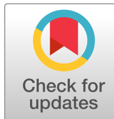

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Choroid plexus is an additional source of melatonin in the brain

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Abstract

The cerebrospinal fluid melatonin is released from the pineal gland, directly into the third ventricle, or produced *de novo* in the brain from extrapineal melatonin sources leading to a melatonin concentration gradient in the cerebrospinal fluid. Despite the interest on this topic, the brain areas capable of producing melatonin are not yet clear. Bearing this in mind, we hypothesized that the choroid plexus (CP) could be one of these melatonin sources. We analyzed and confirmed the presence of the four enzymes required for melatonin synthesis in rat CP and demonstrated that arylalkylamine *N*-acetyltransferase shows a circadian expression in female and male rat CP. Specifically, this enzyme co-localizes with mitochondria in rat CP epithelial cells, an organelle known to be involved in melatonin function and synthesis. Then, we demonstrated that melatonin is synthesized by porcine CP explants, although without a circadian pattern. In conclusion, our data shows that the CP is a local source of melatonin to the central nervous system, probably contributing to its high levels in the cerebrospinal fluid. We believe that in the CP, melatonin might be regulated by its endogenous clock machinery and by the hormonal background.

Introduction

Melatonin, an indolamine mainly released from the pineal gland (PG), is controlled by both an endogenous circadian clock and by the environmental light/dark cycle.[1] The synthesis of melatonin is mediated by the sequential action of four enzymes. These include tryptophan hydroxylase (TPH), aromatic L-amino acid decarboxylase (AADC), arylalkylamine *N*-acetyltransferase (AANAT) and acetylserotonin-*O*-methyltransferase (ASMT).[2] Tryptophan is hydroxylated by TPH forming 5-hydroxytryptophan. This product is then decarboxylated by AADC to form serotonin (5-HT).[3] Finally, AANAT converts 5-HT to N-acetylserotonin, which is then converted to melatonin by ASMT, an enzyme that is now believed to share relevancy with AANAT as rate limiting enzymes.[4] In rodents the nocturnal increase in AANAT mRNA content is responsible for the day/night differences in the production of pineal melatonin.[5,6] Curiously, AANAT localization in pinealocytes seems to be restricted to mitochondria. In

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addition, the high levels of melatonin in mitochondria and the effectiveness in the conversion of serotonin to melatonin when compared to other organelles, led several authors to speculate that melatonin might be synthesized by mitochondria.[7] More recent evidence showing melatonin synthesis and release by mitochondria further supported this hypothesis.[8,9]

Synthesis of melatonin also occurs in other organs and tissues, including the gastrointestinal tract,[10] skin,[11] ovary,[12] retina[13] and cells of the immune system.[14,15] In gut, for example melatonin is produced at even higher concentrations than in the PG.[16] In the last few years there has been considerable interest in possible alternative sources of melatonin in the central nervous system (CNS). In fact, mRNAs encoding AANAT and ASMT have been identified in the rat brain cortex, raphe nuclei and striatum,[10,17] suggesting these may be extrapineal sources of melatonin to the CNS.[18,19] This hypothesis is reinforced by the fact that melatonin concentration in the cerebrospinal fluid (CSF) is higher than in the circulation and that the circadian fluctuation of melatonin levels in the CSF rapidly increase after darkness onset and remain high until morning, showing a higher amplitude.[20] Thus, the high levels of melatonin detected in the CSF might not be only a result of melatonin synthesized by the PG and released directly into the third ventricle via the pineal recess but also a result from production elsewhere in the brain.[17,21] Interestingly, a review of the literature on this topic has proposed a possible release of melatonin from the choroid plexus (CP) to explain the high levels of melatonin in the lateral brain ventricles.[17]

The CP, located within each ventricle of the brain, is primarily responsible for the production of CSF. It is also a source of many biologically active compounds, holds numerous specific transport systems and an array of peptide and receptors.[22,23] It has been reported that melatonin receptors (MT1 and MT2) are present in the CP,[24] suggesting the CP as a target for melatonin. Furthermore, in a previous study from our group we have demonstrated that the CP holds a functional circadian clock that is controlled by the light/dark cycle and sex steroid hormones.[25] More recently, the demonstration that the CP encloses a circadian clock remarkably robust, and with higher amplitude oscillations than the suprachiasmatic nucleus

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(SCN), suggested that it might be an alternative to the master clock to control the circadian rhythm.[26] Although we have demonstrated that CP holds a functional circadian clock,[25] the ability of the CP to synthesize melatonin has not been studied. In this study we assessed the possibility that the CP could be a source of melatonin production, analyzed the expression of melatonin synthesizing enzymes and investigated the intracellular localization and circadian expression of AANAT therein.

Material and Methods

Animals

CPs and PGs collected from Wistar rats (8-10 weeks) in the beginning of the dark period were used in the present study for RT-PCR and Western blot experiments. All animals were housed in appropriate cages at constant room temperature in a 12h light/12h dark photoperiod, and given standard laboratory chow and water *ad libitum*. The dark-to-light transition phase is defined as Zeitgeber time (ZT)-0, and sampling times were defined according to ZTs. When required, adult female and male Wistar rat CPs were collected from both lateral ventricles at ZT1, ZT7, ZT13, or ZT19 (n=4-5 for each time point and each experimental group) for AANAT circadian expression. CPs from newborn Wistar rats (P3-P5) were collected for primary cultures of choroid plexus epithelial cells (CPECs) for immunocytochemistry experiments.

Porcine CPs and PGs were obtained from slaughtered pigs at a local slaughterhouse (Oviger-Alcains, Portugal) and used to determine the levels of melatonin due to the larger size, which enables to reduce the number of laboratory animals. Porcine CPs and PGs were collected from female and male animals (5-6 months) between 13h30 and 15h00.

The experiments on rats were performed in compliance with the NIH guidelines, and the National and European Union rules for the care and handling of laboratory animals (Directive 2010/63/EU). Rat experiments were also carried out according to the Portuguese law for animal welfare and the protocol was approved by the Committee on the Ethics of animal Experiments of the Health Science Research Centre of the University of Beira Interior.

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Choroid plexus epithelial cells

Primary cultures of CPs epithelial cells from rat were prepared as previously described.[27] CPs from lateral ventricles were dissected from newborn rats, previously anesthetized on ice, and digested in phosphate-buffered saline (PBS) containing 0.2% pronase (Fluka) at 37ºC for 5 minutes. Dissociated cells were washed in Dulbecco's modified Eagle medium (DMEM, Life Technologies, Ltd.) with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 100 units/mL of penicillin/streptomycin (Sigma-Aldrich). Cells were seeded into 12 mm culture wells, and cultured in a humidified incubator in 95% air–5% CO_2 at 37ºC. Normal DMEM growth medium, supplemented with 10 ng/mL epidermal growth factor (Sigma-Aldrich), 5 µg/mL insulin (Sigma-Aldrich) and 20 µM cytosine arabinoside (Sigma-Aldrich) was replaced 1 day after the initial seeding. Immunocytochemistry experiments were performed within 4–5 days after seeding.

RT-PCR

Total RNA was isolated from adult rat CP and PG tissues using Trizol Reagent (Sigma-Aldrich) as *per* the manufacturer´s instructions. OD values were measured at 260 nm and 280 nm to determine the concentration and the quality of RNA was evaluated by agarose gel electrophoresis. cDNA was synthesized using NZY First-Strand cDNA synthesis kit (NZYTech Ltd.) according to the protocol supplied by the manufacturer. For the RT-PCR, cDNA was amplified by Dream Taq Hot Start Green PCR Master Mix (Thermo Scientific) and specific primers (Table 1) in a final volume of 25 µL. The PCR protocols comprised application of a 30 seconds denaturation period at 95ºC, 30 seconds annealing period at 58ºC, and 1 minute extension at 72ºC, for 40 cycles. TPH, AADC, AANAT and ASMT PCR products were separated by electrophoresis on 1.5% agarose gels and visualized using *greensafe premium* (NZYTech Ltd.) staining. In addition, PCR bands were purified and Sanger sequenced by Stabvida to verify the sequence identity (Fig. S1).

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Western blotting

AANAT protein expression was assessed by Western blot in CP and PG tissues. Protein extracts were obtained using RIPA lysis buffer (NaCl 150 mM, NP-40 1%, sodium deoxycholate 0.5%, SDS 0.1%, Tris 50 mM) and total protein content was measured using BCA Protein Assay Kit (Thermo Scientific). Samples containing 25 μg of extracts total protein were separated by SDS-PAGE using 12.5% gels and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The membranes were blocked for 1 h 30 min with 5% BSA powder in Tris-buffered saline containing 0.01% Tween 20 (TBS-T), and then incubated overnight with primary antibody rabbit anti-AANAT antibody (1:200; SC-68346). Blots were washed at room temperature with TBS-T before incubation for 1 hour with HRP-conjugated goat anti-rabbit secondary antibody (1:50000; SC-2004). Blots were washed, and antibody binding was detected using the ECL substrate (Chemiluminescent HRP Substrate, Millipore) according to the manufacturer's instructions. Images of blots were captured with the ChemiDoc MP Imaging system (Bio-Rad).

Immunofluorescence

CP epithelial cells were seeded on coverslips for 4-5 days. After removing the medium, cells were washed with PBS and incubated for 30 minutes with MitoTracker Green FM (100 nM Molecular Probes, Life Technologies Ltd.) diluted in cell culture medium. Next, cells were washed and fixed with paraformaldehyde 4% for 10 minutes. Cells were then permeabilized with 0.5% Triton X-100 for 5 minutes, followed by a blocking step in PBS-Tween 0.1% (PBS-T) containing 20% of FBS, at room temperature. Then, cells were incubated with rabbit anti-AANAT (1:50; SC-68346) in PBS-T containing 1% FBS, overnight at 4ºC. In negative controls, the primary antibody was omitted. Cells were washed with PBS-T and incubated with Cy3 donkey anti-rabbit (1:800; Jackson ImmunoResearch Laboratories, Inc.) in PBS-T containing 1% FBS at room temperature for 1 hour. Cells were then incubated with the fluorescent dye Hoechst 33342 (Molecular Probes, Life Technologies Ltd.) to visualize their

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nuclei. After several washes, cells were mounted onto microscope slides and visualized under a confocal microscope LSM 710 (Carl Zeiss).

Real-time quantitative PCR

Analysis of the mRNA expression levels of AANAT and the reference gene Cyclophilin A (CycA) were performed by real-time RT-PCR using the kit Maxima SYBR Green/ROX (Thermo Scientific). Cycling conditions were the following: 95ºC for 3 minutes, followed by 40 cycles of 95ºC for 15 seconds, 56ºC for 30 seconds, and 72ºC for 30 seconds. Real-time quantitative PCR gene-specific primers sequences for AANAT and CycA are shown in Table 1. A validation assay was performed with cDNA serial dilutions for both genes. The ΔCt was calculated using CycA mRNA as the reference gene, and the ΔΔCt was calculated between the normalized ΔCt values from each time point and the average Ct value at all time points tested.[28] Relative amounts of all mRNAs were calculated according to the $2^{-\Delta\Delta Ct}$ method.[29] A melting curve analysis was performed after the final cycle to ensure that a single product was obtained.

Culture medium collection

For *in vitro*-cultured explants melatonin determination, porcine CPs (3 CPs/well) and PG (1 PG/well) obtained at a local slaughterhouse were transported to the laboratory in PBS, transferred to a multi well plate and kept in DMEM for 24 hours in a humidified incubator in 95% air–5% CO_2 at 37ºC. After that time, the media were collected and kept at -80ºC until assayed. To analyze the circadian melatonin release by CP, medium from explants was collected and replaced every 4 hours for 24 hours. Medium was kept at -80ºC until assayed by HPLC.

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Determination of melatonin by HPLC

Medium samples was extracted and concentrated prior to analysis. Briefly, 3 mL chloroform was added to 1 mL of the samples and agitated for 1 hour. Afterwards, samples were centrifuged (4000 rpm; Sigma 4K15) for 7 minutes. The organic phase was collected and evaporated under a gentle stream of nitrogen till dryness. All samples were stored at -18 ºC until analysis.

Prior to the analysis, all samples were suspended in 160 µL of 1:1 of MeOH:H2O (v:v), filtered through a polypropylene membrane (diameter of 13 mm and pore of 0,45 µm; from Membrane Solutions) and analyzed by HPLC-DAD-FLD. The detection limit of (MLD) and quantification (MLQ) of this method was estimated to be 0.10 and 0.29 ppb, respectively.

HPLC analysis was performed on an Agilent Technologies system equipped with a 1260 Quaternary Pump, a 1260 Autosampler, a 1100 diode array detector (DAD) with the wavelength set between 200 and 800 nm and a 1100 fluorescence detector (FLD) set to an excitation of 275 nm and emission of 345 nm.

Melatonin separation was carried out using Chromolith HighResolution RP-18e column with 100x4.6 mm from VWR (Darmstadt) and Onyx SecurityGuard C18 cartridges (5x4.6 mm) from Phenomenex. All HPLC runs were performed at a constant flow rate of 1.0 mL/min, in gradient mode, with the oven set to 30°C. The eluents used were a mixture of MeOH/MiliQ water (solution A: 25/75%; solution B: 75/25%). The gradient run was set to: go from 100% to 50% A in 10 minutes, from 10 to 15 minutes changed to 0% A where it was held constant until 16 minutes, then changed to 100% A until 19 minutes where it was kept constant till 20 minutes. The system re-equilibration was performed for 2 minutes with 100% A. All operations and data analysis were processed by the LC OpenLab software. Calibration was performed with melatonin standard solutions (in 90% MeOH) at concentrations between 0.5 and 15 ppb. The limit of detection (LD) and quantification (LQ) was estimated to be 0.47 and 1.40 ppb, respectively.

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Determination of serotonin by HPLC with coulometric detection

An HPLC system (1260) from Agilent Technologies (Waldbronn) with an auto-sampler and quaternary pump was used. The system was coupled to a Coulochem® III detector from ESA (Dias de Sousa S.A.). Compound separation was performed using a Zorbax 300SB C18 reverse-phase analytical column (5 mm, 250_4.6 mm i.d.) acquired from Soquímica. Electrochemical oxidation of the compounds was achieved using a high sensitivity dual electrode analytical cell (5011A) from ESA. The entire chromatographic system was controlled by Chemstation software supplied by Agilent Technologies. The composition of the mobile phase was 75 mM NaH_2PO_4, 1.7 mM OSA, 0.01% triethylamine (v/v), 25 mM EDTA and 15% acetonitrile (v/v) adjusted to pH 3.5 with orthophosphoric acid. The mobile phase was filtered through a 0.2 μm pore nylon membrane and degassed for 15 minutes prior to use. An isocratic flow of 1 mL/min was applied and the column oven was maintained at 23ºC. The potential of the analytical cell was set at (+) 300 mV in the channel 1 (oxidation channel) and (-) 150 mV in the channel 2 (reduction channel). The temperature of the autosampler was set at 4ºC. The retention time of serotonin, in minutes, can be observed in Fig. S2.

Statistical analysis

Statistical analyses were determined by one-way ANOVA followed by Bonferroni´s test using the GraphPad Prism software and results are reported as mean±SEM.

CircWave v1.4 analyses software (Dr. Roelof A. Hut, http://www.euclock.org) was used to analyze the rhythmicity of AANAT expression, by a harmonic regression method with an assumed period of 24 hours and with alpha set at 0.05. Data were considered statistically significant at a value of $p<0.05$.

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Results

The production of melatonin is controlled by four enzymes involved in its synthesis pathway. Thus, in order to determine whether melatonin could be produced by the CP, the presence of the enzymes TPH, AADC, AANAT and ASMT was examined in the CP and PG. RT-PCR enabled the amplification of the expected 208-bp TPH (Figure 1A), 240-bp AADC (Figure 1B), 158-bp AANAT (Figure 1C) and 198-bp ASMT (Figure 1D) fragments in rat CP and PG. Western blot analysis indicated that AANAT protein (~80 kDa) is present in rat CP and PG (Figure 2A). In addition we also examined the cellular localization of AANAT protein in rat CP epithelial cells. Its immunoreactivity was detected in the cytoplasm and clearly overlapped with MitoTracker staining (Figure 2B), indicating that AANAT protein resides almost exclusively in the mitochondria.

Following this, the mRNA circadian expression of AANAT in female and male rat CP was studied (Figure 3). In females, mRNA levels increased during the dark phase ($p<0.05$, ZT7 vs. ZT13) with a peak at the beginning of the night (ZT13; Figure 3A). Thus, using CircWave analysis AANAT mRNA expression levels were statistically rhythmic in females ($p=0.043$; Figure 3B). In males mRNA levels also increased during the dark phase ($p<0.001$, ZT1 vs. ZT19 and ZT7 vs. ZT19; $p<0.05$ ZT13 vs. ZT19), however, they showed a peak at midnight (ZT19), approximately six hours after the females' peak (Figure 3A). CircWave analysis confirmed statistically significant daily rhythmicity of AANAT in the CP of male rats ($p=5.1\times10^{-5}$; Figure 3B).

The CP capacity to synthesize melatonin was analyzed in porcine CP explants due to the higher size of the CP samples. Melatonin was measured by HPLC in the medium collected from *in vitro*-cultures of porcine CP explants for 24 hours. As shown in Figure 4, porcine CP explants synthesized melatonin, with concentrations ranging 487 ± 63.6 pg/mL. Melatonin levels in the supernatant of porcine PG explants were used as positive control (Figure 4). To investigate the existence of a daily rhythmic pattern in the synthesis of melatonin, we assessed its levels in medium collected from porcine CP *in vitro*-culture explants at six

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different time-points throughout the 24 hour period. Melatonin synthesis did not follow a rhythmic pattern in porcine CP explants (Figure 5).

Discussion

It is generally accepted that the high levels of melatonin in the CSF are produced by the PG and other brain sources. Particularly, it was previously suggested that the CP, located in the cerebral ventricles, could have the capacity to produce melatonin.[17] Therefore, we took on this study in order to validate such hypothesis. The results of this study demonstrated the presence of the enzymes involved in the synthesis of melatonin, and the daily variation of AANAT mRNA expression in rat CP. We also confirmed that porcine explants of CP are capable of producing melatonin.

In the last decade there has been growing interest in extrapineal sources of melatonin in the CNS. A major breakthrough was the demonstration that the developing brain produces its own melatonin even in pinealectomized rats.[30] Although an increasing number of publications have documented the expression of melatonin synthesizing enzymes as well as high levels of melatonin in the brain,[31] melatonin production was only effectively demonstrated in cultured astrocytes[18] and C6 rat glioma cells.[32] Thus, concerns have been raised that question which other non-pineal cells could be responsible for melatonin synthesis.

We detected melatonin synthesizing enzymes (TPH, AADC, AANAT and ASMT) in rat CP. Additionally, the presence of 5-HT, which is a substrate for AANAT and a melatonin precursor, was examined by HPLC (Fig. S2), supporting the evidence that the hormone is synthesized in CP from 5-HT via the action of the two key enzymes (AANAT and ASMT).

Melatonin has been found at high concentrations in mitochondria but how it gets there is still a matter of debate. Some studies suggest that melatonin enters mitochondria by simple diffusion or against its gradient by active transport.[33] However many experts now believe that mitochondria have the capacity to produce its own melatonin.[8,9,33,34]

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We thus assessed whether AANAT, the rate limiting enzyme in melatonin synthesis, is located in the mitochondria, and found that in primary cultures of rat CP epithelial cells, AANAT resides almost exclusively in the mitochondria, just like in the pinealocytes. In fact, in early studies, it was demonstrated that in pinealocytes, AANAT was exclusively localized in the mitochondria.[35] Also, significant alterations are observed in the number and morphology of mitochondria in pinealocytes during the dark period.[35] Thus, the co-localization of AANAT with mitochondria in CP epithelial cells, suggests that melatonin synthesis in CP is likely to take place in the mitochondria.

Western blot analysis revealed a major band of an apparent molecular weight of ~80kDa corresponding to AANAT in rat CP, instead of the expected ~23kDa band. The reasons for these differences are not yet entirely understood. A satisfactory explanation may be attributed to the formation of a complex between AANAT and the dimeric 14-3-3 protein. Those complexes are activated at night regulating AANAT activity.[36] This ~80kDa immunoreactive AANAT band was also identified by Pozdeyev *et al.* in retinal photoreceptor cells further supporting that CP AANAT might be as well complexed with the 14-3-3 protein.[37] Such complex was also described in the PG as a mechanism to protect AANAT from proteolysis and to increase its affinity to serotonin.[33,38,39]

Our study also provides evidence for daily rhythmicity in AANAT expression by comparing mRNA expression at different ZTs in female and male rat CP. In female rat CP AANAT mRNA peaks just after the light-to-dark transition, whereas in males AANAT mRNA increases in the light-to-dark transition but peaks later at night. As in the rat PG, the AANAT mRNA expression in CP increases during night-time both in female and male rats. In PG AANAT expression increases more than 100-fold approximately 2 to 4 hours after lights off,[40] although in the CP the amplitude of day/night variations was much lower (2- to 3-fold). Our results are in good agreement with retinal AANAT daily variations, where lower circadian expression also occurs.[41]

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Finally, in an attempt to confirm if melatonin synthesizing enzymes are functional in the CP, we measured the melatonin content in the medium collected from *in vitro*-cultures of porcine CP explants. We showed that CP is able to synthesize melatonin, but without a circadian oscillation. We were surprised with this result. Given the fact that CP harbors a robust circadian clock[25,26] and taking into consideration its privileged localization in brain ventricles, a possible contribution to CSF melatonin circadian rhythm would be expected. Nevertheless, our findings agree with previous data pointing that circadian variations are not common in extrapineal melatonin sources, with the exception of the retina.[19] In cultured rat cortical astrocytes there was no significant differences between day-time and night-time levels of melatonin.[18] In the cerebral cortex and in the liver, the subcellular concentration of melatonin did not exhibit a 24 hour circadian rhythm, either.[19]

The discovery of a melatonin concentration gradient in the CSF of humans and sheep raises the possibility that the melatonin content in the lateral ventricles might not only be derived from the melatonin that diffuses against the current from the third ventricle but may also be released from the lateral CPs.[17] In addition, it was recently proposed that melatonin present in the third ventricle might function as a regulator of the SCN, controlling the light/dark cycle.[42] The evidence arising from our results support the idea that the lateral CPs are able to synthesize melatonin that could act mainly as an antioxidant agent,[43] or flow through the interventricular foramina to the third ventricle functioning as a chemical signal for photoperiodic alterations.[42,43] These data do not exclude the hypothesis that CP melatonin may be released into the circulation.

Melatonin has been shown to interfere with clock genes expression, modulating circadian rhythms not only in the SCN but also in other extra-SCN clocks.[44] Thus, considering the CP an extra-SCN clock, our research highlighted the importance of this tissue in the crosstalk between melatonin and circadian clock functions.

In summary, these findings put in evidence the CP as an additional source of extrapineal melatonin, possibly to the CSF. Presumably, melatonin synthesis might be regulated by the CP endogenous clock that in turn is modulated by sex steroid hormones[25,45] independently

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of the SCN. In fact there are studies suggesting that sex differences exert an effect on melatonin synthesis, with women exhibiting higher circadian amplitudes of melatonin than men in the blood.[46] It is speculated that melatonin in the CSF might protect the surrounding tissues, like CP and ependyma, from oxidative stress, a component of many neurodegenerative diseases in CNS. Nonetheless, further studies on the current topic are required to elucidate the action of melatonin in this extrapineal tissue and its vicinity.

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Figure Legends

Figure 1. Tryptophan hydroxylase (TPH), aromatic L-amino acid decarboxylase (AADC), arylalkylamine N-acetyltransferase (AANAT) and acetylserotonin-O-methyltransferase (ASMT) are present in rat choroid plexus (CP). RT-PCR analysis demonstrates (A) TPH, (B) AADC, (C) AANAT and (D) ASMT mRNA expression. The molecular sizes are shown in base pairs (bp). PG, pineal gland (positive control); C^-, controls with distilled water instead of cDNA (non-template control).

Figure 2. Detection of AANAT in rat choroid plexus (CP) and pineal gland (PG) by Western blot and in rat choroid plexus epithelial cell (CPEC) cultures by immunocytochemistry. (A) Western blot illustrating AANAT protein expression in rat CP and PG (positive control). Protein product with ~80 kDa was detected. (B) Representative fluorescence images of AANAT and MitoTracker Green FM are shown. AANAT protein is stained red and co-localizes with MitoTracker, suggesting the localization of AANAT in mitochondria. Scale bar, 10 μm.

Figure 3. Arylalkylamine N-acetyltransferase (AANAT) profile across 24 hours cycle. (A) AANAT gene expression profile in female and male rat choroid plexus (CP). The grey area denotes the dark phase. Data from ZT1/25 are double plotted. Each data point represents the mean±SEM, n=4-5 rats per group. (B) Corresponding CircWave analysis of AANAT expression in female and male rat CP (p=0.043 for females and p=5.1x10-5 for males). In the graph, each dot represents the level of expression for each animal.

Figure 4. Melatonin levels in medium collected from porcine choroid plexus (CP) explants. Culture medium was collected after 24 hours of porcine CP *in-vitro* culture explants. Melatonin levels in the supernatant of porcine pineal gland explants were used as positive control. Bar graphs indicate mean±SEM.

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Figure 5. Melatonin levels in medium collected from porcine choroid plexus (CP) explants at different time points. Culture medium was collected for 24 hours with 4 hours intervals (0012; 0016; 0020; 0024; 0400 and 0800) of porcine CP *in-vitro* culture explants. Bar graphs indicate mean±SEM. There was no significant difference between the time points tested of melatonin levels in the cultured porcine CP explants.

Table 1. Primers used for RT-PCR for the enzymes involved in the melatonin synthesis and in quantitative real-time RT-PCR for the AANAT and CycA mRNA expression in rat choroid plexus.

Gene	Primer Fw	Primer Rv	Amplicon size (bp)
TPH	CAAGGAGAACAAAGACCATTC	ATTCAGCTGTTCTCGGTTGATG	208
AADC	TACCCAGCTATGCTTGCAGAC	GCGGATAACTTTAGTCCGAGC	240
AANAT	TGCTGTGGCGATACCTTCACCA	CAGCTCAGTGAAGGTGAGAGAT	158
ASMT	TACGGGGACAGGAAGTTTTG	GTGCCACTTCTGGGTTCATT	198
CycA	CAAGACTGAGTGGCTGGATGG	GCCCGCAAGTCAAAGAAATTAGAG	163

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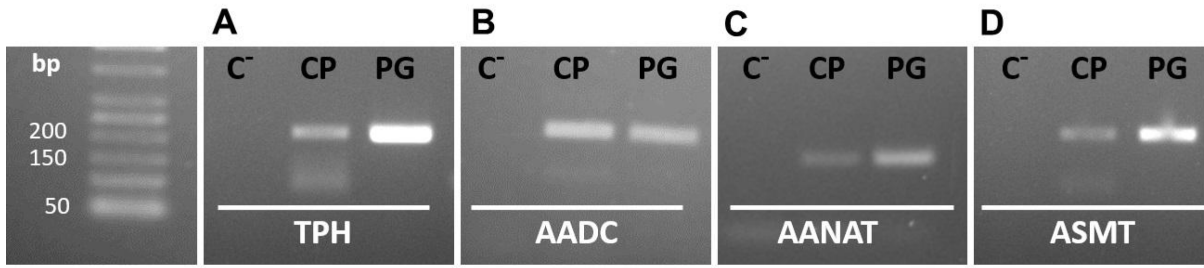

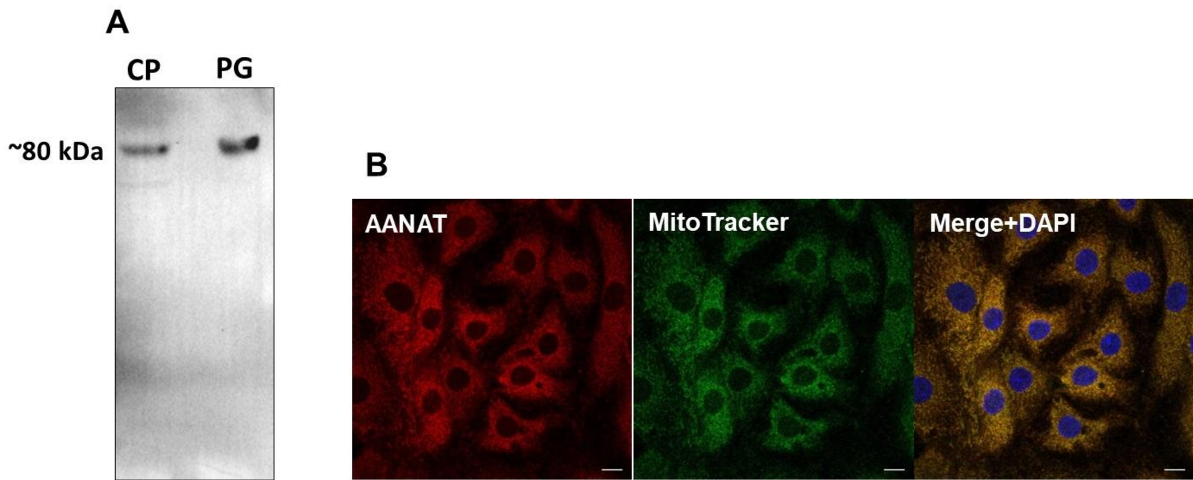

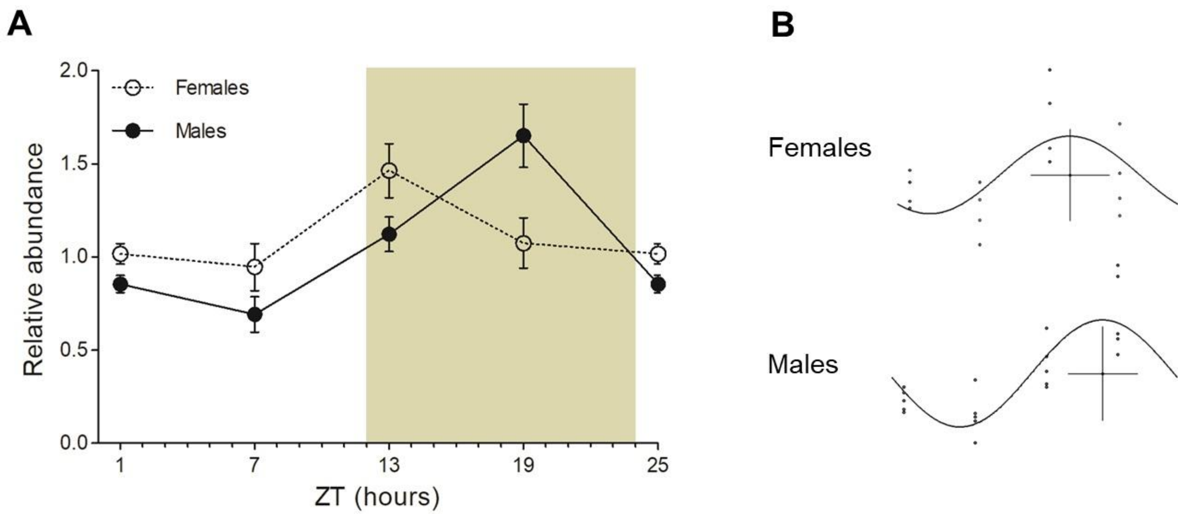

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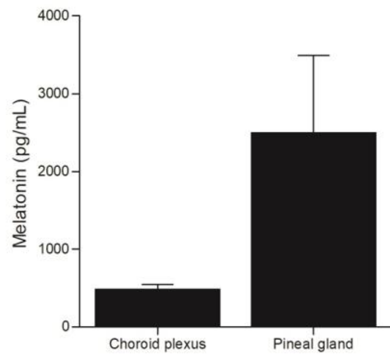

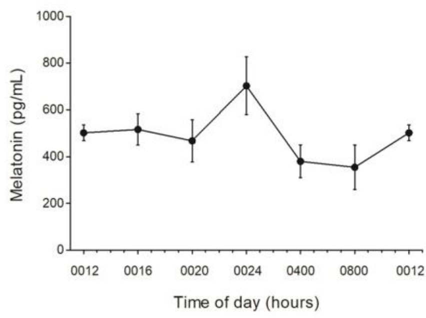

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