

N-acetyltransferase is not the rate-limiting enzyme of melatonin synthesis at night

Abstract: Circadian melatonin production in the pineal gland and retina is under the control of serotonin N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase. Because NAT activity varies diurnally, it has been considered both the melatonin rhythm-generating enzyme and the rate-limiting enzyme of melatonin synthesis. In rats with dramatically reduced NAT activity due to a H28Y mutation in NAT, melatonin levels remained the same as in wildtype controls, suggesting that NAT does not determine the rate of melatonin production at night. Using a combination of molecular approaches with a sensitive *in vivo* measurement of pineal diurnal melatonin production, we demonstrate that (i) N-acetylserotonin (NAS), the enzymatic product of NAT, is present in vast excess in the night pineals compared with melatonin; (ii) the continuous increase in NAT protein levels at late night does not produce a proportional increase in melatonin; and (iii) an increase in NAS in the same animal over several circadian cycles do not result in corresponding increase in melatonin output. These results strongly suggest that NAT is not the rate-limiting enzyme of melatonin formation at night.

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Introduction

Melatonin, the principal secretory product of the pineal gland, is synthesized and secreted by pineal cells with a dramatic circadian rhythm. This rhythmic secretory pattern is conserved in virtually every vertebrate species, and is the result of the precise daily regulation of the melatonin biosynthetic pathway [1, 2]. Melatonin production is catalyzed by two well-characterized enzymatic reactions. First, serotonin, produced at extremely high levels in the pineal gland [3], is converted to N-acetylserotonin (NAS), by the enzyme serotonin N-acetyltransferase (NAT). NAS is subsequently methylated by hydroxyindole-O-methyltransferase (HIOMT) to form melatonin [1, 4].

Early studies investigating the mechanism of melatonin production focused on *in vitro* characterization of the enzymes responsible for its biosynthesis. HIOMT was more easily characterized, principally because it lacks a dramatic diurnal variation in protein and mRNA levels and is relatively stable and thus amenable to biochemical purification. The gene encoding HIOMT was eventually isolated by Ishida et al. [5] by expression cloning. Although molecular studies of HIOMT were made possible early by availability of its sequence, much attention was placed on NAT because of its distinct circadian pattern of enzyme activity. NAT activity peaks during the night, coinciding with the nocturnal production of melatonin [6]. Based on its circadian activity profile, NAT was proposed to be the rate-limiting enzyme in the production of melatonin [7], which is echoed by many others in the field [8–31].

Identification of the gene for rat NAT [27] enabled us to study the precise regulation of NAT gene transcription.

Northern blotting for NAT mRNA demonstrated that production of this pineal-specific transcript is extremely tightly regulated. Remarkably, the NAT mRNA expression and activity overlap over time. Based on this gene expression data and prior studies demonstrating parallel pattern of NAT activity and melatonin formation [32], transcriptional control of NAT appears highly likely to determine the temporal time course of melatonin production in rodents.

Subtractive hybridization analysis of the pineal gland identified many diurnally expressed molecules, which include pineal night-specific ATPase (PINA) [33], patched 1 [34], 3OST2 [35], and many others (J. Borjigin, unpublished data). PINA is an alternative transcript [36] produced from the ATP7B [37] gene that is defective in human patients with Wilson disease (WD) [38]. Long Evans cinnamon (LEC) rat, an animal model for WD [39], contains a large deletion in the C-terminal part of ATP7B [40] and is defective in PINA expression (J. Borjigin, unpublished data).

To understand role of PINA in pineal physiology and WD pathogenesis, we analyzed the pineal gland of LEC rats and discovered abnormally low levels of NAT protein contents and enzyme activity [41]. Further molecular analysis of the LEC pineals identified a point mutation in NAT gene that leads to change of a conserved histidine residue to tyrosine at amino acid position 28. The H28Y mutation was shown to be the cause of low NAT protein expression *in vivo* when segregated away from PINA defects in a new strain of rats. Moreover, the NAT-H28Y protein is less stable when produced in both bacteria and mammalian cells *in vitro* [41]. In this paper, we demonstrate

that melatonin levels in LEC rats are remarkably unaffected by a 10-fold reduction of NAT enzyme activity.

To investigate the role of NAT in melatonin production, we utilized *in vivo* microdialysis for quantitating NAS and melatonin levels in the rat, and correlated these *in vivo* findings with molecular analysis of the pineal. This system was originally developed by Azekawa et al. [42] and automated by Drijfhout et al. [43] using fluorescent detection. Our system utilizes novel minimally invasive surgical implantation of the dialysis probes that allows the long-term (more than 2 wk) stable measurement of pineal secretory products in real time (every 10 min) in individual rats [44]. In this system, we are able to simultaneously measure both NAS and melatonin, thus establishing sensitive, quantitative measurements of the metabolic intermediate and end product of the melatonin synthetic pathway *in vivo*. The system is reproducible and robust, and we are able to obtain identical data from day to day within the same individuals, providing excellent internal controls for experiments. Finally, the data are obtained in real time every 10 min. This is critical in studying circadian processes that can change dramatically over short periods of time [3, 44].

Using on-line pineal microdialysis, we show that NAS, the enzymatic product of NAT, is present in the night pineal gland in vast molar excess compared with melatonin. Our data also reveals that even though NAT protein levels continuously increase during the second half of night, melatonin levels peak much earlier at night. Furthermore, melatonin levels do not vary even when NAS levels increase by more than two-fold in the same animal. Taken together, these data suggest that NAT is not the rate-limiting enzyme in nocturnal melatonin synthesis, and strongly implicate the role of HIOMT as the rate-limiting enzyme of melatonin production at night.

Material and methods

Animals

Adult Sprague–Dawley (SD) male rats were purchased from Harlan (Indianapolis, IN, USA), LEC rats from Charles River (Tokyo, Japan), and Long Evans (LE) rats from Charles River (Wilmington, MA, USA). Rats were housed at 20–25°C with lights on at 06:00 hr [light:dark (LD) 12:12 hr] to measure NAT activity and melatonin. SD rats used for Western analysis and *in vivo* microdialysis were housed in LD 14:10 conditions with lights on at 11:00 hr. Food and water were available *ad libitum* throughout the experiment. Illumination was supplied by white fluorescent lamps (400 lux at cage level). Rats were placed in the LD cycles for at least 2 wk before experiments.

NAT enzyme assay

The NAT activity assay was performed according to the published methods [45]. Briefly, single pineal glands isolated from night (02:00 hr) rats were homogenized individually in 100 μ L of 50 mM phosphate buffer (pH 6.8) on ice. One-tenth of the protein extracts was mixed with trypta-

mine, acetyl CoA, and acetyl 14 C-CoA, and incubated at 37°C for 20 min. The reaction was stopped by addition of 0.2 M borate buffer, pH 10, and the labeled product extracted with 1 mL of chloroform. A portion (500 μ L) of the lower organic phase was transferred to new scintillation vial after two extractions with 0.2 M borate buffer, evaporated to dryness, and radioactivity measured in 10 mL of scintillation fluid after vigorous mixing.

Western blot analysis

The pineal glands were isolated under dim red light from SD rats killed at indicated hours. Total protein extracts from one-tenth of a single gland were loaded in each lane, blotted onto a nitrocellulose membrane, and subsequently probed with a NAT polyclonal antiserum developed in our lab (against N-terminal 15 amino acids). The nonspecific upper bands in each lane (Fig. 3) serve as convenient loading controls.

Melatonin measurement

Pineal total melatonin levels were measured using a modification of the procedure developed by Champney et al. [45]. Pineal glands isolated under dim red light at night (02:00 hr) were sonicated individually in 100 μ L of chilled 50 mM sodium phosphate buffer, pH 6.8 for < 10 s. Half of the volume (50 μ L) was stored at –70°C for NAT assay (see above) and HIOMT assays (not shown) and Western blotting (not shown), and the remaining 50 μ L was mixed with 450 μ L of chilled buffer by vortexing. Duplicate 200 μ L aliquots of the sample were mixed with 2 mL of chloroform in a glass tube and vortexed vigorously. The lower organic phase was extracted once more with 500 μ L of 0.1 M carbonate buffer (pH 10) after the upper aqueous phase was removed. One milliliter of the organic phase (chloroform) was transferred to a new tube and allowed to dry overnight in a fume hood. A quantity of 200 μ L of methanol was used to dissolve the dried samples and one-tenth of which (20 μ L) was injected into a high-performance liquid chromatography (HPLC) column [reversed phase C18 column from Supelco (Bellefonte, PA, USA)] for melatonin measurement using fluorescence detection as described below.

Surgery

The animals were deeply anesthetized with the combination of ketamine (10 mg/mL, 0.5 mL/100 g weight, *i.p.*) and xylazine (2 mg/mL, 0.5 mL/100 g weight, *i.p.*). The head of the animal was shaved and positioned in a stereotaxic instrument. A longitudinal incision (approximately 2.5 cm) is made along the midline in the skin from the base of the skull. The sagittal and lambdoid sutures were then exposed by scraping away the temporal muscles to the periosteum. A circular skull opening (6.8 mm in diameter), centered midline 1.5 mm posterior to the confluence of the superior sagittal and transverse sinuses, was created using a dental disk drill equipped with a shank diamond wheel point (6.8 mm OD, Dremel, Racine, WI, USA). The dura matter was exposed after grinding away the top bone, which was

removed using iris forceps after making a cross cut with a scalpel blade. The underlying membranes were cut open with a scalpel in a T-shaped line in the area below the confluence of the superior sagittal and transverse sinuses, and deflected laterally with two iris forceps. Three stainless steel screws were placed nearby in the parietal and frontal bones to serve as anchors. After the animal's nose was repositioned 30° downward, the dura mater below the confluence of sinuses was then lifted 5 mm with a hook fixed to the stereotaxic frame. The dorsal cerebellum was then pressed downward with a glass blunt tube to expose the pineal gland. The tentorium cerebella, which covers the posterior surface of the pineal and connects to the confluence of the sinuses, was then carefully reflected to visualize the pineal with a micro-hook. The straight sinus is visible in the midline of the pineal posterior surface, which divides the pineal gland into two parts and drains into the confluence of the sinuses. The tip of the guide cannula (CMA/MD, N. Chelmsford, MA, USA) was then positioned adjacent to either side of the exposed pineal before the skull was closed with dental cement. The rats were allowed to recover 24 hr before experimentation.

Microdialysis

Pineal microdialysis was carried out as follows. Immediately before sampling, the rat was anesthetized with halothane briefly; the stylet (or dummy probe) was replaced with a microdialysis probe (CMA12, 20 kDa cut-off, membrane length 4 mm) (CMA/MD) and fixed with plastic glue. The dialysis probe was continuously infused via the microbore PEEK tubing (0.65 mm OD, 0.12 mm ID) at a flow rate of 2 μ L/min with artificial cerebral spinal fluid (CSF; Harvard Holliston, MA, USA). Samples were collected at 10-min intervals via the PEEK tube into a 20- μ L loop of Pollen 8 automatic injector (BAS, West Lafayette, IN, USA), which was on-line with the HPLC system. The sample loop was set to be retained in the load position during the 10-min cycles and was automatically switched to the injection position briefly, after which the cycle was repeated. The rats were linked to the apparatus for dialysis through a quartz dual channel swivel (Instech, Boston, MA, USA) to prevent the tubing from entanglement.

HPLC analysis

The analytical condition for the simultaneous detection of NAS/melatonin was based on Drijfhout et al. [43] with minor modifications. A Shimadzu pump (Shimadzu, Columbia, MD, USA) was used in conjunction with a Shimadzu fluorescence detector (FD, excitation: 280 nm, emission: 345 nm). Samples were injected into the system through a Valco injection valve with a BAS controller and subsequently separated on a reserved phase C18 column (250 \times 4.6 mm; Supelco), set at a constant temperature of 30°C using a Shimadzu column heater, controlled by a Shimadzu system controller. The mobile phase consisted of a mixture of 10 mM sodium acetate, adjusted to a pH of 4.5 with concentrated acetic acid, 0.01 mM Na₂-EDTA, 500 mg/L heptane sulfonic acid and 22% (v/v) acetonitrile.

The flow rate of the HPLC pump was set at 1.5 mL/min throughout the experiment. Standard solutions of NAS and melatonin were used to calibrate the system. The automated control of the HPLC system, the programming of the flow rate, and the handling and storage of the chromatograms were carried out with an external computer using the Shimadzu Class-VP 5.03 chromatography software.

Results

As LEC rats lack PINA, the only other night-specific and pineal-specific molecular identified to date besides NAT, we reasoned that PINA contributes to melatonin synthesis and PINA defects may result in deficiencies in melatonin production. The night pineal glands of LEC and LE rats were assayed for NAT activity and melatonin production (Fig. 1). NAT enzyme activity (open bars) and melatonin levels (black bars) were measured at 02:00 hr. The average NAT activity was 0.13 nmol/pineal/hr for LEC rats (n = 15), 1.4 nmol/pineal/hr for LE rats (n = 14). Melatonin levels of the same cohorts of rats were 1.91 μ g/pineal for LE rats (n = 14) and 1.89 μ g/pineal for LEC rats (n = 15). Thus, a 10-fold reduction in NAT activity did not result in corresponding change of melatonin levels in LEC rats.

Using pineal microdialysis, we found that NAS levels in SD rats are much higher than melatonin levels during the night (Fig. 2). This is true not only for the released NAS and melatonin, but also for total intracellular NAS and melatonin content (not shown). The results shown in Fig. 2 demonstrate a typical tracing from an individual rat 4 days after placement of a microdialysis probe in the pineal. During the day, there is very little production of melatonin or NAS. However, at night, there is a dramatic increase in the amount of NAS and melatonin produced by the pineal. The peak of NAS and melatonin production is achieved within 3 hr of the onset of darkness. Examination of the data in detail (Fig. 2, bottom panel) reveals that melatonin levels never exceed NAS production even early (02:00–04:00 hr) in the circadian cycle. We analyzed more than 300 SD rats (both male and female) and found that in all cases NAS always exceeded melatonin output within 1–2 hr of lights-off at night (data not shown). In addition to SD rats,

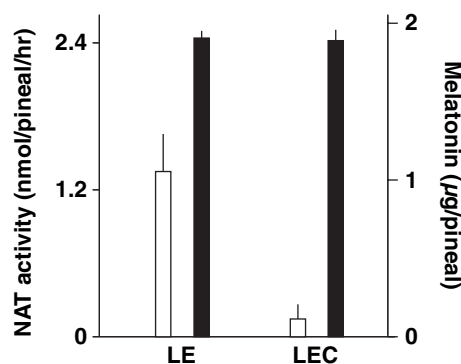


Fig. 1. Melatonin levels in LEC rats. The open bars denote NAT activity, while the black bars indicate levels of melatonin. Results shown were the average of 14 (LE) and 15 (LEC) adult (2 months of age) male rats.

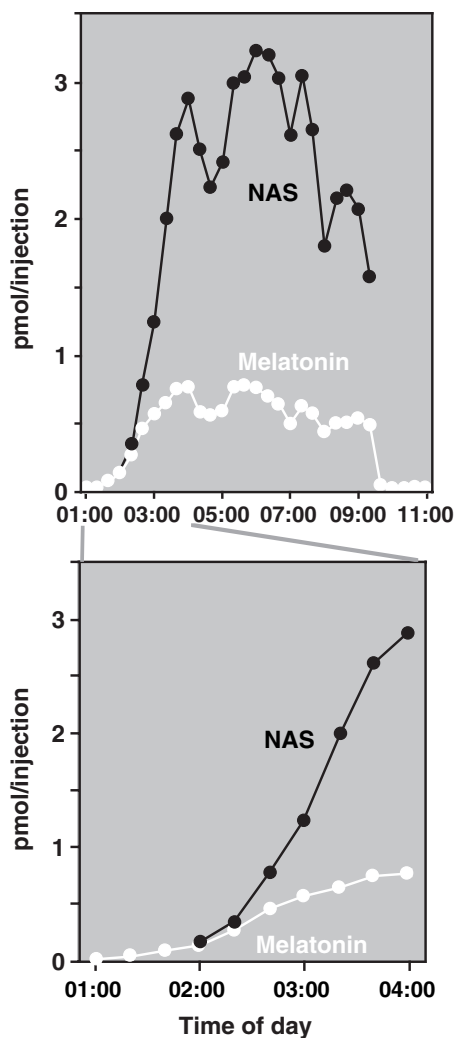


Fig. 2. Real-time determination of pineal N-acetylserotonin (NAS) and melatonin output in SD rats. The upper panel shows a typical tracing of the amount of NAS and melatonin secreted by a rat pineal as a function of time (x-axis; only night value is shown). The amount of product (y-axis; given in fmol detected per 10 min collection period) is calculated by measuring total fluorescence in the appropriate HPLC fraction, standardized to known amounts of NAS and melatonin run in separate experiments. These data represent measurements from a single night in a single rat. NAS and melatonin are shown to peak rapidly at night and remain steady for hours before dropping in the morning. The response was highly reproducible in the same rat. The diurnal timing of secretion is extremely consistent between rats. However, there are differences in the peak amount secreted between rats, due to probe placement variation. In the bottom panel, the early rise in NAS and melatonin is shown in detail to demonstrate that even at very early times, NAS concentration exceeds melatonin.

the same results were also obtained for LE, LEC, PVG, F344, Wistar, and Lewis rats (data not shown).

We reasoned that if NAT is truly the rate-limiting step in melatonin synthesis, the time course of NAT protein production should follow the same general time course as melatonin production measured by microdialysis. That is, there should be a parallel increase in melatonin level with elevation of NAT protein concentration. NAT protein levels determined by Western blotting (Fig. 3) increased

notably from 02:40 to 03:00 hr (lower panel) that coincides with increase of melatonin output. However, NAT protein continues to exhibit dramatic increase from 04:00 to 08:30 hr, before diminishing (lower bands in upper panel). Surprisingly, these patterns do not follow the pattern of nocturnal melatonin levels demonstrated in microdialysis experiments (Fig. 2), where melatonin output peaked by 04:00 hr. The finding that increased NAT in the latter portion of the night fails to elevate melatonin levels provides the third clue that NAT is not the rate-limiting step in melatonin biosynthesis. Rather, HIOMT conversion of NAS to melatonin appears to be the rate-limiting step during the night.

As mentioned, *in vivo* microdialysis provides excellent information on NAS and melatonin output. These data are highly reproducible from day to day after the animal has adapted to the microdialysis probe. At the very beginning of the experiment, however, we consistently note that the pineal has decreased NAS production for the first 2–4 days of measurement. Fig. 4 shows a set of measurements of NAS and melatonin over the initial period of measurement for a single rat, which is reproducibly found in most rats

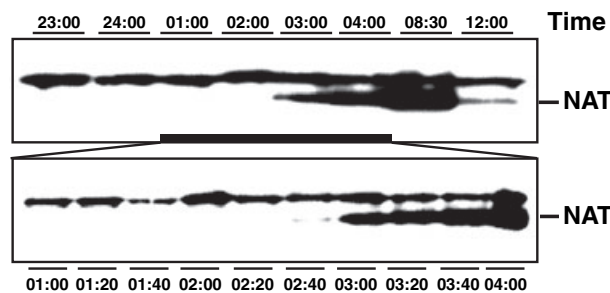


Fig. 3. Circadian rhythm of NAT protein levels. Sprague-Dawley rat pineal glands were collected at indicated time points and cell extracts from one-tenth of a single pineal were analyzed by Western blotting using a NAT-specific antiserum. The NAT proteins are detectable at 03:00 hr and increase steadily until 08:30 hr. A very faint amount of protein is detected at 12:00 hr, 1 hr after lights-on. The NAT band is the lower band. The upper band is nonspecific, used conveniently as a loading control.

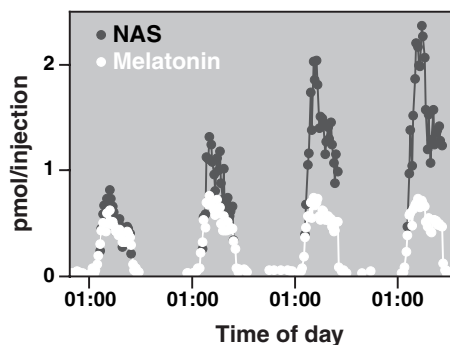


Fig. 4. Simultaneous measurements of NAS and melatonin in the initial days of long-term pineal microdialysis. The traces show four consecutive days of NAS and melatonin measurement in a single SD rat. The total amount of melatonin is much lower than NAS, even immediately after the probe placement. Surprisingly, even though NAS increased steadily over the first 4 days, the melatonin levels failed to change.

analyzed. On day 1, NAS peaks at 850 fmol/10 min, and on subsequent nights, NAS rises gradually to over 2000 fmol/10 min, where it stabilizes for more than a week (not shown). If NAT were the rate-limiting enzyme in melatonin synthesis, one would expect that melatonin increases over the initial 4 days of the experiment, in parallel with NAS increases. In fact, the levels of melatonin are nearly identical throughout the measurement period. This is the fourth piece of evidence that suggests that HIOMT, and not NAT, limits the amount of melatonin synthesized at night.

Discussion

In this paper, we provide four lines of evidence to suggest that NAT does not determine the rate of melatonin production in the living pineal gland at night: (i) the decrease of NAT activity of more than 10-fold did not result in a corresponding reduction in melatonin production; (ii) NAS is present in vast molar excess compared with melatonin within 1 hr of lights-off; (iii) while NAT protein levels continue to increase throughout the night period, melatonin output reaches peak levels within 3 hr of lights-off; and (iv) continuous increase of NAS over consecutive circadian cycles within the same animals in vivo do not produce proportional rise in melatonin output. These findings suggest that modulation of NAT activity over a wide dynamic range may not affect melatonin production.

These data strongly suggest that HIOMT enzymatic activity determines the level of melatonin synthesis in the pineal at night. In this sense, HIOMT, not NAT as previously described, should be considered the rate-limiting enzyme in the synthesis of melatonin during the night. NAT activity is indeed required for melatonin synthesis, and without NAT expression, there is clearly no production of melatonin [46]. However, NAT activity, once turned on, produces such high levels of NAS that is beyond the enzymatic capacity of available HIOMT. Fluctuations in NAT activity and NAS content therefore fail to influence end-product levels. Thus activation of NAT is rate limiting only during the daytime and in the beginning phase of the night (during the first hour of night) when melatonin production is initiating; for the bulk of the night period, HIOMT is the rate-limiting enzyme in vivo.

Identification of HIOMT as a rate-controlling enzyme at night may have important implications for pharmacological treatment of sleep disorders. Drugs that are intended to modulate melatonin levels should be targeted not only to NAT but also against HIOMT. Inhibitors of NAT may be useful in the prevention of melatonin synthesis in the early night, but may be less effective in the late night. Rather, activation or inhibition of HIOMT could be a more effective means of controlling the levels of melatonin synthesis during the bulk of night period.

Our studies also reveal that there is a vast excess of NAT in the pineal at night, resulting in the production of a large quantity of NAS at night. The biological significance of NAS is not clear, but its abundance and circadian rhythm of expression suggest a role in diurnal rhythms. Excessive NAT and NAS ensure that modulation of HIOMT can markedly influence the pineal output of melatonin. HIOMT expression has been shown to have a diurnal rhythm in pineals [47]

whose activity is influenced by a complex set of factors [48, 49]. Indeed, photoperiodic changes in the amplitude of the melatonin peak are driven by HIOMT instead of NAT [50]. While NAT clearly is the diurnal determinant of the melatonin production, the amplitude of nightly melatonin production is most likely controlled by HIOMT in rodents.

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