ORIGINAL ARTICLE

Serotoninergic and melatoninergic systems are expressed in mouse embryonic fibroblasts NIH3T3 cells

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Abstract

OBJECTIVE: Melatonin not only plays an important role in regulating 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. Recently, we reported that cultured rat cortical astrocytes and glioma C6 cells synthesize melatonin. In addition, apolipoprotein E genotype influences melatonin biosynthesis by regulating NAT and MAOA expression in C6 cells.

METHODS: Here, we investigated the expression of genes and enzymes that is responsible for the multistep conversion of tryptophan to serotonin and further to melatonin in mouse embryonic fibroblasts NIH3T3 cells by radioimmunoassay, Immunofluorescence staining, real-time PCR and Western blotting techniques.

RESULTS: Our results showed that cultured NIH3T3 cells could synthesize melatonin and serotonin. Serotonin N-acetyltransferase (NAT), the key enzyme in the pathway of melatonin synthesis, was also detectable using both by western blot and PCR methods. In addition, two other key enzymes, tryptophan hydroxylase (TPH1 and TPH2) for serotonin synthesis and the metabolic enzyme monoamine oxidase A (MAOA) for 5-HT, were present in NIH3T3 cell line.

CONCLUSIONS: In conclusion, we provided evidence that the NIH3T3 cells can synthesize intrinsic serotonin and melatonin and express key enzymes related biosynthetic pathways.

Abbreviations:

NAT	- Serotonin N-acetyltransferase
TPH	- tryptophan hydroxylase
MAOA	- monoamine oxidase A
AADC	- aromatic L-amino acid decarboxylase
HIOMT	- hydroxyndole-O-methyltransferase
5-HIAA	- 5-hydroxyindoleacetic acid
FBS	- fetal bovine serum
DMEM	- Dubucco's modified Eagle's medium
HEPES	- N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
TBS	- Tris Buffered Saline
RIA	- radioimmunoassay

INTRODUCTION

Serotonin is the product of a multistep metabolic pathway that starts with the hydroxylation of the aromatic amino acid L-tryptophan by tryptophan hydroxylase (TPH) (Mockus & Vrana 1998). The resulting metabolite hydroxytryptophan is decarboxylated by aromatic L-amino acid decarboxylase (AADC) to generate the neurohormone serotonin (Kema *et al.* 2000). TPH catalyzes the rate limiting step in setotonin synthesis, and TPH has two isoforms, TPH1 and TPH2 (Walther *et al.* 2003). Besides its specific actions, serotonin also serves as a precursor to melatonin. The biosynthetic pathway of pineal melatonin has been studied thoroughly (Reiter 1991b; Stehle *et al.* 2011). Serotonin is catalyzed by the N-acetyltransferase (NAT) to N-acetyl-5-hydroxytryptamine, which is in turn catalytically converted by hydroxyndole-O-methyltransferase (HIOMT, also known as acetylserotonin methyltransferase, ASMT) to melatonin. However, serotonin can also be oxidized by monoamine oxidase A (MAOA) into 5-hydroxyindoleacetic acid (5-HIAA) in the bypass of melatonin synthesis (Wu *et al.* 2003). Monoamine oxidase has two subtypes, MAOA and MAOB. MAOA is responsible for serotonin metabolisms (Fowler *et al.* 2002).

Circulating melatonin is produced predominantly in the pineal gland and plays an important role in many physiological and pathological functions, including the regulation of circadian rhythms (Reiter 1991a; Reiter 1993), antioxidative protection(Galano *et al.* 2011; Wang *et al.* 2012), improvement of learning and memory (Mares *et al.* 2012; Saxena *et al.* 2010), and immunostimulation (Espino *et al.* 2011). Melatonin synthesis also occurs in extrapineal sites, such as the retina (Gern & Ralph 1979), ovary (Itoh *et al.* 1997; Itoh *et al.* 1999), testis (Tijmes *et al.* 1996), Harderian gland (Djeridane *et al.* 1998), bone marrow (Conti *et al.* 2000; Tan *et al.* 1999), gastrointestinal tract (GIT) (Bubenik 2001), bile (Tan *et al.* 1999), skin (Slominski *et al.* 2002), platelets (Morera & Abreu 2005) and lymphocytes (Carrillo-Vico *et al.* 2004). Recently, we reported that cultured rat cortical astrocytes and glioma C6 cells synthesize melatonin (Liu *et al.* 2007). In addition, apolipoprotein E genotype influences melatonin biosynthesis by regulating NAT and MAOA expression in C6 cells (Liu *et al.* 2012). Furthermore, it was reported that melatonin receptors exist in retina (Reppert *et al.* 1995), ovary (Witt-Enderby *et al.* 1998), lymphocytes (Pozo *et al.* 1997), skin (Slominski *et al.* 2003), platelets (Vacas *et al.* 1992) and astrocytes (Adachi *et al.* 2002). Such a wide distribution suggests a local effect of melatonin in these tissues and cells (Tan *et al.* 2003).

Although melatonin are expressed in many cells, no one reported whether there is expression in mouse embryonic fibroblasts (NIH3T3) cells. In the current study, we investigated whether a serotoninergic and melatoninergic systems was expressed in NIH3T3 cells.

MATERIALS AND METHODS

Animals and reagents

NIH3T3 cell line (mouse embryonic fibroblast) was from ATCC. Dubucco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco. HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), L-glutamine, penicillin, streptomycin, rabbit anti-serotonin, collagenase, and anti-serotonin N-acetyltransferase N-terminal were obtained from Sigma-Aldrich. Monoclonal mouse anti-α-tubulin (TU-02) and FITC-goat anti-rabbit IgG were supplied by Santa Cruze Bio-technology Corporation. RevertAid™ First Strand cDNA Synthesis kit was from MBI. The Taq-DNA-polymerase was from Takara. HRP conjugated goat anti-rabbit IgG was purchased from Promega Corporation. Melatonin Research RIA kit was from Labor Diagnostika Nord (LDN, Germany). The ECL chemiluminescence detection kit for western blots was offered by Amersham Life science.

Cell culture

The NIH3T3 cells were maintained in DMEM supplemented with 10% fetal calf serum, 55 μg/ml sodium pyruvate, 4 mg/ml glucose, 2 mM L-glutamine, 25 mM HEPES, 0.1 mg/L streptomycin, and 100 units/ml penicillin G were added to a 100 mm culture dish at a density of 3.0×10^5 cells/ml. Cells were cultured in a humidified 5% CO_2 incubator at 37 °C. Primary rat pineal cell culture was performed as previously described (Liu *et al.* 2007).

Immunofluorescence staining

Immunofluorescence staining was performed as previously described by our group (Lu *et al.* 2003). The NIH3T3 cells were fixed with 4% paraformaldehyde in TBS (Tris Buffered Saline: 0.05M Tris, 0.9% NaCl, pH 7.6) for 15 min at room temperature, rinsed in TBS (Tris Buffered Saline: 0.05M 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 NIH3T3 cells were incubated with anti-serotonin antibody. Confocal microscopy was performed and the data were processed as described previously(Lu *et al.* 2003).

Real time PCR

Real time PCR was carried out in a final volume of 30 μl, using the SYBR Green PCR kit(Applied Biosystems, Foster City, CA). The sequence of PCR primer for mouse NAT, TPH1, TPH2, MAOA and β-actin used in this study are given in Table 1. Cycling conditions were: 5 min at 95 °C; 40 cycles of 30 sec at 95 °C and 1 min at 60 °C. The data were acquired and processed automatically by ABI Prism 7000 system.

Western blotting

The protocols used for the preparation of the cell lysate and western blotting have been described previously

(Liu *et al.* 2012). For Western blotting, cells were cultured upto cell number to 10^6 in 100mm dishes and harvested, lysed in pre-chilled 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 12,000 rpm 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 N-acetyltransferase N-terminal antibody.

Culture medium collection

The culture media used for melatonin determination in the NIH3T3 cells and pineal cells were collected when the cells were cultured up to a cell number of 10^6. The supernatant of pineal cells were used as a positive control. Fetal bovine serum (FBS)–supplemented medium was used to negative control. Light was avoided when the cell culture medium was collected and the time for collecting the cell culture medium was less than 3 minutes. 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 NIH3T3 cells and pineal cell culture medium and negative controls 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 (CV) was 7.5% and 15.1%, respectively. The sensitivity of the assay is 0.8 pg/ml.

Statistical analysis

Statistical data was analyzed using t-test, which was conducted with SPSS 11.5 for Windows. Values were expressed as mean ± S.E.M. The statistical significance was defined as $p<0.05$.

RESULTS

A low concentration of melatonin in the culture supernatants of NIH3T3 cells were measured by RIA (Figure 1). The melatonin levels measured by RIA were 67.55±4.49 pg/ml in the NIH3T3 cells. A very low amount of melatonin concentration was detected in FBS–supplemented medium (6.51±0.9 pg/ml). The results suggested that the melatonin levels in NIH3T3

Tab. 1. Mouse primers for amplification of cDNA by Q-PCR used in this paper

Gene	Forward	Reverse
NAT	GTCGACTCCTATGAAACAGTCGT	ATCTAAAGTCCTACAGTTCGGGA
TPH1	CACGAGTGCAAGCCAAGGTTT	AGTTTCCAGCCCCGACATCAG
TPH2	GAGTTGCTCCACGCTTTGC	ACACTCAGTCTACATCCATCCC
MAOA	CGGATATTCTCAGTCACCAATG	ATTTGGCCAGAGCCACCTA
β-actin	GCCGATCCACACGGAGTACTT	TTGCCGACAGGATGCAGAA

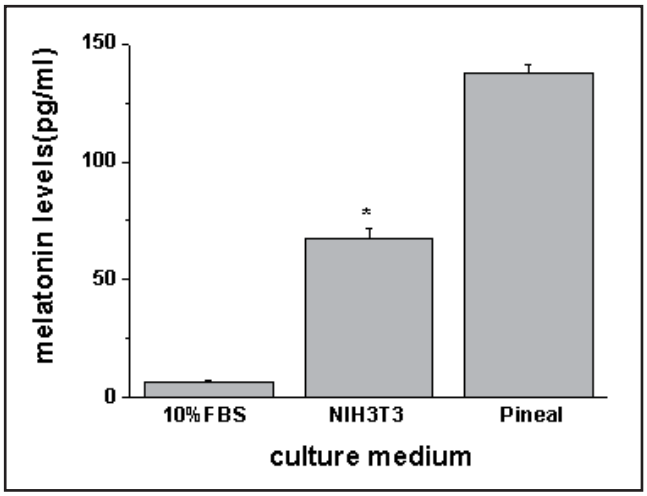

Fig. 1. Melatonin in the supernatant of cultured NIH3T3 cells. Melatonin was determined in the NIH3T3 cells culture medium. All cells were incubated upto a number to 10^6. Melatonin levels in the supernatant of pineal cells were used as a positive control. There was a very low amount of melatonin in the FBS–supplemented medium. Values were expressed as mean ± S.E.M.

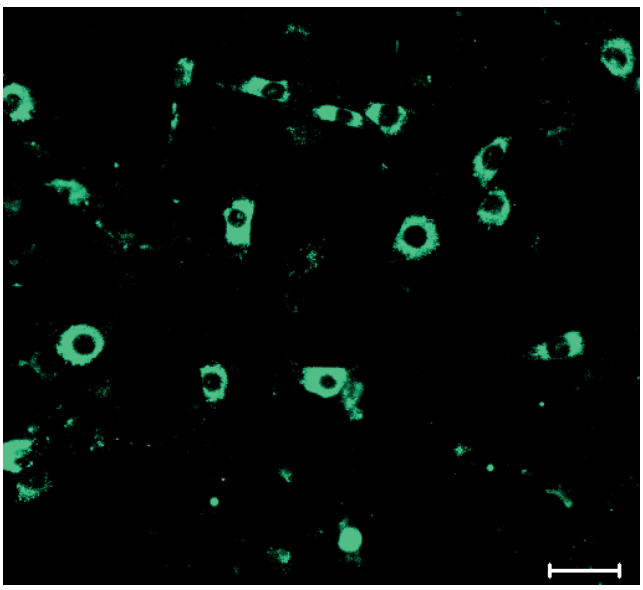

Fig. 2. Serotonin in the cultured NIH3T3 cells. Immunofluoresence was used in order to identify whether NIH3T3 cells expressed serotonin. Serotonin immunoreactivity (green) in the cultured NIH3T3 cells was clearly present. This shows that the melatonin precursor serotonin was present in the NIH3T3 cells.

cells are much higher than those in FBS–supplemented medium($p<0.05$).

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 key enzyme NAT involved in the melatonin pathway in the NIH3T3 cells. Immunofluorescence showed that there were many serotonin containing cells in the NIH3T3 cells (Figure 2). The Quantitative-PCR amplification pattern obtained using the NAT primers revealed the expected expression of the NAT mRNA in the NIH3T3 cells (Figure 3A). Western blotting analysis indicated that NAT protein (23KD) also existed in the NIH3T3 cells (Figure 3B).

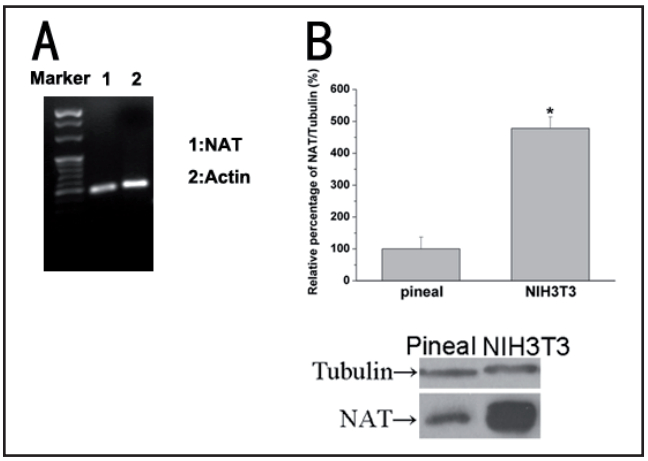

Fig. 3. NAT in the cultured NIH3T3 cells. **(A)** Q-PCR analysis showed NAT mRNA expression in the cultured NIH3T3 cells. To determine the quality of RNA and efficiency of reverse transcription, ß-actin was used as an internal control. DNase was used to treat the RNA in order to avoid false positive results. The expected band of the ß-actin mRNA was amplified in all samples. **(B)** Western blotting analysis showed expression of NAT protein, an enzyme involved in melatonin production, in the cultured NIH3T3 cells. Tubulin was used as an internal control and the NAT in the cultured pineal cell was as a positive control in order to measure the quality of protein.

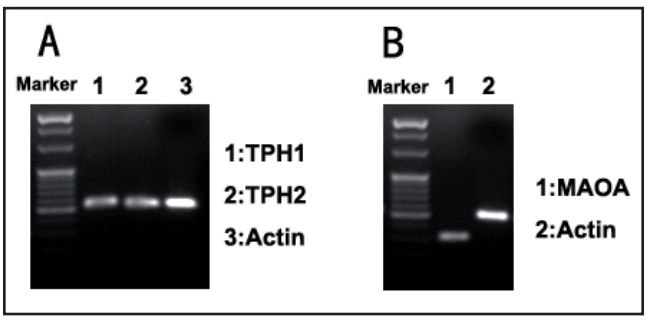

Fig. 4. TPH and MAOA in the cultured NIH3T3 cells. Q-PCR analysis showed TPH1 and TPH2 mRNA **(A)** and MAOA mRNA **(B)** expression in the NIH3T3 cells. To determine the quality of RNA and efficiency of reverse transcription, ß-actin was used as an internal control. DNase was used to treat the RNA in order to avoid false positive results. The expected band of the ß-actin mRNA was amplified in all samples.

To investigate whether a serotoninergic systems was expressed in NIH3T3 cells, the key enzyme tryptophan hydroxylase (TPH) for serotonin synthesis and the metabolic enzyme monoamine oxidase A (MAOA) for 5-HT were tested by Quantitative-PCR. Q-PCR analysis showed that TPH1, TPH2 (Figure 4A) and MAOA (Figure 4B) mRNA was also expressed in NIH3T3 cells.

DISCUSSION

In this study, melatonin was found in cultures of NIH3T3 cells. Our immunofluresence staining results indicated that many serotonin immunoreactive positive cells were found in the cultured NIH3T3 cells. Serotonin is the precursor for melatonin production, and it is catalyzed by the NAT to N-acetyl-5-hydroxytryptamine, which is in turn catalytically converted by HIOMT to melatonin. The presence of the melatonin precursor serotonin and the key enzyme NAT in the NIH3T3 cells show that the NIH3T3 cells are able to synthesize melatonin. In addition, the expression of TPH1, TPH2 and MAOA genes in NIH3T3 cells suggests that NIH3T3 cells can synthesize and metabolize serotonin.

Given the multiple systemic actions of serotonin, as a neurotransmitter, vasodilator, immunomodulator, and growth factor (Buznikov *et al.* 2001; Seuwen & Pouyssegur 1990), and melatonin, as a hormone, neurotransmitter, cytokine, and immunomodulator (Carrillo-Vico *et al.* 2005), the current findings potentially have significant physiologic or pathologic implications. It may provide a local protective or other role by an auto-endocrine or para-endocrine mechanism (Maldonado *et al.* 2007). In addition, melatonin likely has antioxidative actions in fibroblasts as well, as in every other cell type. This function does not require receptors (Fischer *et al.* 2013).

Taken together, these data provide definitive evidence for the capability of NIH3T3 cells to metabolize L-tryptophan to serotonin and further to melatonin. This conclusion is based on the accumulated results of our experiments demonstrating expression of the TPH1, TPH2, NAT, and MAOA genes and metabolism of serotonin to melatonin. The possible physiological function of the melatonin produced in the NIH3T3 cells remains to be determined.

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