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Artificial Induction of Melatonin Rhythms by Programmed Microinfusion

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Abstract. Melatonin was slowly and intermittently infused into rats via an apparatus that allowed it to be discharged from a subcutaneous implant according to a predetermined temporal program. We found that infusion of an aqueous solution of melatonin, mixed with a dye, or of an immiscible fluid lacking melatonin or dye, could be monitored by measuring levels of melatonin or of dye in the rats' urine. We observed a 24-hour rhythm in melatonin excretion which corresponded to the times of its infusion by the apparatus. Approximately 0.1% of infused melatonin was recovered unchanged in the urine. This method of administering exogenous melatonin may facilitate explaining the physiological significance of rhythms in its secretion and rhythms in plasma levels of other hormones.

The rate at which the mammalian pineal synthesizes melatonin *in vivo* has been estimated indirectly by measuring pineal melatonin concentration [10, 21] and the *in vitro* activities of pineal enzymes that catalyze its biosynthesis [7, 27]. It has been inferred that melatonin production varies rhythmically, with its phase and period fixed by the prevailing diurnal lighting schedule. Recently developed analytical methods that permit the assay of melatonin in tissues and body fluids [2, 12] make it possible to demonstrate that rhythmic melatonin biosynthesis is indeed accompanied by rhythmic secretion of the hormone into the blood [11, 20] and cerebrospinal fluid [5] and by its rhythmic excretion into the urine [14].

A number of investigators have shown that cycles in melatonin availability may be more important in mediating its physiological effects than are the average levels of melatonin in the blood. *Fiske and Huppert* [4] discovered that exogenous melatonin can influence the diurnal rhythm of serotonin concentration characteristic of the pineal gland, but that its action is affected by the recipient's photic environment. *Tamarkin et al.* [23] observed that melatonin could induce marked gonadal regression in the hamster if administered daily during a very restricted portion of the light phase of the light-dark cycle. *Reiter* [22] reviewed the literature describing effects of exogenous melatonin and showed that depending on the mode and timing of its administration, both anti- and counterantigonadotrophic effects of the indolamine could be elicited.

These findings all suggest the probable usefulness of new research strategies to allow experimental evaluation of phase relationships between the rhythmic secretion of melatonin and other processes possibly affected by melatonin. This report describes a new method for experimentally inducing rhythms in plasma melatonin levels and presents additional evidence that urinary melatonin content, sampled at frequent intervals, is a good index of plasma melatonin.

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Materials and Methods

Experimental Animals

Animals used in these studies were intact and surgically prepared (pinealectomized or sham-operated) male Sprague-Dawley rats weighing 150–200 g (Zivic-Miller Laboratories, Inc., Allison Park, Pa.). Rats were housed individually in metabolism cages and given free access to food (Charles River Rat, Mouse, and Hamster Original Formula) and water. Unless otherwise indicated, they were exposed

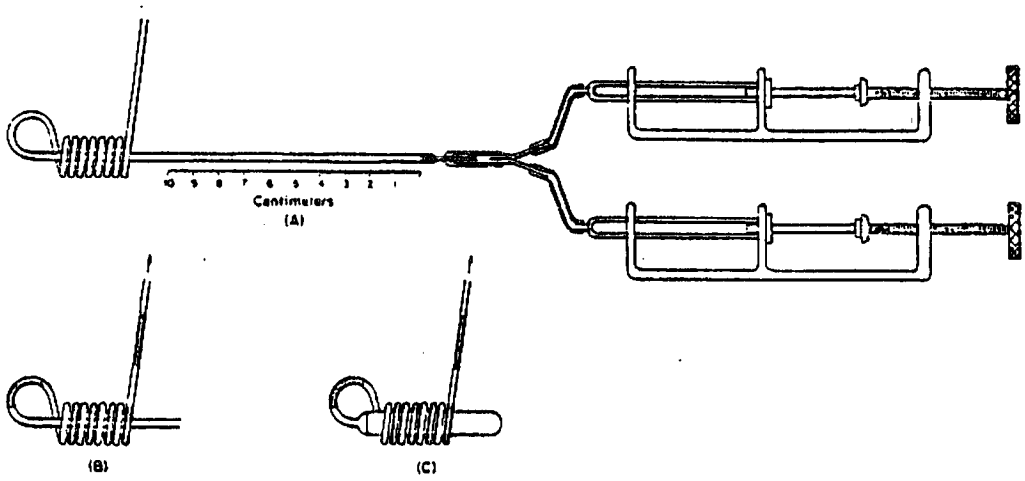

Fig. 1. Programmed microinfusion apparatus. A Individual components of the infusate program are forced from microsyringes, via a manifold, into the straight feeder portion of a thermoformed capillary tubing forming the linearly arrayed program. B The program is then driven, with additional vehicle, into the coiled portion of the tubing. C The feeder portion of the tubing is cut off, a saline-filled osmotic minipump is attached, and the assembly is ready for implantation.

to 12 h light per day, from 09.00 to 21.00. 'Cool white' fluorescent tubes yielding 100 $\mu W/cm^2$ at the animals' level provided the light. Urine specimens were collected during the indicated time intervals and stored frozen until they could be assayed for melatonin content. Pinealectomized animals were examined when killed to establish that the pineal was completely removed.

Temporal Pattern of Rat Urine Production

To characterize the temporal pattern of urine production in rats maintained under diurnal lighting (LD, 12:12), 2 intact rats (400–450 g) were housed in metabolism cages mounted over an automatic fraction collector. For 4 consecutive days, collecting tubes under each cage were changed every 10 min. We noted the time of appearance and the volume of each urine specimen.

Programmed Microinfusion of Melatonin

To infuse melatonin into a rat according to a predetermined temporal program, an Alzet Osmotic Minipump [24] (Alza Corp., Palo Alto, Calif.) filled with physiologic saline discharged the contents of a subcutaneously implanted capillary tube that had been precharged with a linearly arrayed infusate program. The program consisted of alternating segments of a melatonin solution (melatonin dissolved in phenolsulfonphthalein (PSP) solution; Hynson, Westcott & Dunning, Inc., Baltimore, Md.) and an immiscible control fluid (melatonin-free light mineral oil). The minipump, designed to discharge at the rate of 1 μl/h when implanted subcutaneously, was attached to the program-containing capillary tubing by a catheter flow moderator (supplied with the pump). Thus, as the contents of the tubing were gradually displaced by saline solution from the pump, the various components of the programmed infusate were discharged sequentially from the opposite end of the tubing. Because the melatonin was in PSP solution, its infusion could be correlated with the appearance of this red indicator substance in the urine.

The tubing, thermoformed into a spiral coil (to facilitate implantation) was attached to a 3-way manifold which was, in turn, attached via capillary tubing to two screw-driven microsyringes, each containing one component of the infusate program (fig. 1A). The tubing used (polyethylene, PE-60 Intramedic; Clay Adams, Parsippany, N.J.) was transparent and of uniform bore so that infusate could readily be programmed in the tube's straight 'feeder portion' by referring to an external scale (e.g., a conventional metric ruler). The linearly arrayed infusate program was then forced into the coiled portion of the tubing (fig. 1B), and the 'feeder portion' of the tubing was cut off. A saline-filled minipump was then attached via the catheter flow moderator (fig. 1C). In this configuration, the programmed microinfusion assembly was primed (i.e., flow initiated) in a saline bath at 30°C. Immediately before implantation, the uncoiled portion of the tubing was adjusted to a suitable length to discharge the infusate at a remote subcutaneous site. The entire assembly was then implanted subcutaneously into lightly ether-anesthetized rats through a 2-cm dorsal midline incision.

Infusion of Graded Doses of Melatonin

To assess the relationship between the amount of melatonin infused subcutaneously and the amount recovered unchanged in the urine, we prepared programmed microinfusion assemblies, as described above, that would deliver graded doses of melatonin in PSP solution (0–10 μg/6 h) clearly separated by intervals during which melatonin-free light mineral oil would be infused. Two pinealectomized rats weighing 400–450 g were equipped with such implants and then housed in metabolism cages mounted over an automatic fraction collector. Sequential 1-hour urine samples were collected from each animal for 10 days. The urine collections were reduced to 12-hour pools, according to the presence or absence of PSP, to yield specimens corresponding to the various components of the program. Melatonin extracted from each pool was measured by RIA.

Rhythmic Infusion of Melatonin

3 pinealectomized and 3 sham-operated rats weighing 540–750 g that had been housed under constant light for 105 days, were equipped with microinfusion implants programmed to provide rhythmic infusions of 10 μg melatonin in PSP solution for 6-hour intervals, separated by 18-hour infusions of melatonin-free light mineral oil (i.e., yielding a circadian period). Each animal was maintained in a metabolism cage under continuous light, and sequential 6-hour urine specimens were collected; 12-hour urine pools from each animal were prepared according to the presence or absence of PSP in the urine (fig. 3), and their melatonin contents measured.

RIA of Urinary Melatonin

The rat urine pools were extracted directly into chloroform. The organic extracts were then subjected to aqueous washes and evaporated to dryness. The residue was dissolved in ethanol and the mela-

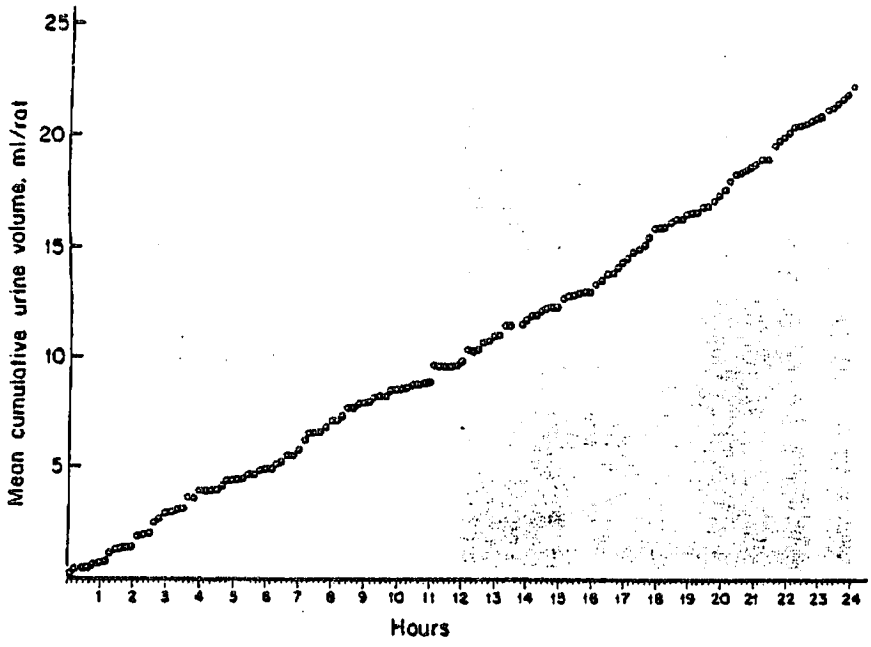

Fig. 2. Urine excretion by rats. Sequential 10-min urine samples were collected from each of 2 rats over a 4-day period. The shaded area represents the 12-hour dark period.

tonin, isolated by thin-layer chromatography, was measured by RIA [9, 12]. This assay method's specificity depends on the physical isolation of melatonin from urine extract [19]. To maximize specificity in the present studies, a narrow band (4–5 mm wide) was excised from the silica gel thin-layer plate, after chromatography, using corresponding chromatographic displacement of visually detectable quantities of authentic melatonin mixed with urine extract as a reference. Recoveries of authentic melatonin added to urine samples were 50–55%; melatonin values reported here were corrected for recovery.

Results

Temporal Pattern of Rat Urine Production

To determine when and how frequently rats normally urinate when maintained under diurnal lighting, urine excretions of two rats were measured at 10-min intervals for 4 consecutive days. Figure 2 represents the mean cumulative urine volume excreted per rat during 24 h. These results substantiate earlier observations [13] on a larger population of comparable animals: although the rats consume 3–5 times as much water at night, only 55–58% of the total daily urine volume is excreted during the 12-hour dark period and 42–45% during the 12-hour light period. Furthermore, rats urinate at frequent intervals throughout the day, 7–9 times during the light period, and 13–16 times during the dark period.

Recovery of Infused Melatonin

Table I summarizes the relationship between the amounts of infused and of unmetabolized melatonin recovered in the urine. The portion of subcutaneously infused authentic melatonin that appeared unchanged in the urine was approximately 0.1%. Over a 10-fold variation in doses, however, an excellent correlation was observed between the quantities of melatonin infused and those recovered from the urine ($r = 0.99$, $p < 0.01$).

Table I. Recovery of graded infusions of melatonin in the urine of pinealectomized rats maintained in constant light

Melatonin infused, ng/segment	Melatonin recovered in corresponding urine pools, ng/pool			Melatonin recovered, %
	rat 1	rat 2	mean ± SE	
0	0.2	0.3		
1,000	1.8	1.6	1.7±0.1	0.15
2,500	2.9	2.6	2.8±0.2	0.10
5,000	5.9	4.1	5.0±0.9	0.10
10,000	9.8	10.6	10.2±0.4	0.10

Basal levels of melatonin excretion (ng/12 h) before implantation of the programmed microinfusion apparatus, between melatonin infusions, and after the infusate program was exhausted, were: rat 1, 0.21 ng/12 h; rat 2, 0.25 ng/12 h (SE = 0.02).

Rhythmic Infusion of Melatonin

Programmed microinfusion assemblies designed to provide circadian cyclic infusions of 10 μg melatonin in PSP solution were implanted in each of three pinealectomized and three sham-operated rats previously maintained for

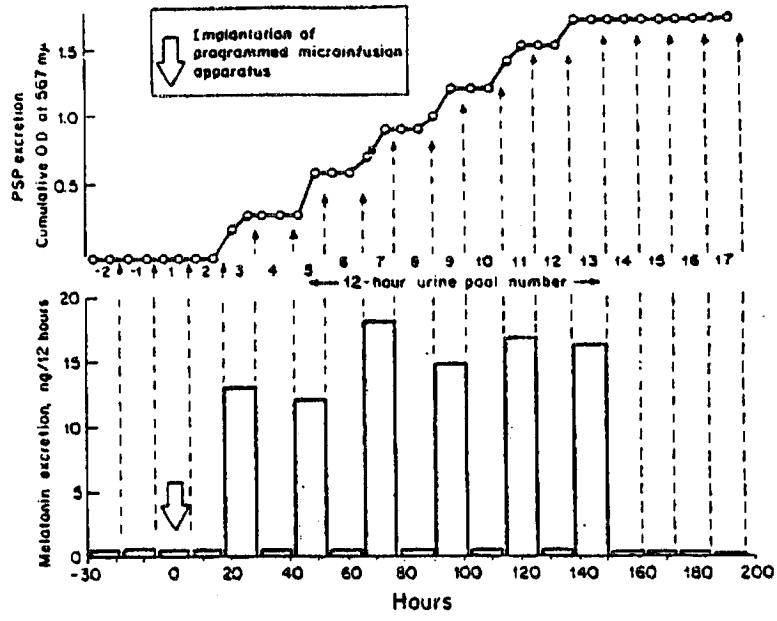

Fig. 3. Rhythmic infusion of melatonin. A programmed infusate, consisting of 10 μg melatonin in PSP solution alternating with melatonin-free mineral oil, was implanted in a pinealectomized rat (rat C). 6-hour urine samples were collected and pooled according to the cyclic appearance of PSP, and the melatonin content of the urine samples was measured.

Table II. Recovery of melatonin in the urine of pinealectomized and sham-operated rats maintained in constant light

	Urinary melatonin concentrations, ng/12 h		
	basal levels[1]	melatonin infusion[2]	body weight, g
Pinealectomized			
Rat A	0.26 ± 0.02	13.69 ± 0.79	540
Rat B	0.28 ± 0.02	9.38 ± 1.24	610
Rat C	0.28 ± 0.02	15.33 ± 0.95	745
Sham-operated			
Rat F	0.60 ± 0.04	18.93 ± 1.45	750
Rat G	0.26 ± 0.02	9.62 ± 0.54	615
Rat H	0.20 ± 0.01	12.00 ± 0.72	565

Each animal received six 10-μg melatonin infusions on a circadian schedule (see fig. 3, rat C). Basal levels of melatonin excretion are based on the amounts of melatonin in 12-hour urine collections taken before, between, and after the infusions.

[1] 12-hour periods during which melatonin-free oil was infused and no phenolsulfonphthalein (PSP) appeared in the urine (mean ± SE).

[2] 12-hour periods during which phenolsulfonphthalein (PSP) appeared in the urine.

105 days under continuous light. (Pinealectomy or exposure to continuous environmental illumination abolishes the daily rhythm of endogenous melatonin excretion [13, 16].)

Urine specimens from each animal were collected every 6 h for 7 days. The urine samples were adjusted to uniform volume and made alkaline by adding sodium hydroxide solution. The optical density of each sample, measured at PSPs absorption maximum of 567 nm, yielded a 'stair-step' curve when plotted cumulatively against time (fig. 3). The magnitudes and slopes of the rises reflect the quantities and rates, respectively, of melatonin infusion during the melatonin-in-PSP segments of the infusate program, while the tread lengths reflect the durations of melatonin-free oil segment infusion.

The 6-hour urine collections from each animal were reduced to 12-hour pools and their melatonin contents measured (fig. 3; table II). Several 12-hour urine specimens (some collected before the programmed infusion apparatus was implanted and some collected after the infusate program was exhausted) were also analyzed for melatonin content, to document the absence of endogenous variations in the melatonin excretion rate and to establish the 'basal level' of melatonin excretion from these animals.

With the exception of rat F, the 'basal level' of melatonin excretion (0.2–0.3 ng/12 h in all animals) approaches the RIAs sensitivity limit, as it was applied in these studies. These levels might more precisely be called 'background measurements', since we cannot be sure that they entirely reflect the authentic methoxyindole. In previous studies, however, comparable amounts of melatonin were found in 12-hour urine collections from pinealectomized rats [17] and from intact animals exposed to continuous illumination [1], by RIA and by a more specific bioassay [12].

The fraction of the infused melatonin that appeared unchanged in the rat's urine varied substantially from one

animal to another (e.g., 9.38 ng/12 h for rat B vs. 18.93 ng/12 h for rat F).

The proportion recovered from each of six successive pulses of 10 μg melatonin, however, was quite characteristic for each animal and the mean coefficient of variation in urinary melatonin following the 10-μg infusions was 16%.

Discussion

These studies show that (a) a programmed microinfusion apparatus can induce circadian melatonin rhythms of any desired amplitude within the range investigated in pinealectomized or light-exposed rats; (b) while the quantities of unmetabolized melatonin found in rat urine after its slow, intermittent infusion are meager, they do bear a relatively consistent relationship (approximately 0.1%) to the quantities infused, and (c) because of the uniformity in the urine production rate and the frequency with which rats urinate, urinary melatonin levels provide temporally proximal indications of its renal clearance and blood levels.

Rhythmic infusions of melatonin into rats whose endogenous rhythmic melatonin production had been suppressed (by pinealectomy or exposure to continuous illumination), caused parallel rhythms in melatonin excretion (fig. 3). The fraction of infused melatonin that appeared unchanged in any particular rat's urine varied by as much as 2-fold from one animal to another, but was quite constant for any given rat. We do not know the mechanisms underlying variations between animals (table II) in the proportion of infused melatonin excreted unchanged, but they may be related to those causing similar variations in urinary melatonin levels in human subjects [14, 25].

Studies of the physiological disposition of radioactively labeled melatonin, administered to rats as a bolus injection [8, 15, 28], have shown that the hormone disappears rapidly from plasma and tissues by first-order decay, and is excreted in urine primarily as glucuronic acid and sulfate conjugates of 6-hydroxymelatonin. Apparently these studies did not measure unchanged melatonin in urine. A 44-year-old male human subject, given a single oral dose of 4.3×10^5 nmol of melatonin, excreted 0.3% of the dose given during the first 6.5 h and 0.1% during the next 17.5 h [26].

The relationship between plasma melatonin levels and the melatonin excretion rate can be measured directly in human subjects because their blood can be sampled frequently, and because they can empty their bladders more-or-less at will. Such studies show a remarkable correlation between circulating melatonin levels and the melatonin excretion rate [11, 25]. Earlier studies showed that intact rats maintained under diurnal lighting (LD 12:12) normally excrete about 0.5 ng melatonin during the 12-hour light period and about 1.5 ng during the dark period [1, 18]. Also, plasma from animals killed at the midpoint of the daily dark period contains more melatonin (75 pg/ml) than that from animals killed at the midpoint of the light period (6.3 pg/ml) [6]. The fact that the level of melatonin in the urine of pinealectomized rats was not zero is consistent with earlier studies showing that pinealectomy does not completely eliminate plasma and urinary melatonin [6, 17]. Whether the melatonin content of a particular animal's urine reflects plasma melatonin levels during the urine sampling interval depends primarily on the frequency with which the animal empties its bladder (compared with the frequency of significant changes in plasma melatonin levels). The current results show that the normal rate of urine production among rats is fairly uniform during a 24-hour period (fig. 2), despite the animal's nocturnal way of life; they also show that rats urinate at frequent intervals during both the light and dark portions of the day. Thus, measurement of melatonin content in timed urine collections can provide an accurate index of changes in circulating melatonin levels in rats.

Clearly, neither bolus injections nor slow, continuous infusions of melatonin (from a Silastic capsule or a beeswax pellet) are adequate for reproducing the physiologic effects of endogenously secreted melatonin [4, 22, 23] or of certain other hormones [3] that normally exhibit rhythmic variations in plasma levels. Rather, the precise timing of the rise or fall in hormone levels is critically important.

We hope that the strategies and apparatus described here may help investigators to characterize melatonin's true physiologic roles, by determining which functions, if any, can be attributed to melatonin rhythms when these rhythms are altered.

Acknowledgments

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