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Pineal melatonin synthesis and release are not altered throughout the estrous cycle in female rats

Abstract: Melatonin times reproduction with seasons in many photoperiodic mammalian species. Whether sexual hormones reflect on melatonin synthesis is still debated. The aim of this work was to study, using a large panel of technical approaches, whether the daily profile of pineal melatonin synthesis and release varies with the estrous cycle in the female rat. The mRNA levels and enzyme activities of the melatonin synthesizing enzymes, arylalkylamine *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase were similar at the four stages of the rat estrous cycle. The endogenous release of melatonin, followed by transpineal microdialysis during six consecutive days in cycling female rats, displayed no significant variation during this interval. Taken together, the present results demonstrate that there is no regular fluctuation in the pineal metabolism leading to melatonin synthesis and release throughout the estrous cycle in female rats.

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Introduction

The pineal gland, via the rhythmic secretion of melatonin, is essential for transducing seasonal changes in day length (photoperiod) into physiological responses, such as seasonal reproduction [1]. Interestingly, it has been proposed that the gonadal steroids may have a negative feedback effect on the pineal gland, especially on the melatonin-synthesizing enzymes arylalkylamine *N*-acetyltransferase (AANAT) and hydroxyindole-*O*-methyltransferase (HIOMT) [2]. In rats, however, various studies reported contradictory results concerning the possible alteration in melatonin synthesis and release throughout the estrous cycle. Quay [3] showed a trend in reduction of melatonin during the diestrus. Ozaki et al. [4] reported a sudden reduction of melatonin during proestrus, whereas Johnson et al. [5] observed a reduction during the estrous phase of the cycle. Additionally, other studies with rats reported estrous cycle-dependent variations in HIOMT activity with the highest and lowest activities being, respectively, at diestrus and estrus [6], diestrus and proestrus [7] or estrus and proestrus [8]. No significant difference in AANAT activity was found associated with the estrous stages [9, 10]. In other species (hamster, ewe and monkey), pineal or plasma melatonin level do not appear to vary as a function of the reproductive state [11–13].

This brief survey of the literature shows that there is no clear evidence of estrous cycle-dependent variation in the daily rhythm of pineal melatonin in female rats. The purpose of this study was to use new methods including a combination of microdialysis, radioenzymatic and *in situ* hybridization techniques in an attempt to solve this controversy. Using pineal microdialysis it is possible to follow the endogenous release of melatonin in the same animal throughout its estrous cycle, overcoming any possible interference of inter-individual variations. Secondly, AANAT and HIOMT activities and melatonin content were assayed at night in the same pineal of female rats at different stages of the estrous cycle. Finally, pineal AANAT and HIOMT mRNAs transcript levels were measured at the various stages of the estrous cycle using *in situ* hybridization.

Materials and methods

Animals

Adult female Wistar rats weighing 240–270 g were kept on a 12-hr light/12-hr dark cycle in a temperature-controlled room (21 ± 2°C), with water and food *ad libitum* for at least 2 wk before the experiments. Vaginal smears were taken daily in the morning (or 6 hr after lights off, for the

pineal microdialysis experiment) to monitor estrous cycles. Only animals exhibiting at least two consistent 4-day estrous cycles were used in the following studies. All experiments with animals were performed in accordance with 'Principles of Laboratory Animal Care' (NIH publication no. 86-23, revised 1985) as well as in accordance with the French national laws.

Experimental design

Experiment 1

Eleven animals were used to follow the daily profile of the endogenous pineal melatonin release as a function of the estrous cycle, using transpineal microdialysis. The animals were housed in a reversed 12-hr light/12-hr dark cycle (lights off from 10:00 to 22:00 hr). The dark onset (10:00 hr) corresponded to Zeitgeber time ZT-12. The animals were kept under these conditions for 3 wk before surgery (see below). The experiment lasted 6 days during which the pineal gland was continuously perfused with a Ringer's solution and the rats were checked for the estrous cycle stages.

Experiment 2

Forty female rats were used to measure nocturnal pineal melatonin content, AANAT and HIOMT mRNAs and activities at the four stages of estrous cycle. The animals were housed for 3 wk (checked daily for their estrous stage) before the experiment. They were sacrificed in the dark (dim red light) at ZT-19 (7 hr after lights off). Vaginal smears were taken 1 hr before being killed and the rats were distributed into four groups according to the estrous stage (proestrus, P; estrus, E; metestrus, M, and diestrus, D). In half of these animals (five per estrous stage) the pineal gland was removed to assay melatonin and to measure AANAT and HIOMT enzymatic activities. In the remaining animals (five per estrous stage) the brain was removed with the pineal attached, to perform *in situ* hybridization.

Surgery and dialysis

Animals were anaesthetized with Equithesin i.p. (0.4 mL/100 g body weight). The implantation of the microdialysis probe was performed as already described [14]. The microdialysis probe (0.22 mm i.d., 0.27 mm o.d., 10,000 molecular weight cutoff) was prepared from saponified cellulose ester membrane (Cordis Dow Medical International, Oosterwolde, the Netherlands). The surface of the probe was coated with silicone glue (CAF3, Rhône Poulenc, France) except for a 2-mm wide zone. The microdialysis probe bearing a tungsten wire with a sharpened point was fastened horizontally in a transverse position in a holder mounted a stereotaxic apparatus (David Kopf Instruments, Roucaire, Les Ulis, France). One hole was drilled on each side of the temporal bone, 1.6 mm ventral to the skull and 0.7 mm posterior to lambda according to the atlas of Paxinos and Watson [15], and the probe was pushed laterally through the pineal gland. Both inlet and outlet tubes were fixed on the skull in a vertical position with dental cement. The rats were allowed to recover from the surgery for at least 4 days in individual cages. During experiments, the inlet of the probe was connected to a microinjection pump (Pump 22, Harvard Biosciences, Les Ullis, France) *via* a fluid swivel (375/22, Instech Laboratories, Plymouth Meeting, PA, USA) with polyethylene tubing. The probe was perfused with Ringer's solution at a flow rate of 3 μL/min. The composition of the Ringer's solution was 147 mmol/L of NaCl, 4 mmol/L of KCl, 1.2 mmol/L of $CaCl_2$ and 1.0 mmol/L of $MgCl_2$. The outlet connection consisted of microbore PEEK tubing (0.13 mm i.d., 0.51 mm o.d.) connected to a 1.5 mL polypropylene microvial. Collected samples were stored at −20°C until assayed by radioimmunoassay (RIA). Samples were collected hourly from ZT-11 to ZT-1 and every 2 hr from ZT-1 to ZT-11, during 6 days. At the end of the experiment, rats were decapitated and the brains, dissected with the pineal glands, were frozen at −20°C. Cryostat sections (25 μm) of the brain/pineal were stained with cresyl violet in order to determine the location of the microdialysis probe.

Melatonin radioimmunoassay

Melatonin was measured in pineal glands and pineal microdialysates by RIA [14, 16]. Single pineal glands were sonicated in 110 μL sodium phosphate buffer (0.05 M, pH 7.9), centrifuged and 25 μL of the supernatant was assayed for melatonin whereas the remaining supernatant was used for enzymatic activity and protein assays. Melatonin was assayed using a specific antiserum (R19540, INRA, Nouzilly, France) at a final dilution of 1:200,000 and [^{125}I]-2-iodomelatonin as a tracer. Sheep anti-rabbit antiserum (INRA, Nouzilly, France) was used to separate the bound and free tracer. Protein content was measured in 20 μL tissue homogenate following the protocol of Lowry with BSA as standard [17]. Melatonin concentration in the pineal gland was expressed as pg/μg of protein.

Melatonin concentrations in dialysates were determined directly without extraction in duplicate 25 μL samples. The assay was validated for pineal dialysates as already reported by Barassin et al. [14]. The limit of sensitivity of the assay was 0.5 pg/tube. Melatonin in the pineal microdialysate was expressed as pg/25 μL microdialysate.

Pineal AANAT and HIOMT activity radioenzymatic assay

Arylalkylamine *N*-acetyltransferase activity was assayed by measuring the amount of ^{14}C–*N*-acetyltryptamine formed from ^{14}C–acetyl CoA (^{14}C–ACoA) and tryptamin. HIOMT activity was assayed by measuring the amount of melatonin formed from *S*-adenosyl-L-[^{14}C–methionine] and *N*-acetylserotonin (NAS). Immediately after the sacrifice of the animals, individual pineal glands were sonicated in 110 μL sodium phosphate buffer (0.05 M, pH 7.9), and AANAT and HIOMT activities were determined as previously described [18, 19] in the same pineal homogenate.

For AANAT activity, 30 μL of the tissue homogenate were incubated for 20 min at 37°C with 80 mM tryptamine and 32 mM ^{14}C–ACoA [44.1 mCi/mmol, New England Nuclear (NEN)-Dupont, Le Blanc Mesnil, France] in a

final volume of 60 μL (pH 6.8), then the reaction was stopped by the addition of 1 mL chloroform. Newly synthesized *N*-acetyltryptamine was measured after extraction in 1 mL of water-saturated chloroform and counting of the radioactivity after evaporation of the organic solvent.

For HIOMT activity, 25 μL of the tissue homogenate were incubated for 30 min at 37°C with 1 mM NAS and 43.8 μM *S*-adenosyl-L-[^{14}C–methionine] (59.3 mCi/mmol; NEN-Dupont) in a final volume of 100 μL (pH 7.9), then the reaction was stopped by the addition of 200 μL sodium borate buffer (12.5 mM; pH 10). Newly synthesized melatonin was measured after extraction in 1 mL water-saturated chloroform and counting of the radioactivity after evaporation of the organic solvent. Enzymatic activities were expressed as picomoles of reaction product formed per hr/μg of protein.

In situ hybridization of AANAT and HIOMT mRNAs

Coronal sections of frozen brains (20 μm thick) were thaw-mounted onto gelatin-coated slides. For each animal, the pineal AANAT and HIOMT mRNA levels were quantified by quantitative *in situ* hybridization performed on two separate sets of sections according to a protocol previously described (HIOMT: [20], AANAT: [16]).

The sense and antisense riboprobes were synthesized from the linear pBluescript-CMV phagemid and pBluescript plasmid containing the cDNA encoding AANAT (1311 bp, [21]) or HIOMT (1542 bp, [22]) with either T7 or T3 RNA polymerase (MAXIscript transcription kit, Ambion, Montrouge, France; α[^{35} S]-UTP, 46.3 TBq/mmol, NEN-Dupont). The AANAT and HIOMT probes were hydrolyzed by an alkaline treatment to generate 200-bp-long fragments. The AANAT and HIOMT mRNA analysis was performed according to the same protocol as follows. All the prehybridization steps were carried out at room temperature. Sections were incubated in 4% paraformaldehyde/1 × phosphate-buffered saline (PBS) for 15 min. They were washed successively into 1 × PBS and 2 × sodium saline citrate (SSC) for 2 min each. Sections were then acetylated with 0.5% acetic anhydrate/0.1 M triethanolamine (pH 7.4) for 10 min and rinsed in 2 × SSC and 1 × PBS for 2 min each. They were then incubated for 30 min in 0.1 M glycine/0.1 M Tris (pH 7.0) and rinsed in 2 × SSC and 1 × PBS before being dehydrated in graded ethanol solutions (70, 90, 95, and 100%, 1 min each) and dried at room temperature.

For hybridization, probes were dissolved in a hybridization solution containing 2 × SSC/20% Dextran sulfate/50% deionized formamide/10 mM dithiothreitol/1 × Denhardt's solution. Dehydrated brain sections were overlaid with 80 or 50 pM of the antisense or sense AANAT or HIOMT probes, respectively, and incubated overnight at 54°C in a moist chamber.

Posthybridization treatment consisted of washing the sections for 10 min at room temperature in 2 × SSC before being incubated for 30 min at 37°C with 0.02 Kunitz unit/mL ribonuclease type X-A (from bovine pancreas, Sigma, Saint Quentin Fallavier, France) in 0.5 M NaCl/10 mM Tris (pH 7.4)/10 mM EDTA. The sections were then washed three times (5 min each) in 2 × SSC at room temperature before dehydration in graded ethanol solutions (70, 90, 95 and 100%, 1 min each) and air dried.

The slides were then exposed to autoradiographic films (Hyperfilm MP, Amersham, Les Ulis, France) for 48 hr at room temperature. Quantitative analysis of the autoradiograms was performed using the BIOCOM computerized analysis program RAG200. Specific hybridization was determined by densitometry as the difference between total (antisense) and non-specific (sense) hybridization.

Statistical analysis

For microdialysis experiment, each individual melatonin profile was characterized by its onset time (IT50), its offset time (DT50) and the peak amplitude (Y_{ampl}). These values were determined by fitting a logistic peak with the following equation:

$$Y = Y_0 + \frac{Y_{ampl}}{(1 + e^{2.91(IT50-x)})(1 + e^{2.77(x-DT50)})}$$

where Y was the nth data point, x the time point of the nth point, Y_0 basal level during daytime, and Y_{ampl} the amplitude of the nocturnal peak. IT50 was defined as the time point at which 50% of the increase in melatonin level was reached and DT50 as the time at which 50% of the decrease occurred. The non-linear regression analysis was performed with SigmaPlot software (SPSS ASC GmbH, Erkrath, Germany) and fitted through datapoints of each experimental day of each animal [14]. The results from the individual regressions gave a mean Y_0 close to zero, thus in further analyses, only the three parameters IT50, DT50 and Y_{ampl} were considered to characterize the pattern of the melatonin peaks. To compare the variations in melatonin peak among the four estrous cycles an analysis of variance–covariance was carried out considering the variances and residues of variance given by the regressions characterizing the melatonin peak. We only considered the first occurrence of one of the four stages observed in each animal.

In the experiment 2, results on melatonin content, AANAT and HIOMT activity and mRNA expression are expressed as mean ± S.E.M. and were computed using GraphPad Prism data analysis and graph package (version 3.01, GraphPad Software Inc., San Diego, CA, USA). Data, distributed according to the estrous cycle phase of each animal just before the sacrifice, were compared using the one-way ANOVA procedure.

The level of probability was fixed at 0.05 for statistical significance.

Results

In experiment 1, all the 11 female rats had the probe correctly implanted within the pineal gland but only three of them recovered in displaying a regular 4-day estrous cycle. Therefore the individual profile of melatonin production has been followed during 5–6 consecutive days in three cycling female rats with raw values of melatonin release presented as a function of ZT for each animal (Fig. 1). No regular pattern of variation in the melatonin profile is observed among the four stages of the estrous cycle in any of the female rats. The statistical analyses

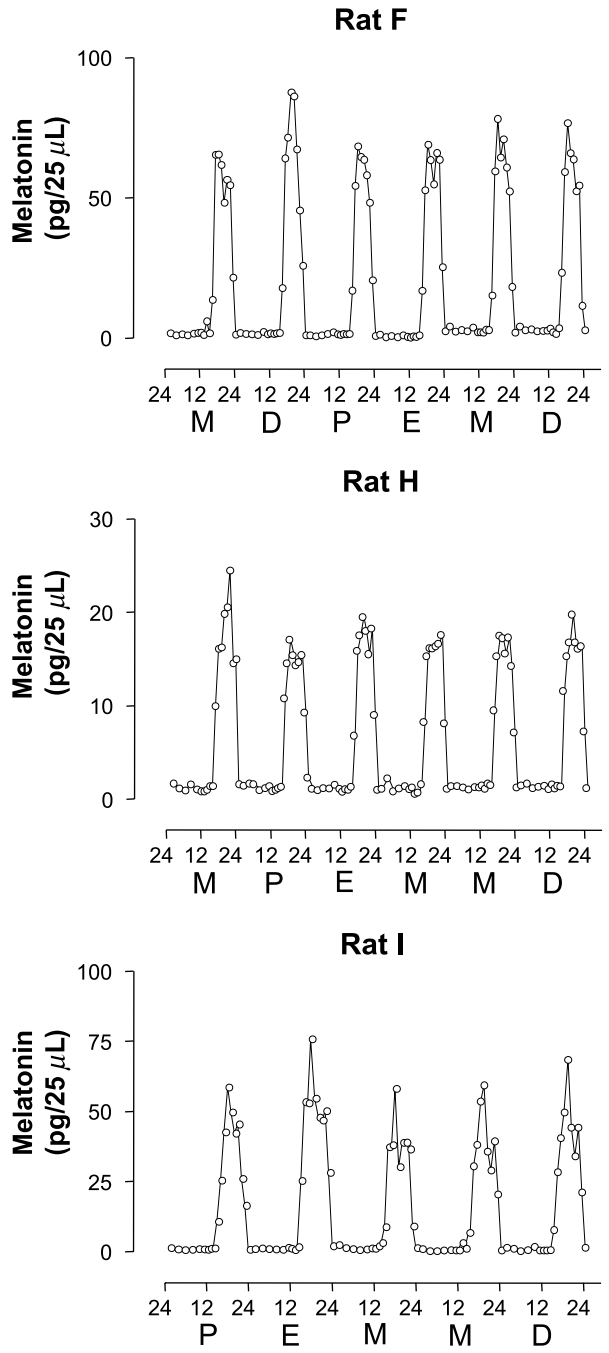

Fig. 1. Daily pattern of melatonin release measured in pineal microdialysates for five to six consecutive days in three cycling female rats. Female rats (F, H, I) were kept under a 12-hr light/12-hr dark cycle with lights off at 10:00 am and were checked everyday for estrous stage (P, E, M, D). Pineal microdialysates were sampled every 1 hr (night) or 2 hr (day), and assayed for melatonin.

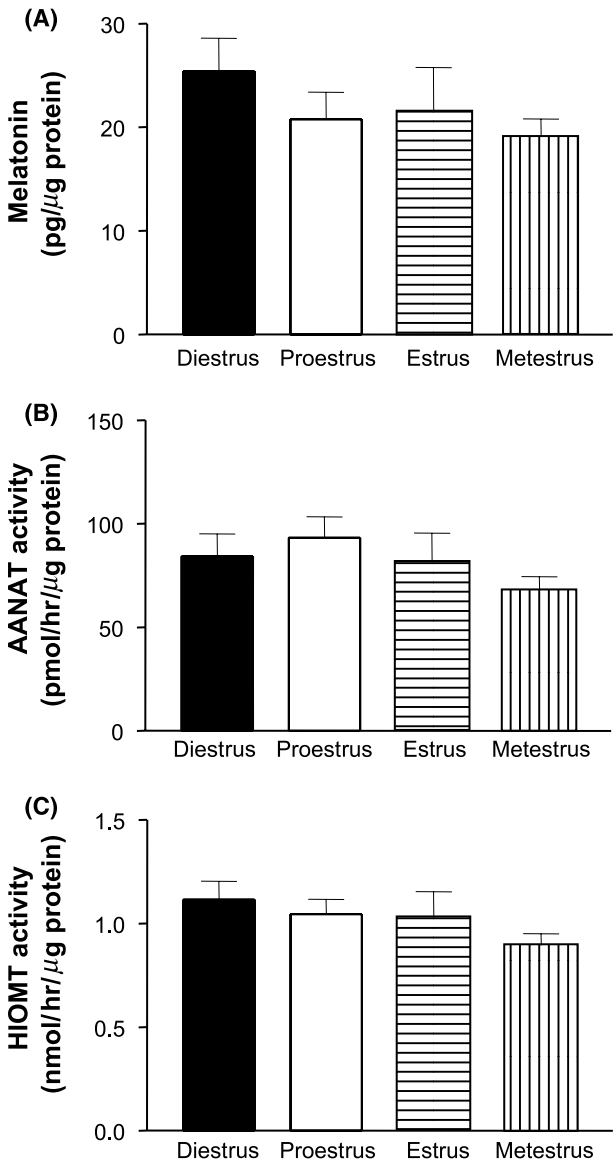

Fig. 2. (A) Melatonin content, (B) arylalkylamine *N* acetyltransferase (AANAT) activity and (C) hydroxyindole-*O*-methyltransferase (HIOMT) activity in the pineal gland of female rats at each of the four stages of the estrous cycle. Animals were sacrificed 7 hr after lights off. Data are given as mean ± S.E.M. of n = 5 animals.

carried on the parameters characterizing the melatonin peak (onset, amplitude, offset) revealed no significant difference related to the estrous stage ($F^{9}_{191} = 0.818$, $P = 0.6$).

In experiment 2, melatonin content (Fig. 2A), AANAT (Fig. 2B) and HIOMT (Fig. 2C) activities, and protein level were measured in the same pineal gland of female rats, at either of the four estrous stages, sacrificed 7 hr after dark onset (when the melatonin peak amplitude is at its maximum). No significant difference in nocturnal melatonin content or enzyme activity is found as a function of the stage of the estrous cycle (melatonin values: D = 25.43 ± 7.15 pg/μg protein, P = 20.78 ± 5.84 pg/μg protein, E = 21.61 ± 9.37 pg/μg protein, M = 19.11 ± 3.82 pg/μg protein, ANOVA, $P = 0.53$; AANAT values: D = 84.44 ± 24.07 pmol/hr μg protein, P = 93.30 ± 22.78 pmol/hr μg protein, E = 82.07 ± 30.01 pmol/hr μg protein, M = 67.85 ± 14.84 pmol/hr μg protein, ANOVA, $P = 0.64$; HIOMT values: D = 1.12 ± 0.20 nmol/hr μg protein, P = 1.05 ± 0.16 nmol/hr μg protein, E = 1.03 ±

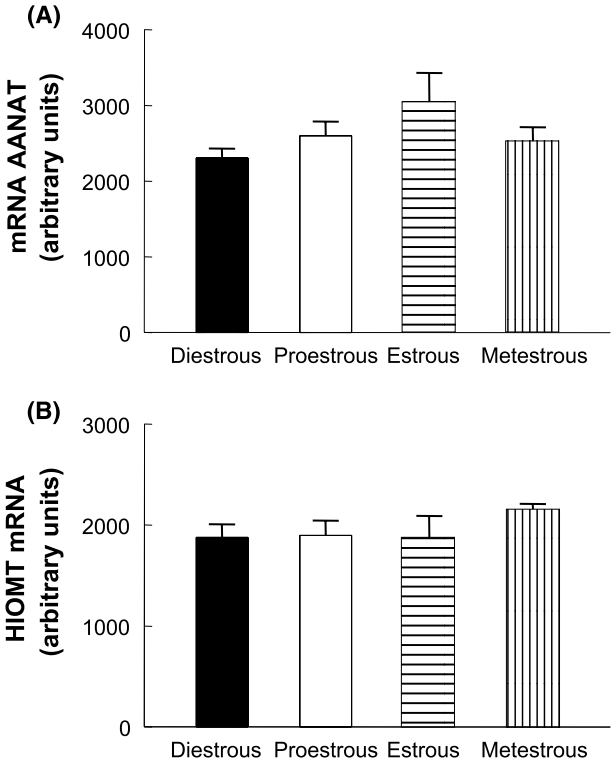

Fig. 3. (A) arylalkylamine *N*-acetyltransferase (AANAT) mRNA and (B) hydroxyindole-*O*-methyltransferase (HIOMT) mRNA levels measured by *in situ* hybrodization in the pineal gland of female rats at each of the four stages of the estrous cycle. Animals were sacrificed 7 hr after lights off. Data are given as mean ± S.E.M. of n = 5 animals.

0.28 nmol/hr μg protein, M = 0.89 ± 0.13 nmol/hr μg protein, ANOVA, $P = 0.39$; mean ± S.E.M. of five animals per stage).

The expression of AANAT (Fig. 3A) and HIOMT (Fig. 3B) mRNAs was measured in the same pineal gland of female rats, at either of the four estrous stages, sacrificed 7 hr after dark onset. No significant difference has been found in the amount of either AANAT mRNA (D = 2308.94 ± 124.06 dpm, P = 2601.26 ± 187.38 dpm, E = 3048.40 ± 384.47 dpm, M = 2530.54 ± 185.55 dpm; ANOVA, $P = 0.23$; mean ± S.E.M. of five animals per stage) or HIOMT mRNA (D = 1881.40 ± 126.65 dpm, P = 1902.49 ± 143.10 dpm, E = 1867.96 ± 226.94 dpm, M = 2159.37 ± 51.45 dpm; ANOVA, $P = 0.40$; mean ± S.E.M. of five animals per stage) throughout the estrous cycle.

Discussion

This paper shows that there is no significant variation in the daily pattern of pineal melatonin release throughout the estrous cycle in female rats. This result is strengthened by the demonstration that gene expression and activity of the two melatonin-synthesizing enzymes AANAT and HIOMT did not vary as a function of the estrous stage.

These data are in agreement with those obtained for ewe and hamster melatonin contents, but they are in contrast with some results obtained for rats. The data showing a strong reduction of melatonin during proestrus [4] were obtained from urine samples. Another study, monitoring urinary 6-sulphatoxymelatonin excretion, showed a significant variation during the estrous cycle, with higher values on proestrus [23]. An explanation for these results could be an estrous cycle variation in the hepatic ability for metabolizing melatonin or, even more likely, a greater or smaller ability of the kidney to excrete metabolic melatonin products according to the estrous stages. Quay [3] showed a small reduction in melatonin during the diestrus whereas another study [5] reported a reduction of pineal melatonin during proestrus. It seems possible that the inconsistencies of finding reproducible variations in melatonin secretion throughout the rat estrous cycle is because of large inter-individual differences.

To overcome these putative inter-individual variations we used the transpineal microdialysis technique which allowed for the monitoring of endogenous melatonin release for several consecutive days in the pineal gland of a single animal. We have previously validated this technique and demonstrated that for each animal, the melatonin profile is stable up to 5 consecutive days with very low intra-individual variability [14].

The endogenous melatonin secretion from the pineal gland of three cycling female rats was followed for 5–6 consecutive days. No consistent or regular variation of the nycthemeral melatonin profile was detected that could be strictly associated to any of the four stages of the estrous cycle. Occasionally, one melatonin peak appeared of higher amplitude but with no significant relation with the estrous cycle (during the diestrus in rat H, during the metestrus in rat F, during estrus in rat I). These inter-individual variations could explain the inconsistent results found in the literature. Therefore, the absence of a significant variation in the melatonin pattern in relation with the estrous stage of three female rats exclude any regular or coherent variation of pineal melatonin synthesis depending on the hormonal physiologic environment because of the estrous cycle.

To strengthen these observations, gene expression and enzyme activity of AANAT and HIOMT were examined during the night at each of the estrous stages. Enzyme activities were already examined according to the reproductive status in mammals, but, to our knowledge, this study is the first to analyze the effect of sexual hormones on pineal enzyme gene expression. Nocturnal pineal AANAT mRNA and activity displayed no significant variation associated with the four estrous stages. This observation is in agreement with data in the literature reporting no difference in AANAT activity throughout the estrous cycle [9, 10]. Similar to AANAT, nocturnal HIOMT mRNA level and activity displayed no significant variation during the estrous cycle. This is in contrast to data reported in the literature [7, 8, 10] although these three studies reported contradictory results. Therefore, it is not surprising that, in fact, we found no significant difference in HIOMT gene expression and enzyme activity in relation to the estrous cycle.

Our results, however, do not exclude an effect of gonadal steroids *per se* on pineal metabolism. The pineal gland

accumulates specifically estradiol and testosterone [24, 25] and contains nuclear binding sites for estradiol, testosterone [26–28]. Furthermore, testosterone exhibits stimulatory effects and castration reduces cAMP concentrations [29], AANAT activity [30] and melatonin secretion [31]. In female rats, estradiol displays an inhibitory effect on the $\alpha 1\beta 1$-AR-induced cAMP and Ca^{2+} levels, AANAT activity and melatonin production while ovariectomy leads to a significant increase in the cAMP/AANAT/melatonin pathway [32–37]. Similarly, an increase in nocturnal melatonin secretion (associated with an increase in AANAT not HIOMT activity in rat pineal) is observed during menopause in relation to the existence of a low estrogen environment in rat [37] and human [38].

In summary, this is the first time that a potential effect of estrous cycle on pineal metabolism has been studied in such a thorough way looking at all steps of melatonin synthesis (enzyme gene expression, enzyme activities, melatonin content and melatonin release). This study clearly demonstrates that the melatonin synthesis pathway is not altered during the estrous cycle in female rats, which is similar to the results reported for hamster [12], sheep [11] and monkey [13].

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