize pain and suffering of the animals. C6 cell line was from ATCC.

Dubucco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). HEPES (*N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), L-glutamine, penicillin, streptomycin, rabbit anti-serotonin, collagenase, ascorbic acid, and anti-serotonin NAT N-terminal were obtained from Sigma-Aldrich (St Louis, MO, USA). Monoclonal mouse anti-α-tubulin (TU-02) and FITC-goat anti-rabbit IgG were supplied by Santa Cruze Bio-technology Corporation (Santa Cruze, CA, USA). RevertAid^{TM} first strand cDNA synthesis kit was from MBI (Hanover, MD, USA). The Taq-DNA-polymerase was from Takara (Otsu-Shiga, Japan). Rabbit anti-glial fibrillary acidic protein (GFAP) was obtained from DAKO (Santa Barbara, CA, USA). Rabbit anti-microtubule-associated protein 2 (MAP2) was from Chemicon Corporation (Temecula, CA, USA). The Live-Dead viability/cytotoxicity assay kit was purchased from Molecular Probes (Eugene, OR, USA). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG was purchased from Promega Corporation (Madison, WI, USA). Melatonin Research RIA kit was from Labor Diagnostika Nord (Nordhorn, Germany). Prestained protein molecular weight marker was provided by Fermentas (Hanover, MD, USA) and the ECL chemiluminescence detection kit for western blots was offered by Amersham Life science (Cleveland, OH, USA).

Cell culture

The primary pineal cell cultures were performed as previously described [37]. Pineal glands were isolated from neonatal rats under sterile conditions at day 1 postnatally. After incubation with 0.25% trypsin solution at 37°C for 20 min, the dispersed pineal cells were maintained in DMEM supplemented with 10% fetal calf serum. Sodium pyruvate (55 μg/mL), ascorbic acid (0.1 mg/mL), glucose (4 mg/mL) streptomycin (0.1 mg/L), and penicillin G (100 units/mL) were added to a six-well cell culture cluster at a density of 3.0×10^5 cells/mL. Cells were cultured in a humidified 5% CO_2 incubator at 37°C.

Cortical astrocytes used for the primary cultures were also isolated from neonatal rats at day 1 postnatally [38, 39]. Briefly, the brains removed and transferred into prechilled D-Hank's under sterile condition. To avoid contamination with pineal gland cells, the rat cortex and the pineal gland were carefully removed separately. Then the cortex was chopped into pieces and resuspended in 3 mL 0.25% trypsin solution. After 20 min incubation at 37°C, FBS was added to stop the action of trypsin. The cells were dispersed gently and centrifuged at 1000 rpm for 2 min. The cell pellets were then resuspended in DMEM supplemented with 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at a density of 3×10^5 cells/mL, respectively. Cells were seeded onto 100 mm dishes. The cultures were incubated in a humidified incubator at 37°C under 5% CO_2. Astrocytes were identified by GFAP, the astrocyte marker. Almost all astrocytes had a positive immunocytochemical staining for GFAP after 10 days of culture.

C6 cells were maintained at 37°C in a humidified 5% CO_2 incubator in DMEM supplemented with 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Live–dead assay

The cell viability of cultured rat cortical astrocytes was measured by the live–dead assay. Samples were processed according to the manufacturer's recommendations. Briefly, the cells were incubated with 500 μL combined live/dead assay reagents for 30 min in a 37°C incubator after washing with warm phosphate-buffered saline (PBS). After another wash with PBS, the labeled cells were studied under the fluorescence microscope.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described by our group [40]. Rat astrocytes and C6 cells were fixed with 4% paraformaldehyde in TBS (Tris Buffered Saline: 0.05 M Tris, 0.9% NaCl, pH 7.6) for 15 min at room temperature, rinsed in TBS (Tris Buffered Saline: 0.05 M Tris, 0.9% NaCl, pH 7.6) for 3 × 10 min, and treated with 0.3% hydrogen peroxide in TBS for 30 min to quench endogenous peroxidase activity. Subsequently, the astrocytes were incubated with anti-GFAP, MAP2 or the serotonin antibody. Confocal microscopy was performed and the data were processed as described previously [40].

RT-PCR

Rat primary cortical astrocytes, pineal cells and the C6 cells were cultured and harvested for the total RNA isolation using Trizol Reagent. Total RNA was extracted with phenol–chloroform, precipitated with isopropanol, washed with 75% ethanol and dissolved in RNase-free distilled water. OD values were measured at 260 nm and 280 nm to determine the concentration and quality of RNA. cDNA was synthesized using the kit according to the manufacturer's protocol. The primers for rat NAT, HIOMT and β-actin PCR used in this study are shown in Table 1. For all the PCR reactions, 5 μL of reverse transcription product was amplified after a 10-min denaturation procedure in a final volume of 25 μL using 2.5 U Taq-DNA-polymerase. Thirty-five PCR cycles were performed (94°C, 1 min; 62°C, 30 s; 72°C, 30 s), followed by a final 10-min extension at 72°C.

Table 1. Rat primers for amplification of cDNA by RT-PCR used in this paper

Gene	Forward	Reverse
NAT	GGAGGTTTGTC CCCACACTTC	GAGGCTCCCA AGAACCAGG
HIOMT	GGCAAGACCCA GTGTGAGGTT	GCAAGAATGAA GAGGTCAGCG
β-actin	ACCGTGAAAAG ATGACCCAGAT	GTAACCCTCATA GATGGGCACA

NAT, *N*-acetyltransferase; HIOMT, hydroxyndole-*O*-methyltransferase.

Western blotting

The protocols used for the preparation of the cell lysate and western blotting have been described previously [41]. For Western blotting, cells were cultured upto cell number to 10^6 in 100 mm dishes and harvested, lysed in prechilled lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet p-40, 0.5% sodium deoxycholate, and a mixture of protease inhibitors) for 30 min and centrifuged at 13,000 *g* for 15 min. The supernatant was collected as the total protein of cells, which was mixed with the same amount of sample buffer. After boiling for 5 min, the protein sample was resolved on 15% SDS-PAGE gels and subsequently transferred onto nitrocellulose membranes and detected by anti-serotonin NAT N-terminal antibody.

Culture medium collection

The culture media used for melatonin determination in the rat cortical astrocytes, the C6 cells and the rat pineal cells were collected when the cells were cultured up to a cell number of 10^6. For the measurement of a possible melatonin diurnal rhythm, the astrocyte culture medium was collected at 12:00 hr and 16:00 hr during daytime, and 0:00 hr and 4:00 hr at night. Light was avoided when the cell culture medium was collected and the time for collecting the cell culture medium was <3 min. Subsequently, the medium was centrifuged for 10 min at 12,000 rpm at 4°C. The supernatant was collected and kept at −80°C until assayed.

Melatonin determination (RIA)

The melatonin concentrations in the rat cortical astrocytes, the C6 cells, and the pineal cells culture medium were measured by a direct radioimmunoassay (RIA). The assay was conducted according to the procedure of the commercial kit. The standard of melatonin levels in the melatonin research RIA Kit range from 0 to 1000 pg/mL. The standard curve of melatonin assay was highly reproducible, with an average correlation coefficients of 0.999. Each sample was measured in duplicate. The cross reactivity between melatonin and *N*-acetyl-serotonin, serotonin was 0.8% and $<0.01\%$, respectively. The average intra- and inter-assay coefficient of variation was 7.5% and 15.1%, respectively. The sensitivity of the assay is 0.8 pg/mL.

Statistical analysis

Statistical data were analyzed using *t*-test, which was conducted with SPSS 11.5 (Chicago, IL, USA) for Windows. Values are expressed as mean ± S.E.M. The statistical significance was defined as $P < 0.05$.

Results

The Immunofluorescence staining for GFAP and MAP2 was performed on 10-day cultured rat astrocytes as shown in Fig. 1. Almost all cells expressed GFAP (Fig. 1A) and there were no MAP2 immunoreactive positive cells after 10-day culture in vitro present under our culture condition (Fig. 1B). The live–dead staining was used to investigate the cell viability in cultured cortical astrocytes. The cultured cortical astrocytes appeared to be nearly 100% living cells (green-stained) while dead cells (red-stained) were not observed. It suggested that cells grew well after 10-day culture when the rat cortical astrocytes culture medium was collected (Fig. 1C).

A low concentration of melatonin in the culture supernatants of cortical astrocytes and the C6 cells were measured by RIA (Fig. 2). The melatonin levels measured by RIA were 54.9 ± 3.39 pg/mL in the cultured rat cortical astrocytes and 60 ± 2.54 pg/mL in C6 cells. The melatonin levels

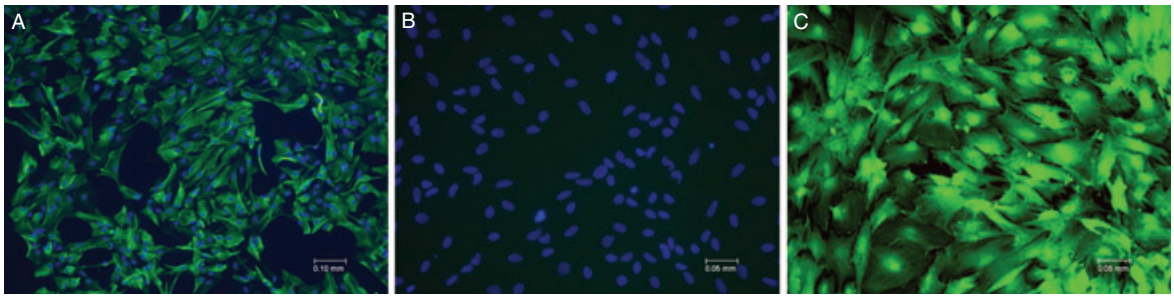

Fig. 1. Identification of the glial cell type by immunofluorescence staining and measurement of the cell viability by live–dead assay. (A) The anti-glial fibrillary acidic protein immunofluorescence (green) showed that the major cell type in the 10-day cultured cortical cells was astrocyte. DAPI staining (blue) was used to identify nuclei. (B) We used MAP2 immunofluorescence to determine whether neurons were present between the astrocytes. The blue represents the nucleus of all the cells. The MAP2 immunostaining result (green) indicated that there was no neuron present between the astrocytes. (C) The live–dead assay showed the cell viability in the 10-day cultured rat cortical astrocytes. The green color represents living cells and no dead (red) cells were present.

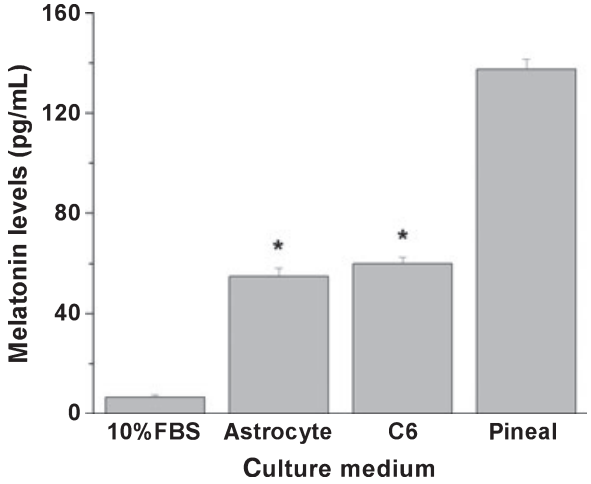

Fig. 2. Melatonin in the supernatant of cultured rat cortical astrocytes and the C6 cells. Melatonin was determined in the rat cortical astrocytes culture medium. All cells were incubated upto a number to 10^6 after 10 days in vitro. Melatonin levels in the supernatant of pineal cells were used as a positive control. There was a very low amount of melatonin in the fetal bovine serum supplemented medium. Values were expressed as mean ± S.E.M., $*P < 0.001$, significant differences compared with pineal cells (n = 11).

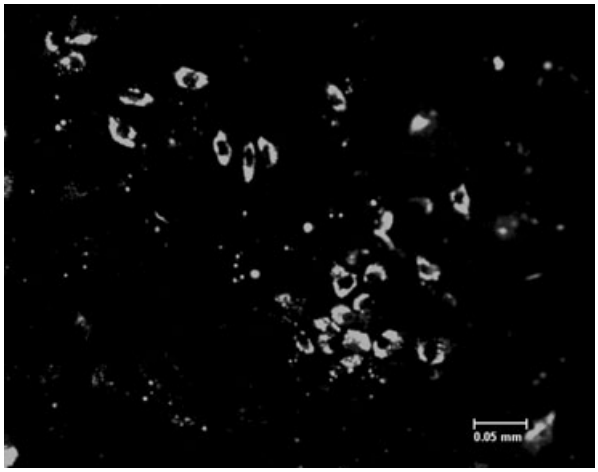

Fig. 3. Serotonin in the cultured rat cortical astrocytes. Immunofluoresence was used to identify whether cultured rat cortical astrocytes expressed serotonin. Serotonin immunoreactivity (green) in the cultured rat cortical astrocytes was clearly present. This shows that the melatonin precursor serotonin was present in the 10-day cultured rat cortical astrocytes.

in cultured pineal cells (137.55 ± 3.98 pg/mL) were used as a positive control. The melatonin concentration in the rat cortical astrocyte culture supernatants was thus approximately two-fifth of that of the pineal cultures. A very low amount of melatonin concentration was detected in FBS-supplemented medium (6.51 ± 0.9 pg/mL).

The synthesis of melatonin requires the presence of the precursor serotonin and the enzymes involved in its synthesis pathway. Thus, we investigated the presence of serotonin, the two key enzymes, NAT and HIOMT, involved in the melatonin pathway in the astrocytes. Immunofluorescence showed that there were many serotonin containing cells in the cultured rat cortical astrocytes after 10-days of culture (Fig. 3). The RT-PCR amplification pattern obtained using the NAT primers revealed the expected expression of the NAT mRNA both in the cultured astrocytes and the C6 cells (78 bp, Fig. 4A). Western blotting analysis indicated that NAT protein (23 KD) existed both in the cultured astrocytes and the C6 cells (Fig. 4B). RT-PCR analysis showed that HIOMT mRNA was also expressed both in the cultured astrocytes and C6 cells (77 bp, Fig. 5).

The melatonin concentrations in the cultured rat cortical astrocytes measured by RIA in the day-time (12:00 hr, 16:00 hr) and night-time (0:00 hr, 4:00 hr) were 50.19 ± 3.56 pg/mL, 47.08 ± 4.94 pg/mL, 39.6 ± 4.46 pg/mL and 45.36 ± 4.69 pg/mL, respectively (Fig. 6). The melatonin concentrations were thus independent on clock time and statistical analysis suggested that there was no significant difference between day-time and night-time concentrations of melatonin in the cultured rat cortical astrocytes (n = 15, $P = 0.085$).

Discussion

In this study, a low amount of melatonin was found in the cultures of rat cortical astrocytes for the first time. To exclude the possibility that melatonin synthesized by the pineal gland in vivo may have accumulated in the astrocytes before they were isolated, we collected the astrocyte culture medium only after a 10-day incubation period. In addition, since the half-life of melatonin is about 20 min in the rat plasma [42], the accumulated melatonin should be

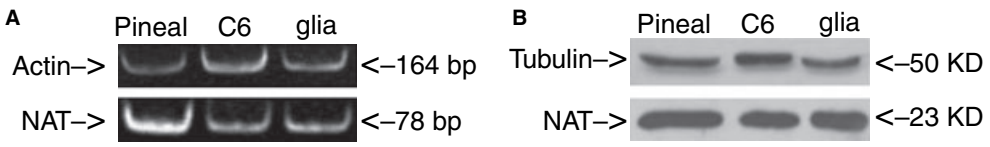

Fig. 4. *N*-acetyltransferase (NAT) in the cultured rat cortical astrocytes and the C6 cells. (A) RT-PCR analysis showed NAT mRNA expression (78 bp) in the cultured rat cortical astrocytes and the C6 cells. To determine the quality of RNA and efficiency of reverse transcription, β-actin was used as an internal control. DNase was used to treat the RNA to avoid false positive results. The expected band (164 bp) of the β-actin mRNA was amplified in all samples. The NAT in the cultured pineal cell was as a positive control. (B) Western blotting analysis showed expression of NAT protein, an enzyme involved in melatonin production, (at 23 KD) in the cultured cortical astrocytes and the C6 cells. Tubulin (50 KD) was used as an internal control and the NAT in the cultured pineal cell was as a positive control to measure the quality of protein.

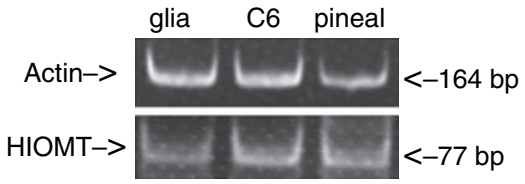

Fig. 5. Hydroxyndole-*O*-methyltransferase (HIOMT) in the cultured rat cortical astrocytes and the C6 cells. RT-PCR analysis was used to determine whether HIOMT was expressed in the cultured rat cortical astrocytes and the C6 cells. It indicated that the cells expressed indeed HIOMT mRNA in the expected band (77 bp). β-actin (164 bp) was as used an internal control and the HIOMT in the cultured pineal cell was used as a positive control. We used DNase to treat the RNA to avoid false positive results.

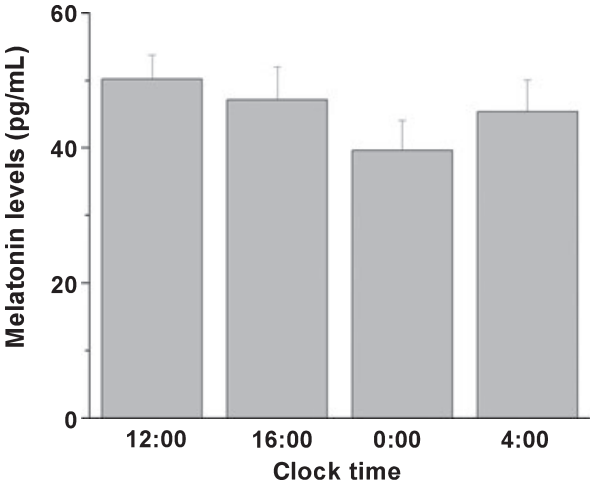

Fig. 6. Lack of diurnal melatonin changes in the cultured rat cortical astrocytes. To determine whether the cultured rat cortical astrocytes had diurnal rhythm, the day- (12:00 hr, 16:00 hr) and night-time (0:00 hr, 4:00 hr) melatonin concentrations in the cultured rat cortical astrocytes were measured by radioimmunoassay. Statistical analysis suggested that there was no significant difference between day-time and night-time levels of melatonin in the cultured rat cortical astrocytes (n = 15, $P = 0.085$).

catabolized completely at the time when the astrocyte culture medium was collected. So, we conclude that melatonin released in the astrocytes culture medium was synthesized by these cells.

Our immunofluorescence staining results indicated that many serotonin immunoreactive positive cells are found in the cultured rat cortical astrocytes. Serotonin is the precursor for melatonin production, and it has also been demonstrated that 5-HT1 receptor subtype is expressed in the cultured astrocytes in vitro [43]. The presence of the melatonin precursor, serotonin, the enzyme NAT and the last enzyme HIOMT in the cultured rat cortical astrocytes show that the rat cortical astrocytes are able to synthesize melatonin. In addition, it suggests that rat cortical astrocytes synthesize melatonin via the traditional synthetic pathway. Together these data clearly demonstrate that the rat cortical astrocyte is a physiological source of melatonin.

It was reported that melatonin concentrations in the CSF of the third ventricle are much higher than in blood [15]. It is generally assumed that the pineal gland is the main resource for melatonin of the central nervous system. Although the level of melatonin produced in the rat cortical astrocytes was about two-fifth of the rat pineal melatonin in the same culture conditions, cortical astrocyte secretion of melatonin will most probably contribute considerably to the high melatonin concentrations in the CSF in vivo, considering the large amount of astrocytes in the brain. Further more, the wide expression of NAT and HIOMT mRNAs in rat extrapineal tissues indicated the importance of extrapineal melatonin production [44]. The exact function of the local melatonin is not known at the present. Melatonin concentrations in bone marrow, bile, and lymphocytes are much higher than the levels in blood. It has been reported that melatonin synthesized by human lymphocyte involved in modulating IL-2/IL-2 receptor system [45]. It is worthwhile to note that the cortical astrocytes express the melatonin membrane receptor [34]. One may presume, therefore, that melatonin, synthesized by cortical astrocytes, may provide a local protective or other role by an auto-endocrine or para-endocrine mechanism [46].

Previous studies have demonstrated that melatonin plays an important role in the regulation of circadian rhythms under light/dark (LD) cycles [2]. The rhythms in the in vivo rat pineal melatonin persists even under constant darkness (DD) conditions [47]. In the present study, we investigated the possibility that melatonin synthesized by cultured rat cortical astrocytes may also show a diurnal rhythm. This appeared, however, not to be the case. The data rather suggest a role of the melatonin from astrocytes in trophic support to neurons or modulation of synaptic activity [48, 49].

In conclusion, we found for the first time that cultured rat cortical astrocytes and the C6 cell line were able to synthesize low amounts of melatonin without a diurnal rhythm. In addition, the melatonin precursor serotonin, the enzymes NAT and HIOMT were expressed in cultured rat cortical astrocytes. The possible physiological function of the melatonin produced in the cultured rat cortical astrocytes remains to be determined.

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