Lack of effect of melatonin on sexual maturation in female rats*

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Summary. Daily subcutaneous injections of 100 µg melatonin given to prepubertal female rats housed in 14L:10D or 12L:12D failed to delay puberty as evidenced by the age at which vaginal opening occurred; neither the Sprague–Dawley nor the Wistar strain rats were responsive to melatonin treatment. Reproductive organ weights (ovaries and uteri) at vaginal opening were unaffected by such treatment. Administration of melatonin through the drinking water in doses of 100, 500 or 1000 µg/day did not alter the timing of puberty or the reproductive organ weights in rats of the Sprague–Dawley or Long–Evans strains (housed in 12L:12D). Our experimental methods are identical to a previous report and we have no explanation for our failure to reproduce the earlier results.

Keywords: melatonin; puberty; vaginal opening; rat

Introduction

Melatonin is known to have an inhibitory effect on the reproductive axis of mammals (Minneman & Wurtman, 1975). Daily injections of melatonin to adult hamsters results in gonadal regression and decreased testicular and accessory organ weights in males (Reiter *et al.*, 1976; Tamarkin *et al.*, 1976) and cessation of oestrous cyclicity in females (Tamarkin *et al.*, 1976). However, the role of melatonin in the reproductive function of non-seasonal breeders such as the rat remains relatively obscure. The reproductive system of the adult male rat is unresponsive to melatonin (Lang *et al.*, 1983), but some influence has been noted in adult females: daily injections of melatonin result in some disruption of the oestrous cycle (Chu *et al.*, 1964) while injections given during the 'critical period' on the day of pro-oestrus result in the blockade of ovulation (Collu *et al.*, 1971; Ying & Greep, 1973; Sorrentino, 1975). In addition, Reiter *et al.* (1980) have demonstrated that removal of the olfactory bulbs induces sensitivity to melatonin in immature female Sprague–Dawley rats.

More dramatic effects have been reported when melatonin treatment begins prepubertally (for reviews see Rivest *et al.*, 1986; Lang, 1986). In female rats, daily melatonin injections result in a delay in vaginal opening, decreased ovarian weight, a lower level of pituitary GnRH receptors and a decreased incidence of oestrus after vaginal opening. Male rats treated similarly between Days 20 and 40 of life have decreased testicular and seminal vesicle weights, lower concentrations of plasma LH, FSH and testosterone and fewer pituitary GnRH receptors, as compared to controls. The influence of melatonin on the sexual maturation of the rat is believed to be via the hypothalamus by affecting GnRH release (Rivest *et al.*, 1986; Lang, 1986).

Common to both the hamster and the rat is the importance of the time of injection with respect to the photoperiod. There appears to be a diurnal pattern in the sensitivity of the reproductive axis

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to melatonin such that sensitivity is greatest in the late afternoon or shortly before the onset of darkness (Rivest *et al.*, 1986; Lang, 1986). Morning melatonin injections are without effect. Diurnal variations in gonadotrophin release are clearly of importance in the attainment of reproductive competence (MacKinnon *et al.*, 1978; Ojeda *et al.*, 1986). Jacobson & Wilkinson (1986) have suggested that circadian variations in brain neurotransmitter binding sites (receptors; see also Wirz-Justice, 1987), and in particular hypothalamic opioid receptors, may entrain prepubertal hormone secretion. Since melatonin is now thought to be a timekeeping hormone (Karsch, 1986; Reiter, 1987), we wished to know whether the anti-pubertal effects of melatonin (Rivest *et al.*, 1986; Lang, 1986) might be expressed as a disruption of hypothalamic opioid receptor binding rhythms. Unfortunately we have been unable to replicate the report by Rivest *et al.* (1985) that daily melatonin injections delay rat vaginal opening. We also include in this paper experiments with different rat strains as well as attempts to use melatonin in the drinking water.

Materials and Methods

Animals. Female rats were obtained from Canadian Hybrid Farms, Halls Harbour, Nova Scotia (Sprague-Dawley) or from Charles River Canada Ltd, St Constant, Quebec (Wistar and Long-Evans) and were housed 4–6 per cage under controlled temperature ($20^{\circ}\text{C} \pm 1$) and controlled lighting (fluorescent 34 W Cool White; General Electric) of either 14 h light:10 h dark (14L:10D), lights on at 07:00 h (Exps 1 and 2) or 12L:12D, lights on at 06:00 h (all remaining experiments). Food and water were available *ad libitum*. Rats arriving in litters were used so that each litter provided rats for treatment and control groups. Rats were always weaned on Day 22 of life. Starting at Day 29 rats were checked daily for vaginal opening. On the day of vaginal opening rats were killed and uteri and ovaries were removed and cleaned of associated fat. Corpora lutea were counted and organs weighed.

Injections. Starting on Day 15 of life (except Exp. 1) melatonin ($100\text{ }\mu\text{g}$) was injected subcutaneously every day until vaginal opening. Control rats received the vehicle solution. For Exps 1 and 2 performed with a 14L:10D schedule, injections were given at 16:30 h (9.5 h after lights on) while for the other experiments in 12L:12D, injections were given at 17:00 h (11 h after lights on). Melatonin was dissolved in saline-ethanol (9:1 v/v; 0.9% NaCl and 100% ethanol). Samples of 1–5 mg were stored in plastic vials at -4°C and fresh solutions were made daily immediately before injections. Melatonin was obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.) and from Aldrich Chemical Co., Inc. (Milwaukee, WI, U.S.A.). Animals were treated with melatonin from Sigma, while that from Aldrich was purchased for the purpose of comparing melatonin from the two sources by thin-layer chromatography in order to ensure purity of the treatment drug.

Melatonin administration via drinking water. Melatonin was administered to prepubertal female rats via their drinking water in doses of 100 to 1000 $\mu\text{g}/\text{rat}/\text{day}$. On the basis of previously determined water consumption of 25 ml/rat/day, melatonin was dissolved in ethanol and diluted with water acidified to a pH of 2.5 using concentrated HCl. The use of acidified water eliminated the need to change the drinking bottles daily (Hoag *et al.*, 1965; Tober-Meyer & Bieniek, 1981). Water bottles were also covered with aluminium foil to prevent any adverse effects of light on the melatonin. Control rats received acidified water with ethanol. All rats receiving melatonin via drinking water were housed under 12L:12D.

Statistical analysis. Group means were compared using Student's *t* test. Differences were considered significant when a *P* value of 0.05 or less was obtained.

Results

Experiments 1 and 2

Methods. Female Sprague-Dawley (SD) rats were obtained at 21 (Exp. 1) or 9 (Exp. 2) days of age and were housed under 14L:10D. Daily melatonin injections were begun on Day 22 (Exp. 1) or Day 15 (Exp. 2).

Results. Melatonin treatment beginning on Day 22 (Exp. 1) was without effect on the age at which vaginal opening occurred. Treatment starting at Day 15 (Exp. 2) resulted in a significant 1.6-day delay in vaginal opening (Fig. 1). Melatonin treatment had no effect on uterine weight in either experiment but rats in Exp. 2 showed a reduction in ovarian weight (Fig. 1) (27.8 ± 1.1 and $24.4 \pm 0.9\text{ mg}/100\text{ g}$ body weight in control and experimental rats, respectively).

Fig. 1. The age at vaginal opening and ovarian and uterine weights/100 g body weight (at vaginal opening) of control rats (open bars) and rats treated prepubertally with melatonin (shaded bars) via daily 100 µg subcutaneous injections. The conditions of each experiment are detailed in the 'Results' section. Values are mean $\pm$ s.e.m.; N = 7–12 for control rats and 10–16 for melatonin-treated rats. * $P < 0.05$, ** $P < 0.025$, *** $P < 0.005$.

Experiment 3

Methods. Female SD rats were received at 9 days of age, housed in 12L:12D and given melatonin injections from Day 15.

Results. There was no delay in vaginal opening in melatonin-treated females and no effect on ovarian or uterine weights (Fig. 1).

Experiment 4

Methods. SD rats were born at the University Animal Care Center in a photoperiod of 14L:10D. At 4 days of age female young and their mothers were transferred to 12L:12D. Melatonin was administered by s.c. injections starting on Day 15.

Results. Vaginal opening was significantly advanced by 1.5 days in rats treated with melatonin (32.5 ± 0.4 vs 34.0 ± 0.3 for controls) (Fig. 1). This was the only incidence of melatonin significantly advancing vaginal opening. It was also the only occasion of melatonin-treated rats having heavier ovaries than controls (30.8 ± 1.5 vs 27.3 ± 1.1 mg/100 g body weight, $P < 0.05$). Uterine weights were similar in both groups.

Experiments 5 and 6

Methods. The procedures of Exp. 3 were followed except that the rats were of the Wistar strain.

Results. Although the conditions were identical for Exps 5 and 6, melatonin treatment did not show the same effects in both experiments: vaginal opening was delayed in Exp. 6 (36.2 ± 0.6 vs 34.1 ± 0.6 days) but not in Exp. 5. Uterine weight was unaffected in each experiment. Ovarian weight was reduced in Exp. 6 (29.3 ± 2.5 vs 39.8 ± 3.1 mg/100 g body weight) and unchanged in Exp. 5.

Experiment 7

Methods. Following the exact procedures used by Rivest *et al.* (1985), 12-day pregnant Wistar rats were obtained and the young were born into a photoperiod of 12L:12D. Melatonin injections (100 µg, s.c.) were begun on Day 15 of age.

Results. There were no changes in the age at vaginal opening, uterine weight or ovarian weight in melatonin-treated rats compared to controls (Fig. 1).

Experiment 8

Methods. Female SD rats obtained at 21 days of age were given melatonin in the drinking water at doses of 100 or 500 µg/rat/day.

Results. Neither dose of melatonin affected the day of vaginal opening when compared to controls (Fig. 2). Uterine weight was unaffected by either dose but the dose of 500 µg resulted in a reduction of ovarian weight (29.9 ± 1.4 vs 36.9 ± 2.0 mg/100 g body weight) (Fig. 2).

Experiment 9

Methods. These were the same as those in Exp. 8 except that the doses of melatonin were increased to 500 and 1000 µg/rat/day and begun on Day 21.

Results. No effects were observed on vaginal opening, uterine weight or ovarian weight (Fig. 2).

Experiment 10

Methods. Long-Evans rats were obtained at 21 days of age and given melatonin via the drinking water at doses of 500 or 1000 µg/rat/day, starting on the day of arrival.

Results. A change in vaginal opening was observed only in the rats treated with 500 µg melatonin daily (Fig. 2); vaginal opening was advanced by 1.5 days (32.9 ± 0.4 vs 34.4 ± 0.8). No effects were seen in uterine or ovarian weights in either of the treatment groups (Fig. 2).

Discussion

Rivest *et al.* (1985) have reported that daily injections of 100 µg melatonin administered 11 h after the onset of light to female Wistar rats, housed under 12L:12D and starting on Day 15 of life results in a 10-day delay of vaginal opening. After careful consideration of all of these factors and the performance of thin-layer chromatography to ensure purity of the compound, our results failed to show the same effect of melatonin. Since this is unlikely to be due to a strain difference, the most evident explanation seems to be that the sensitivity to melatonin varies between rat colonies. If this is so it weakens the case for the importance of melatonin in the sexual maturation of the female rat.

Fig. 2. The effect of administering melatonin through the drinking water on age at vaginal opening and ovarian and uterine weights/100 g body weight (at vaginal opening). The doses varied from 100 to 1000 µg/day. See 'Results' for the conditions of each experiment. Values are mean $\pm$ s.e.m.; N = 10 for control rats and N = 10 or 12 for melatonin-treated rats. * $P < 0.05$, *** $P < 0.005$.

In our experiments, vaginal opening did occur slightly later in the melatonin-treated rats, but, except in Exp. 4 in which there was a significant advance in the time of vaginal opening, delays in the age at vaginal opening were statistically significant only in Exps 2 and 6. However, whenever the time of vaginal opening was statistically affected by treatment, the change was never more than 6% of control values. Modifications of such limited magnitude may be interpreted as merely due to natural variation rather than to a significant physiological effect of melatonin. Nevertheless, when melatonin did alter timing of vaginal opening there was a corresponding and significant change in ovarian weight. Delays in vaginal opening seen in Exps 2 and 6 were accompanied by significant decreases in ovarian weight while the earlier age of vaginal opening in Exp. 4 was associated with increased ovarian weight. Uterine weight was unaffected by melatonin treatment in all experiments. The mean number of corpora lutea per ovary was between 6 and 7 for all experiments and there were no differences between control and melatonin-treated rats.

One can readily see this basic variability in the results of the identical Exps 5 and 6 (Wistar rats). Significant changes in time of vaginal opening and ovarian weight were observed in Exp. 6 but not in Exp. 5. There is no evident explanation for this observation but it again suggests that the observed differences are no more than inherent variation and have no physiologically significant cause. Discrepancies such as these have been reported previously and thus are not surprising. Adams *et al.* (1965) reported that daily injections of 100 µg melatonin between Days 21 and 35 of life delayed vaginal opening in one experiment and not another and Collu *et al.* (1971) found that ovarian weight was reduced in intraventricular injections of melatonin (starting at age 25 days) in one experiment but not another. Rivest *et al.* (1985) also commented that time of vaginal opening in some of the rats (40%) was unaffected by 100 µg melatonin injections (given 9 h after lights on in 12L:12D).

Treatment of rats with melatonin in the drinking water effected changes in only two instances: ovarian weight was significantly reduced in Sprague–Dawley rats receiving a daily dose of 500 µg, and vaginal opening occurred earlier (by 4–4%) in Long–Evans rats receiving this same dose. The doses of 100 µg and 1000 µg produced no effects in any of the experiments. Why 500 µg reduced ovarian weight in Exp. 8 but not in Exp. 9 is unclear. Melatonin administered via the drinking water has resulted in gonadal atrophy in male hamsters (Pévet & Haldar-Misra, 1982), indicating that the melatonin can be absorbed unchanged through the gastrointestinal tract.

We can think of no simple explanation to account for our inability to replicate the work of Rivest *et al.* (1985). The three rat strains available to us appear to be generally unresponsive to melatonin. This suggests that the Swiss colonies used by Rivest *et al.* (1985) are somehow more sensitive to the anti-pubertal effect of melatonin. It would be useful if these rats could be made available in North America.

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