

An Inhibitory Effect of Melatonin on the Estrous Phase of the Estrous Cycle of the Rodent

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ABSTRACT. Microgram doses of melatonin decrease the incidence of vaginal smears that demonstrate estrous phases in Sprague-Dawley rats and C3H/HeN mice. This property is not shared by the precursors of melatonin, serotonin and N-acetylserotonin, or its major metabolite, 6-hydroxymelatonin.

The minimum effective dose of melatonin is decreased when daily melatonin injections are started prior to gonadal maturation. Pinealectomy is followed by an increase in the incidence of estrus; this increase is inhibited by melatonin treatment. (*Endocrinology* 75: 238, 1964)

MELATONIN (5-methoxy N-acetyltryptamine) (1) is synthesized in the pineal glands in all species of mammals and birds (2-4) thus far examined. The synthesis is effected through the action of an enzyme, hydroxyindole-O-methyl transferase, which is found in the pineal gland only (5). While melatonin has not yet been identified in blood or urine, its normal release from the pineal gland into the circulation has been inferred (6) from its presence in peripheral nerves (7). This nervous tissue cannot synthesize melatonin (5) but can concentrate melatonin from the blood (6, 8). When radioactive melatonin is administered it is also concentrated in a variety of endocrine organs, especially the ovary (8). The high uptake in the ovary is not the result of circulatory factors, but of an intrinsic property of ovarian tissue (8).

It has been shown that melatonin administration influences the functioning of several endocrine organs: small doses (1-10 mg) depress the growth of the immature rat ovary and the subsequent

incidence of estrous vaginal smears in the adult rat (6); larger doses (150-500 mg) affect the uptake of ^{131}I by the thyroid (9), and the weight of the seminal vesicle (10). When rats are kept in constant light there is an increase in the incidence of estrous phases. A single dose of melatonin counteracts this effect of light (6). This report analyzes in further detail the relation between melatonin and the estrous cycle of the rodent. We have found a) that the effect of melatonin varies with the age of the animal and the method of administration, b) that it can be elicited in more than one species, c) that it is chemically specific, and d) that melatonin inhibits the increase in the incidence of estrous phases which follows pinealectomy.

Materials and Methods

Because of the wide variation in the duration of the various phases of the estrous cycle in a single rat and among rats in a group, the experiments were designed so that the data obtained would be amenable to statistical analysis. Groups of 8-12 rats were subjected to a variety of treatments; vaginal smears were taken daily at approximately the same time (1-4 PM), stained by the Papanicolaou

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technique (11) and examined cytologically. The estrous cycle was divided into 4 phases: proestrus, estrus, metestrus and diestrus. The cellular picture of the different phases was similar to that reported previously (12). The estrous cycle of the mice could be divided into the same phases as that of the rats. Vaginal smears taken during the first 3 phases were grouped together as "estrous smears." The "incidence of estrus" was determined for all the rats in a treatment group for each day. The over-all incidence of estrous smears was similarly determined for each week of treatment, or for the entire length of the treatment period. Differences in the incidence of estrous smears between treatment and control groups were subjected to a Chi-square analysis (13). In some experiments animals served as their own controls, receiving diluent injections for a period before and after 2-6 weeks of administration of the substance to be tested. In other experiments, groups of litter mate rats were divided initially into control and treated groups. Animals were kept in normal diurnal lighting (12 hr of light daily), except as otherwise noted.

The rats were of the Sprague-Dawley strain; mice were C3H/HeN strain; both were obtained from the Animal Production Center of the National Cancer Institute. All animals were housed in plastic cages and left in air-conditioned rooms. They were fed laboratory chow and had access to tap water as drinking water *ad lib*.

The following solutions of melatonin¹ were used for injection: 1) A solution in aqueous medium (water or saline), which was suitable only for very low concentrations of material and had to be prepared fresh every few days because of the tendency of melatonin to crystallize out of water. 2) A solution in absolute ethanol with a subsequent 1:100 dilution with water. 3) A solution in glacial acetic acid with subsequent 1:100 dilution with water. 4) A solution in chloroform with subsequent 1:50 dilution with sesame oil. In experiments in which melatonin and other substances were to be compared, all compounds were dissolved in the same vehicle. No difficulties were encountered in dissolving melatonin precursors or metabolites.

¹ Melatonin, N-acetylserotonin and 6-hydroxymelatonin were obtained from Regis Chemical Company, Chicago, Illinois.

TABLE 1. Effect of melatonin or serotonin on the incidence of estrus in rats

Treatment	No. of smears		% Estrus
	Estrus	Total	
Diluent	103	195	52
Serotonin	93	202	46
Melatonin	70	187	37*

Groups of 11 28-day-old rats were given daily ip injections of serotonin creatinine sulfate (50 μ g), melatonin (20 μ g), or diluent for 28 days. Vaginal smears were taken daily, following spontaneous vaginal opening. "Estrus" included proestrus, estrus and metestrus.

* $p < 0.005$ compared to diluent group.

Pinealectomy and sham-operations were performed, and constant illumination was provided, essentially as described previously (14, 15).

Results

1. *Effect of melatonin administration to immature rats upon the subsequent incidence of estrous smears in the mature animals.* Groups of 28-day-old rats were given 0.2 ml containing melatonin (20 μ g), serotonin creatinine sulfate (50 μ g), or aqueous diluent intraperitoneally daily for 28 days. Spontaneous vaginal opening occurred in all groups between days 37 and 42. Daily vaginal smears were then taken until the animals were 56 days old. The melatonin-treated animals had a decreased incidence of estrous phases, while the incidence of estrous phases of serotonin-treated rats did not differ significantly from that of the animals given the aqueous diluent (Table 1).

Studies of the effect of melatonin on ovary weight indicated that a greater reduction appeared after subcutaneous administration than after other routes of administration (6). Hence, another experiment was performed in which groups of ten similar rats were given 2 μ g of melatonin or aqueous diluent subcutaneously every day. Treatment was started on the twenty-eighth day of life and was continued for eight weeks. Daily

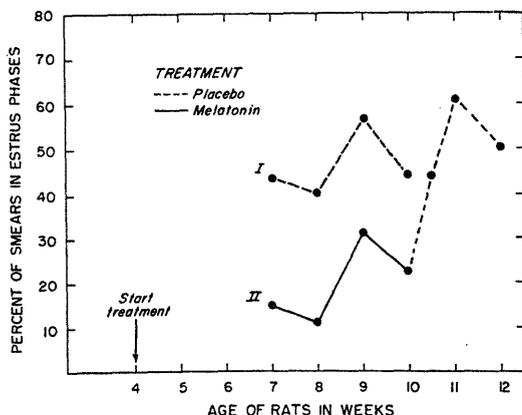


FIG. 1. Effect of melatonin on the incidence of estrous phases. Rats were given daily injections of 2 μg of melatonin, or diluent, starting on their 28th day of life, for 8 weeks. Daily vaginal smears were taken after the animals were 7 weeks old. When the animals were 10 weeks old, diluent was substituted for melatonin.

vaginal smears were taken starting when the animals were seven weeks old; these were grouped and the incidence of estrus was plotted at weekly intervals. After six weeks of treatment, animals which had been receiving melatonin were then given injections of diluent. Melatonin treatment was associated with a 20% incidence of estrus (Fig. 1); litter mate animals treated concurrently with diluent showed an estrus incidence of 45%. Within three days after melatonin treatment was withdrawn, the incidence of estrus rose to 44% and then to 62%. Subsequently these animals returned to normal estrous levels.

2. *Effect of melatonin, N-acetylserotonin and 6-hydroxymelatonin on the incidence of estrus in adult rats.* It was not possible to demonstrate an effect of melatonin on the estrous cycles of adult rats using daily doses of less than 10 μg . At a dosage level of 10 μg or above, melatonin was most effective when administered in sesame oil. In a typical experiment, ten adult rats weighing 160 g were given

melatonin in 1% chloroform-sesame oil for three weeks; the incidence of estrus during this period was 27%. Subsequently the animals were given only the vehicle for three weeks; the incidence of estrus rose to 52% ($p < 0.005$). Finally, animals were returned to melatonin for two weeks; thereupon the incidence of estrus fell to 38% ($p < 0.01$).

Melatonin is synthesized *in vivo* in the pineal gland by the O-methylation of N-acetylserotonin (2). Its major pathway of metabolism involves initial 6-hydroxylation by the liver (16). To determine whether the anti-estrus property of melatonin was specific, or was shared by its precursors or metabolites, groups of seven to ten rats were given N-acetylserotonin (10 or 50 μg), melatonin (10 μg), 6-hydroxymelatonin (10 μg), or the vehicle alone daily subcutaneously for four to eight weeks. All compounds were dissolved in ethanol, which was diluted to 1% with water. Only melatonin depressed the incidence of estrus ($p < 0.005$) (Table 2).

3. *Effect of pinealectomy and melatonin on the incidence of estrus.* Groups of six to eight 28-day-old rats were subjected to pinealectomy or sham-operation. Two

TABLE 2. Effect of N-acetylserotonin, melatonin and 6-hydroxymelatonin on incidence of estrus in adult rats

Treatment	No. of smears		% Estrus
	Estrus	Total	
Diluent	169	370	46
N-acetylserotonin			
10 μg	57	119	49
50 μg	65	161	40
Melatonin			
10 μg	80	269	29*
6-Hydroxymelatonin			
10 μg	57	105	54

Groups of 7-10 mature rats were given drugs daily for 4-8 weeks. "Estrus" included pro-estrus, estrus and metestrus.

* $p < 0.005$ compared to diluent group.

months later these animals were treated alternately for two to three weeks with daily injections of melatonin (1 or 10 μg) dissolved in 1% acetic acid, or other diluent, and vaginal smears were taken. Pinealectomy was associated with an enhanced incidence of estrus. This was significantly depressed by melatonin (Table 3). Sham-operated animals were less sensitive to melatonin than pinealectomized litter mates. The reduction in the incidence of estrus in sham-operated rats produced by 10 μg of melatonin was not statistically significant, possibly because of the small number of smears examined.

4. *Effect of melatonin on the incidence of estrus in adult C3H mice.* Ten 2-month-old C3H/HeN mice were placed under constant illumination for one month to enhance the incidence of estrus. Daily vaginal smears were then checked for two weeks; estrous smears constituted 68% of the 120 smears examined. The animals were then kept in light and given alternating two- to three-week courses of melatonin (10 μg daily dissolved in 1% ethanol) or diluent. Melatonin administration was associated with a significant depression of the incidence of estrus (40% of 150 smears, $p < 0.005$); mice given the vehicle alone showed a 61% incidence (110 smears). The effect of melatonin in C3H/HeN mice kept in diurnal lighting was not examined.

Discussion

Melatonin has an inhibitory effect on the incidence of vaginal estrus in the rat. This effect is not shared by its precursors, serotonin or N-acetylserotonin, or its principal metabolite, 6-hydroxymelatonin. The estrus-inhibiting effect can also be demonstrated in the mouse, and is enhanced by pretreatment of rats with the substance prior to gonadal matura-

TABLE 3. Effect of pinealectomy and of melatonin on the incidence of estrus in rats

Treatment	No. of smears		% Estrus
	Estrus	Total	
Sham-operated			
Diluent	34	70	49
Melatonin			
1 μg	29	63	45
10 μg	23	63	36
Pinealectomized			
Diluent	41	54	76*
Melatonin			
1 μg	32	54	59
10 μg	19	42	45**

Groups of 6-8 immature rats were subjected to pinealectomy or sham-operation. Two months later animals were treated with alternate courses of melatonin or diluent, and vaginal smears were taken. "Estrus" includes proestrus, estrus and metestrus.

* $p < 0.005$ compared to sham-operated diluent group.

** $p < 0.005$ compared to pinealectomized diluent group.

tion. Since circulating melatonin is taken up by the brain and is concentrated by the ovary and pituitary (8), it is not possible at this time to define the locus of its anti-estrous action. The effect of melatonin is only statistical: some animals show no response to this drug, just as certain rats show no increase in the incidence of estrus when placed in constant illumination.

It appears likely that melatonin exerts a modifying influence on the rodent gonad, rather than having a primary effect like the pituitary gonadotropins. The observations that pinealectomy is associated with an increased incidence of estrus, and that this increase is inhibited by melatonin administration, indicate that endogenous melatonin release may play a role in the estrous cycle. Pinealectomy in the immature rat is also followed by enhanced ovarian growth; this hypertrophy is blocked by the administration of bovine pineal extracts (17). When pineal extracts are prepared as described (18) from lyophilized bovine

pineals to which radioactive melatonin (16) has been added, 10 to 30% of the added material is present in the extracts (Wurtman and Axelrod, unpublished observations). It is probable that even more endogenous melatonin persists, since the pineal has been shown to bind melatonin by a temperature-dependent concentrating mechanism (8). Hence it is possible that one ovarian-hypertrophy-inhibiting factor in bovine pineal extracts may be melatonin.

The amount of melatonin that the rat pineal is capable of synthesizing per day is of the same order of magnitude as the minimum doses used here (19). It has been shown that the activity of hydroxyindole-O-methyl transferase, the enzyme in the rat pineal which makes melatonin, varies markedly with environmental illumination: constant lighting decreases melatonin-synthesizing ability, while darkness enhances it (19). Interruption of the sympathetic innervation of the pineal blocks this effect of lighting (20). Exposure to light for as little as 24 hours produces a marked inhibitory effect upon melatonin synthesis (20). It has been suggested that there is a diurnal cycle to *in vivo* melatonin synthesis, and that this cycle may influence other biologic rhythms, such as the estrous cycle (19).

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