Mechanisms regulating the marked seasonal variation in melatonin synthesis in the European hamster pineal gland

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Mécanismes régulant la variation saisonnière de la synthèse de la mélatonine dans l'encéphale de hamster européen

MELATONIN PRODUCTION in the pineal gland displays marked, predictable, and reproducible daily and seasonal variations allowing most organisms to anticipate and adapt their behavioral and physiological functions to the cyclic variations of their environment (51). In wild species, the seasonal variation of circulating melatonin is a strong signal synchronizing for the timing of seasonal functions, especially those related to reproduction, with the annual variations of light, temperature, humidity, and food availability (13, 17, 44, 50). To date, however, it is not clear what drives the seasonal variations in pineal melatonin synthesis and release.

Current knowledge on the cellular and molecular regulation of pineal melatonin synthesis mainly relies on studies made using the nonseasonal laboratory rat. Melatonin is synthesized from serotonin following acetylation by the enzyme arylalkylamine N-acetyltransferase (AA-NAT) and methylation by hydroxyindole-O-methyltransferase (HIOMT) (25). Pineal metabolic activity is mainly under the control of the endogenous biological clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus, its activity being primarily synchronized by the light-dark variation transmitted by the retinohypothalamic tract (27).

Daytime inhibitory and nighttime stimulatory SCN outputs reach the pineal gland through the superior cervical ganglia whose sympathetic fibers release norepinephrine in the pineal gland only during the night (7, 21). Norepinephrine binds to ß-adrenergic receptors to cause a large cAMP-induced transcriptional and posttranscriptional activation of the unstable (t1/2 = 3 min) AA-NAT (9, 26, 58). Although HIOMT gene expression is also stimulated by norepinephrine, its effect on HIOMT activity is only observed on a long-term seasonal scale (53–55, 57). Norepinephrine also binds to α-adrenergic receptors that potentiate via Ca2+-induced PKC activation, the cAMP pathway (68, 69, 71). Other neurotransmitters, especially neuropeptides such as vasopressin (60), vasoactive intestinal peptide (VIP) (23), pituitary adenylate cyclase-activating peptide (PACAP) (62), or neuropeptide Y (NPY) (40, 61), are able to modulate the noradrenergic stimulation of melatonin synthesis (59). As soon as it is synthesized, melatonin is released into the blood stream. Therefore, the nocturnal norepinephrine-induced increase in AA-NAT activity drives the nocturnal rise of circulating melatonin, an important day/night cue for synchronization of the daily rhythms.

In addition, the nocturnal synthesis and release of melatonin display marked seasonal variations. In most species, the duration of the melatonin peak enlarges with the night duration in short photoperiod (SP) or in winter (51). In the rat, we showed that the increase in the duration of the melatonin peak is driven by Aa-nat mRNA and AA-NAT activity (53), probably as a conse-
quence of prolonged release of norepinephrine under SP. Although it has been demonstrated that the variations in melatonin peak duration are a crucial hormonal signal for encoding season (2), numerous species, especially when raised outdoors, also display a large seasonal variation in the amplitude of the melatonin peak. This has been reported for sheep (1), tamar (31), goat (24), mule (5), European hamster (Cricetus cricetus) (73, 74), deer (43), Siberian hamster (34, 57, 66), and horse (14). These observations have led to the suggestion that factors, other than photoperiod, which display annual variations, may be integrated and transmitted via melatonin (44, 47, 72).

Surprisingly, in all the species studied so far, the increase in the amplitude of the melatonin peak is not associated with AA-NAT because, on the contrary, a decrease in the amplitude of enzyme mRNA and/or activity has been observed in the Siberian hamster (18, 57), rat (53), Arvicanthus (Garidou, Simonneaux, and Vivien-Roels, unpublished data), and Syrian hamster (Garidou and Simonneaux, unpublished data). In the Siberian hamster, we recently demonstrated that the twofold increase in melatonin peak amplitude observed in animals raised under SP compared with those in long photoperiod (LP) was not correlated to AA-NAT activity (it was lower under SP) but rather to HIOMT activity [2-fold higher under SP (57)]. We postulated that the SP-induced increase in HIOMT activity results from an increased rate of HIOMT synthesis with more Hiomt mRNA being synthesized under long nights, which, in turn, drives the increase in melatonin peak amplitude (53, 54, 57).

Of the species cited above, the European hamster (Cricetus cricetus) displays the largest seasonal variation in melatonin peak amplitude ranging from a 1.5- to 2-fold nocturnal increase in May-June to a 10- to 20-fold increase in November-December (73). In addition, the pineal indole 5-methoxytryptophol (5-ML) synthesized from serotonin through another metabolic route ending with HIOMT also exhibits a seasonal variation (73). During autumn, from October to December, pineal HIOMT increases by 80% together with a large increase in NPY immunoreactivity (36, 56). These data suggest that, as observed in the Siberian hamster, HIOMT could be an important factor in the seasonal/photoperiodic regulation of pineal melatonin and 5-ML synthesis in the European hamster.

The aim of the present study was to compare the daily variation in pineal metabolism (Aa-nat mRNA, AA-NAT and HIOMT activities, melatonin and 5-ML contents) in European hamsters kept in natural LP (June) and SP (November) conditions. In addition, the pineal metabolism of hamsters raised indoors under artificial SP was compared with that of hamsters kept outdoors under natural SP (November) to assess the possible involvement of seasonal factors different from photoperiod. Finally, a number of in vivo and in vitro experiments were performed to investigate the neurotransmitters and cellular mechanisms that could be implicated in the regulation of pineal melatonin synthesis.

**Materials and Methods**

**Animals**

European hamsters (Cricetus cricetus) were born and raised in our breeding colony. They were either kept outdoors in natural photoperiod and thermoperiod, or indoors, at 20 ± 1°C, under SP (8:16-h light-dark cycle, with lights off at 1800) with food and water supplied ad libitum. In winter, animals hibernating outdoors were awakened 3 days before the experiments.

All experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and follow the recent “Guiding Principles For Research Involving Animals and Human Beings” by the American Physiological Society. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

**Experimental Protocols**

**Seasonal variation in the daily profile of pineal metabolism.** Male European hamsters, raised outdoors, were killed at different times of the day in June (darkness from 2130 to 0500; sampling at 1200, 2330, 0130, 0330, 0700) or in November (darkness 1700 to 0800; sampling at 0900, 1300, 1700, 2100, 2400, 0300, 0600). In addition, female European hamsters were brought indoors and kept for 8 wk under artificial SP before being killed in mid-November (darkness from 1800 to 1000; sampling at 1400, 2000, 2400, 0400, 0800). For each time point, five brains with the pineal attached were frozen in −20°C isopentane and then stored at −80°C until slicing of the pineal for Aa-nat mRNA in situ hybridization and HIOMT assay; and five pineal glands were dissected, frozen in liquid nitrogen, and rapidly assayed for AA-NAT activity, melatonin, 5-ML, and protein content.

In vivo regulation of melatonin content in the European hamster pineal gland. To alter melatonin synthesis, various protocols with adrenergic agonists and antagonists were performed in vivo conditions.

**Propranolol injection at night under SP.** In November, groups (n = 7–8 per group) of female European hamsters were injected intraperitoneally either with the β-adrenergic antagonist propranolol (20 mg/kg, dissolved in Ringer, Sigma, St. Louis, MO) or with vehicle at 1800 and 2200 and killed at 0200; a control group was killed at 1800.

**Isoproterenol injection during the day or night under LP.** In June, groups (n = 5 per group) of European hamsters were injected intraperitoneally 1) at 1100 either with isoproterenol (5 mg/kg, Sigma dissolved in Ringer) or with vehicle and then killed at 1300 or 2) at 0100 either with isoproterenol (5 mg/kg, dissolved in Ringer) or with vehicle and then killed at 0300. For each experiment, the pineal gland was immediately dissected, frozen in liquid nitrogen, and stored at −20°C until assay for melatonin.

**In vitro regulation of melatonin release from European hamster pineal cells.** Various drugs were used to stimulate melatonin release from dissociated pineal cells maintained in primary culture according to Simonneaux et al. (62). Several cultures were made in April, May, November, and December. The pineal gland of 6-mo-old male and female hamsters was rapidly dissected and dissociated in a saline solution (137 mM NaCl, 5 mM KCl, 0.7 mM NaH2PO4, 0.35 mM CaCl2, 10 mM glucose, 25 mM HEPES, and 0.1% bovine serum albumin) containing 20 mg collagenase (Serva, Paris, France), 3 mg trypsin (Sigma), and 40 µg DNase (Sigma) in 25 ml. Dissociated cells were resuspended in culture medium con-
taining DMEM (Sigma) enriched with 10 mM HEPES, 45 mM NaHCO3, 10% calf serum (GIBCO, Cergy Pontoise, France), and 0.04 mg/ml gentamycin (Sigma) at a density of 150,000 cells/well and incubated at 37°C under a water-saturated 5% CO2-95% air atmosphere. After 3 days of culture, cells were washed and then incubated for 5 h with various drugs (dissolved in serum-free culture medium): isoproterenol (10 μM), phenylephrine (an α-adrenergic agonist, 10 μM, Sigma), dibutyryl cyclic AMP (DBcAMP; a efficacious analog of cAMP, 1 mM, Sigma), PMA (a protein kinase C activator, 1 μM, Sigma), pituitary adenylate cyclase-activating peptide (PACAP; 0.1 μM, Bachem, Voisin-le-Bretonneux, France), VIP (0.1 μM, Bachem), NPY (0.1 μM, Bachem), somatostatin (0.1 μM, Bachem), and Leu-enkephalin (0.1 μM, Bachem). In one experiment, cells were cultured for up to 9 days with the medium changed every 2 days. At the end of the incubation, the medium was sampled and directly assayed for melatonin.

Ex vivo regulation of melatonin release from the European hamster pineal gland. Analysis of melatonin release from perfused female European hamster pineal glands was performed according to Simonneaux et al. (63) in February. Pineal glands were rapidly dissected in an oxygenated ice-cold Krebs-Ringer solution between 0800 and 0900 and then settled in perfusion columns (3 glands/column). Starting at 1000, the pineal glands were perfused continuously with a 37°C oxygenated Krebs-Ringer solution running at a flow rate of 0.1 ml/min. The perfusate was collected every 30 min from 1130 to 2100. Starting at 1300, the pineal glands were perfused for 8 h with Krebs-Ringer, 10 μM isoproterenol, 1 mM DBcAMP, or 0.1 mM tryptophan (Trp; substrate for serotonin synthesis, Sigma). All drugs were dissolved in Krebs-Ringer. Melatonin was assayed, directly in the perfusate of all tubes from 1130 to 1300 (these values giving the basal release) and in every second tube taken thereafter.

**Aa-nat In Situ Hybridization**

Coronal sections (20 μM) of hamster brain were sliced in a cryostat at −16°C and thaw-mounted onto gelatin-coated slides. The slides were stored at −80°C until hybridization with the Syrian hamster Aa-nat riboprobes according to a protocol previously described (12). Briefly, radioactive antisense and sense riboprobes were transcribed from the linearized pCR-script cloning vector containing the cDNA encoding the Syrian hamster Aa-nat (1,045 bp) with T3 (antisense) or T7 (sense) RNA polymerase (MAXIscript transcription kit; α[35S]-UTP, 1,250 Ci/mmol, NEN-Dupont, Leblanc-Mesnil, France) and hydrolyzed by alkaline treatment for 27 min to generate 200-bp fragments. Brain sections were submitted to prehybridization treatments (fixation, acetylation, glycine treatment, and dehybridization) before an overnight incubation at 54°C in a medium containing 80 amol riboprobe/μl. After hybridization, slides were washed with X-A ribonuclease (0.02 kunitz U/ml, Sigma) and 2× sodium saline citrate to remove most of the nonspecific binding. Finally, slides together with [35S] standards (laboratory made) were exposed to an autoradiographic film (Hyperfilm MP, Kodak, Orsay, France) for 3 days. Quantitative analysis of the autoradiograms was performed using the computerized analysis system Biocom-program RAG 200. Specific labeling of the riboprobe was determined as the difference between total (antisense) and nonspecific (sense) hybridization, both being run parallel in each experiment.

**Enzyme Activity Assays**

*AA-NAT and HIOMT activities were measured separately in in vitro experiments. AA-NAT activity. Each pineal gland was sonicated in 110 μl phosphate buffer (0.05 M, pH 6.8) containing 0.35 mM acetyl-CoA (Sigma). Tissue homogenate was split for the different assays into 40 μl (AA-NAT activity), 20 μl (protein), 20 μl (melatonin), and 20 μl (5-ML). AA-NAT activity was assayed as described in Gariou et al. (10). Tissue homogenate was incubated for 20 min at 37°C in the presence of 10 mM tryptamine as substrate and [14C]-acetyl-CoA (44.1 mCi/mmol; NEN-Dupont; final specific activity 5.06 μCi/μmole) in a final volume of 80 μl. Enzymatic reaction was stopped by addition and extraction in 1 ml ice-cold water-saturated chloroform. Radioactivity was measured after evaporation of 800 μl chloroform and addition of 3.5 ml scintillation medium.

*HIOMT activity. After slicing for Aa-nat in situ hybridization, the remaining pineal gland was sonicated in 100 μl phosphate buffer (0.05 M, pH 7.9) and split into 50 μl for the HIOMT assay and 40 μl for the protein assay. HIOMT activity was assayed as described in Ribelayga et al. (54). Tissue homogenate was incubated for 30 min at 37°C with 1 mM N-acetylserotonin and 43.8 μM S-adenosyl-l-[14C-methionine] (59.3 mCi/mmol; NEN-Dupont) in a final volume of 100 μl (pH 7.9), and 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.

**Indole Assays**

Melatonin radioimmunoassay. Melatonin was measured without extraction in pineal tissue homogenate, pinealocyte culture medium, and pineal perfusate. The radioimmunoassay used rabbit antiserum [IR 19540, Institut National de la Recherche Agronomique (INRA), Nouzilly, France] at a final dilution of 1/200,000, laboratory-made [125I]melatonin as radiolabel, and sheep anti-rabbit antiserum (INRA) to separate the bound and free tracer following protocols previously described (54, 62, 63, 73).

5-ML radioimmunoassay. Tricine buffer (pH 6, 200 μl) was added to 20 μl of pineal supernatant, and 5-ML was assayed using a sheep antiserum (batch n° 1320, Stockgrand, University of Surrey, Guildford, UK) at a final dilution of 1/8,000, laboratory-made [125I]5-ML as radiolabel and dextrose-charcoal to separate bound and free 5-ML following a protocol previously described (64).

Serotonin assay by HPLC. Serotonin was assayed in the pineal perfusate according to Miguez et al. (35). Perifusates were diluted (1/1) with an antioxidant solution (0.4 M perchloric acid, 0.4 mM sodium metabisulfite, 0.1 mM EDTA), and 20-μl aliquots were injected into the chromatographic system (M590 pump from Waters, C18 ODS column from Beckman, Coulochem 5100 detector from ESA Bedlord).

**Protein Assay**

Protein content in 20 μl pineal tissue homogenate was determined following the protocol of Lowry et al. (29) with bovine serum albumin as standard.

**Data Analyses**

*Aa-nat* mRNA content is expressed in disintegrations per minute (dpm) using internal standards. AA-NAT and HIOMT activities are expressed in picomoles per hour per
microgram of protein. Melatonin and 5-ML contents are given in picograms per microgram of protein (except for melatonin in pg/gland for in vivo experiments); melatonin release is given in picograms per minute for pineal perfusion experiments and in picograms per 15,10^4 cells for pineal cell culture experiments.

All data are given as means ± SE of n = 5–7 animals or replicate. Statistical analyses between conditions were performed using Student-Newman-Keuls multicomparison test following one-way ANOVA. The differences were considered statistically significant for *P < 0.05.

RESULTS
Seasonal Variation in the Daily Profile of Pineal Metabolism in the European Hamster

When raised outdoors, the European hamsters displayed a marked seasonal variation in pineal melatonin content with nighttime peak values varying from 0.4 ± 0.1 pg/µg protein (82 ± 14 pg/gland, n = 5) in June up to 3.1 ± 0.5 pg/µg protein (355 ± 50 pg/gland, n = 5) in November (Fig. 1, a1 and a2). This seasonal

Fig. 1. Seasonal and photoperiodic variation in pineal metabolism in the pineal gland of European hamsters. Animals were either raised outdoors and studied in June [1: natural long photoperiod (LP)] or in November [2: natural short photoperiod (SP)] or kept indoors under an artificial SP (3: 8:16-h light-dark cycle with lights off at 1800). Two groups of hamsters were killed (2 × 5 animals/time point) at the indicated times. In 1 group, the pineal was assayed for melatonin, 5-methoxytryptophol (5-ML), protein (prot) contents, and for arylalkylamine N-acetyltransferase (AA-NAT) activity. In the second group, the brain with the pineal attached was removed, half of the pineal was sliced for Aa-nat in situ hybridization, and the other half was assayed for hydroxyindole-O-methyltransferase (HIOMT) activity and protein content. Values are means ± SE.

*P < 0.05 compared with midday values. Gray area indicates darkness.
change in melatonin synthesis appears to be driven by Aa-nat gene expression since a similar large seasonal variation was observed in Aa-nat mRNA and AA-NAT activity with the highest nighttime amount of Aa-nat mRNA, 496 ± 82 dpm \((n = 5)\) in June rising up to 1,524 ± 269 dpm \((n = 5)\) in November (Fig. 1, b1 and b2); and AA-NAT activity, \(2.2 ± 0.4\) pmol·h\(^{-1}\)·μg protein\(^{-1}\) \((n = 5)\) in June increasing to 10.8 ± 1.2 pmol·h\(^{-1}\)·μg protein\(^{-1}\) \((n = 5)\) in November (Fig. 1, c1 and c2). Conversely, the mean HIOMT activity measured over 24 h was not significantly altered between June (mean value of 0.58 ± 0.07 pmol·h\(^{-1}\)·μg protein\(^{-1}\)) and November (0.53 ± 0.03 pmol·h\(^{-1}\)·μg protein\(^{-1}\)), Fig. 1, d1 and d2).

Regarding pineal 5-ML content, the daily pattern was inverse compared with melatonin with lower 5-ML values during the night. In addition, there was a marked seasonal variation in daytime 5-ML content with higher values in November (ranging between 0.10 ± 0.03 and 0.25 ± 0.08 pg/μg protein) compared with June (ranging between 0.03 ± 0.01 and 0.06 ± 0.01 pg/μg protein), with the nighttime values being similar ~0.03 pg/μg protein in both conditions (Fig. 1, e1 and e2). This may be related to higher basal pineal metabolism in November compared with June since like 5-ML, daytime melatonin content was higher in November (0.28 ± 0.02 pg/μg protein) compared with June (0.04 ± 0.01 pg/μg protein). In contrast, daytime AA-NAT and HIOMT activities were identical at both times of the year (1.14 ± 0.02 and 0.61 ± 0.08 pmol·h\(^{-1}\)·μg protein\(^{-1}\) for AA-NAT and HIOMT, respectively).

To make a distinction between seasonal and photoperiod cues involved in the regulation of melatonin synthesis, the daily profile of pineal metabolism in European hamster raised under natural SP (November, outdoors) was compared with animals raised in artificial SP (8:16-h light-dark cycle indoors), the main difference between these two conditions being the external temperature. The indoor group was brought into the animal facilities from mid-September until mid-November. The mean protein content per pineal between outdoor (168 ± 10 pg/gland) and indoor (169 ± 11 pg/gland) hamsters was equal. The amplitude of the nocturnal melatonin peak was significantly lower in indoor hamsters (maximum of 2.1 ± 0.2 pg/μg protein) compared with outdoor hamsters (maximum of 3.1 ± 0.5 pg/μg protein) (Fig. 1, a2 and a3). These variations do not appear to be driven by AA-NAT since maximal nighttime values of Aa-nat mRNA (Fig. 1, b2 and b3) and AA-NAT activities (Fig. 1, c2 and c3) were similar in both conditions. On the contrary, HIOMT activity was found to be lower in indoor hamster (24-h mean 0.35 ± 0.05 pmol·h\(^{-1}\)·μg protein\(^{-1}\), \(n = 19\)) than in outdoor animals (24-h mean 0.53 ± 0.03 pmol·h\(^{-1}\)·μg protein\(^{-1}\), \(n = 30\), \(P < 0.05\)); Fig. 1, d2 and d3). Daytime values of melatonin and 5-ML content, Aa-nat mRNA, and AA-NAT activity were similar in both groups.

In Vivo Regulation of Melatonin Synthesis in the European Hamster Pineal Gland

To assess the contribution of norepinephrine in the daily regulation of melatonin synthesis, the effect of in vivo injections of adrenergic agonists and antagonists on pineal melatonin content was determined.

Propranolol (a β-adrenergic antagonist) injection in the early night significantly reduced nighttime melatonin content (Fig. 2A). This experiment was repeated once. In contrast, daytime injection of isoproterenol (a β-adrenergic agonist) did not increase pineal melatoni-
nin content (Fig. 2B) nor AA-NAT activity (vehicle: 105.6 ± 13.4 pmol·h⁻¹·gland⁻¹; isoproterenol: 150.3 ± 25.2 pmol·h⁻¹·gland⁻¹). To find out whether European hamsters may be responsive to an adrenergic agonist only at night, the effect of a nighttime injection of isoproterenol in June (natural LP when the nocturnal peak of melatonin is small) was tested. This nighttime isoproterenol injection further increased nocturnal melatonin content (Fig. 2C) and AA-NAT activity (vehicle: 282.2 ± 48.1 pmol·h⁻¹·gland⁻¹; isoproterenol: 445.6 ± 48.9 pmol·h⁻¹·gland⁻¹; \( P < 0.05 \)) up to values observed in November.

**In Vitro Regulation of Melatonin Release from European Hamster Pineal Cells**

To better understand the mechanisms that might be involved in the regulation of melatonin synthesis, various drugs were tested on dispersed pineal cells cultured for 3 days. None of the drugs [10 μM isoproterenol with and without 10 μM phenylephrine (α-adrenergic agonist); 1 mM DBCAMP with and without 1 μM PMA (PKC activator); 0.1 μM PACAP with and without 10 μM isoproterenol; 0.1 μM NPY with and without 1 μM isoproterenol; 0.1 μM Leu-enkephalin with and without 1 μM isoproterenol; and 0.1 μM somatostatin with and without 1 μM isoproterenol] incubated for 5 h with the pineal cells increased the melatonin release that stayed at a basal value of 60 pg/15.10⁴ cells (data not shown). Because the pineal cells may take longer to recover from the cell dissociation procedure, 10 μM isoproterenol was also tested on 5-, 7-, and 9-day-long cultures. Isoproterenol had no additional effect on these conditions.

**Ex Vivo Regulation of Melatonin Release from European Hamster Pineal Gland**

The loss of tissue integrity during pineal cell dissociation could explain the inability to stimulate melatonin synthesis in the previous experiments. Therefore, 8-h infusions of isoproterenol, DBCAMP, or Trp, the substrate for serotonin synthesis, were performed on ex vivo hamster pineal glands, sampled in the early morning and then settled in a perifusion system. Neither isoproterenol nor DBCAMP induced melatonin (Fig. 3A) or serotonin (Fig. 3B) release. Trp, in contrast, caused a marked increase in serotonin release (Fig. 3B) as well as a smaller increase in melatonin release (Fig. 3A).

**DISCUSSION**

The European hamster displays a marked variation in the duration and amplitude of the nocturnal melatonin peak in response to seasonal changes in the environment (73, 74). Here we demonstrate that these seasonal variations are mainly driven by Aα-nat gene transcription and enzyme activity, probably via the hypothalamic endogenous clock-controlled release of norepinephrine.

In addition to the European hamster, other rodents and nonrodent species also exhibit seasonal variations in melatonin synthesis (47, 72). In the Siberian hamster, the variation in melatonin peak duration was shown to be driven by AA-NAT activity (setting the on/off of nocturnal melatonin synthesis), whereas the variation in peak amplitude was driven by HIOMT (tuning the rate of melatonin synthesis with photoperiodic variation) (57). To assess whether this would also be the case in the European hamster, we assessed the daily variation in pineal metabolism under natural LP (June) and SP (November) conditions. The marked annual variation in the nocturnal peak of pineal melatonin content was confirmed with a small peak (5-fold increase at night; 4-h duration) in June and a large one (15-fold nocturnal increase; 9-h duration) in November, confirming our previous observations (73). Surprisingly, this variation was associated and probably dependent on a similar variation in Aα-nat mRNA and AA-NAT activity but not on a change in HIOMT activity. This result indicates that, in this species, AA-NAT is the limiting enzyme for the seasonal variation in
both the duration and amplitude of the nocturnal peak of melatonin. In support of an earlier study (73), the pineal indole 5-ML displayed a seasonal variation in daytime (but no nighttime) values, with higher daytime content of 5-ML in November. Because neither daytime AA-NAT nor HIOMT activities are significantly altered with the seasons, this 5-ML variation may depend on substrate availability/synthesis. This could be assessed by analyzing the seasonal variation in serotonin content or tryptophan hydroxylase activity.

To determine which neurotransmitters might be involved in the regulation of melatonin synthesis, various in vivo and in vitro experiments were carried out. A \( \beta \)-adrenergic antagonist was able to markedly reduce the nighttime increase in pineal melatonin as observed in many rodent (6, 11, 67) or nonrodent species (4, 37). Surprisingly, however, an acute injection of a \( \beta \)-adrenergic agonist had no effect on daytime pineal melatonin [which is in contrast to the rat (6)], whereas it was able to further increase the low nighttime melatonin content in the pineal of European hamster studied in June. These in vivo observations demonstrate the involvement of norepinephrine in the nocturnal stimulation of melatonin synthesis in the European hamster pineal and suggest a nocturnal gating of this effect. This gating hypothesis is strengthened by the inability of various adrenergic agonists and second messenger analogs to stimulate melatonin production in in vitro conditions. Similar gating has previously been reported in the Syrian hamster (52). Gating mechanisms may be explained by various mechanisms such as the presence of inhibitory factor(s) during the day and/or presence of stimulatory factor(s) during the night that still need to be investigated. Among the other neurotransmitters that might be involved in the regulation of melatonin synthesis, NPY is a likely candidate because it originates, like norepinephrine, primarily from the superior cervical ganglia and displays a marked seasonal variation in the European hamster pineal gland (36). Addition of various peptides in the presence or absence of a \( \beta \)-adrenergic agonist, however, could not induce melatonin synthesis and release from dissociated hamster pinealocytes, probably because of the gating mechanism suggested above. Other approaches (for example in in vivo experiments or on ex vivo pineal glands sampled at night) will be necessary to assess the putative role of other neurotransmitters in the regulation of melatonin in the European hamster.

Regarding the marked seasonal variation of melatonin in the European hamster, several points need to be addressed: 1) which environmental factors are the seasonal cues controlling this variation? 2) which structure(s) read and transmit the seasonal variation to the pineal gland? 3) which neurotransmitters are involved in the seasonal regulation of melatonin?

In this study, we first analyzed the seasonal variation of pineal metabolism in animals raised outdoors and therefore exposed mainly to changes in light and temperature. In addition, we compared the pineal metabolism of animals raised under SP indoors and outdoors. The overall pattern of pineal metabolism was similar between indoor and outdoor hamsters demonstrating that photoperiodic variation is the main driving force for the seasonal regulation of pineal activity. We observed, however, that the nocturnal peak of melatonin was lower under artificial SP (animals kept at 22°C) compared with natural SP (mean temperature of 4.9°C). This observation is not surprising because we previously showed that the maximal amplitude of the melatonin peak occurs in November-December (night duration of 15–16 h) in outdoor European hamsters (73) but at a schedule of 14:10-h light-dark cycle in indoor animals (74). Similarly, the amplitude of the melatonin peak under SP is higher in outdoor than in indoor Syrian hamsters (3). These results suggest that outside temperature may influence melatonin production as already implied by the observation that the amplitude of the melatonin peak is higher in European hamsters kept indoors at 10 or 20°C compared with 30°C (74). Surprisingly, we found no alteration of the amplitude of \( Aa-nat \) mRNA and AA-NAT activity peak associated with that of melatonin, suggesting that in these conditions, the “temperature-dependent” variation in melatonin peak amplitude is not driven by AA-NAT. In contrast, the mean HIOMT activity over 24 h was significantly lower under artificial SP (0.35 ± 0.05 nmol·h\(^{-1}\)·\( \mu \)g protein\(^{-1}\)) compared with natural SP (0.53 ± 0.03 nmol·h\(^{-1}\)·\( \mu \)g protein\(^{-1}\)). This finding is in agreement with earlier data reporting a significant decrease in pineal HIOMT activity in rats exposed to lower temperature (38). The higher HIOMT activity may account for the increase in melatonin peak amplitude observed in SP animals exposed to low outdoor temperature. It will be important to test this hypothesis by measuring pineal HIOMT activity in hamsters kept indoors under a constant photoperiod schedule but with varying temperatures. The effect of temperature is probably of physiological importance since lowering temperature has been reported to accelerate Syrian and Siberian hamster gonadal regression under SP (16, 28, 46). Other factors may also explain the difference in melatonin peak amplitude observed between outdoor and indoor SP hamsters. The indoor hamsters may not be in phase with their endogenous circannual clock (30) or they may have kept the memory of the previous photoperiod to which they were exposed (74).

The present in vivo experiments indicate a major role for norepinephrine in the daily regulation of melatonin synthesis, although the intracellular mechanisms still need to be determined. The seasonal variation in melatonin synthesis is probably also regulated by norepinephrine because the lower \( AA-NAT \) activity and melatonin content in June appear dependent on a reduced noradrenergic stimulation of melatonin synthesis. In addition, we recently reported that another neurotransmitter, NPY, exhibits a marked seasonal variation with maximal NPYergic innervation occurring in late autumn (36). This increase was associated with a significant increase in HIOMT activity and 5-ML production (56), suggesting that seasonal cues may be integrated by the pineal gland through various enzymatic steps by different neurotransmitters.
Although it is well established that the pineal gland is a primary site for building the seasonal endocrine message, it is not yet known where the seasonal information is encoded. Several recent studies suggest that the circadian biological clock may also be a seasonal clock (15, 45, 70) since light-induced FOS immunoreactivity in the SCN depends on photoperiod history (70, 75); clock gene expression in the SCN displays melatonin-independent photoperiodic variation (32, 33, 39) and the daily profile of vasopressin mRNA in the SCN differs in LP and SP (19). Our observation that the low nocturnal melatonin content in hamsters in June was markedly increased by an exogenous injection of a β-adrenergic agonist up to values similar to those observed in hamsters in November suggests that the low melatonin production in summer is due to reduced noradrenergic input. This finding would suggest a weaker SCN clock output to the pineal gland in summer compared with winter and thus implies a marked seasonal variation in SCN clock activity. In contrast to this hypothesis, however, is the observation of a stronger daily locomotor rhythm in summer compared with winter (76), which can be entrained by a light stimulus (47). It is also possible that, instead of the clock itself, efferent structure(s) between the SCN and pineal gland may integrate the seasonal variation in the environment. In addition, the contribution of specific pineal mechanisms to the seasonal variation in melatonin synthesis should not be excluded. Photoperiodic variation of a pineal inhibitory transcription factor known to reduce Aa-nat transcription has been demonstrated in the rat (8, 49, 65). In addition, in European hamsters studied in May-June, when the nocturnal peak of melatonin is very small, a marked daily variation of pineal serotonin content is still evident (48). First, this observation indicates that some aspect(s) of pineal rhythmicity are conserved (not altered?) in LP, and second, it suggests that there might be seasonal regulation of Aa-nat only, independent of serotonin. This would also explain the observation that in perfused hamster pineals, infusion of the serotonin substrate Trp induces a large increase in serotonin release compared with melatonin, whereas in the rat, Trp infusion induces both a large increase in serotonin and melatonin release (41, 42).

To establish whether the European hamster SCN clock is a site for integration of seasonal information that may drive the large seasonal variation in melatonin synthesis and release, it will be necessary to determine whether the daily rhythm in clock gene expression is markedly modified between summer and winter and to verify if other clock outputs such as corticosterone or leptin release (20, 22) exhibit similar large seasonal variations.

In conclusion, this study shows that the large photoperiodic variation in melatonin synthesis and release in the European hamster is driven primarily by Aa-nat gene transcription and enzyme activation but does not exclude the participation of HIOMT, which may relay temperature information, in the regulation of the melatonin peak amplitude. The possibility that this seasonal variation depends on seasonal alteration of the SCN clock circadian activity and is transmitted by norepinephrine requires further investigation.

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