Effect of exogenous melatonin on neuroendocrine–reproductive function of middle-aged female rats

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The possible role of melatonin in the regulation of the reproductive system of female rats during ageing was investigated in middle-aged female rats showing irregular duration of the oestrous cycle ($n = 30$). Blood samples were obtained by jugular venepuncture during the oestrous cycle in control rats. After this experiment was completed, the female rats were treated with melatonin for 2 months and blood samples were obtained at different stages of the oestrous cycle. Plasma LH, FSH and prolactin concentrations were significantly increased in the afternoon of the day of pro-oestrus after melatonin treatment compared with control rats. Moreover, FSH concentrations too were significantly increased on the morning of pro-oestrus and oestrus in melatonin treated rats compared with control rats. Similarly, oestradiol concentrations were significantly higher on the morning of pro-oestrus in melatonin treated rats compared with controls. Another group of rats showing irregular duration of the oestrous cycle was used to study the possible effect of melatonin treatment on the timing of pro-oestrous surges of LH and FSH. The results showed that LH and FSH peak values occurred at 5 h after melatonin treatment. Pituitary responsiveness to LHRH in a 90 min test was also studied in middle-aged rats showing irregular duration of the oestrous cycle that had been injected for 1 month with either melatonin or saline. Prolactin response was unaffected by exogenous melatonin, but a stimulatory effect of melatonin on LH and FSH pituitary responsiveness to LHRH was observed. The results indicate an improved function of the neuroendocrine–reproductive axis in middle-aged rats after melatonin treatment.

Introduction

The pineal gland is involved in animal and human reproduction through the action of its hormone, melatonin. Prolonged and quantitatively increased circulating melatonin concentrations have been observed in association with amenorrhoea (Berga *et al.*, 1988; Laughlin *et al.*, 1991) and decreased fertility in women (Kauppila *et al.*, 1987). Exogenous melatonin administration alone or in combination with a synthetic progestin caused a significant decrease in LH, oestradiol and progesterone in women, and consequently this combination was shown to be an effective oral contraceptive (Voordouw *et al.*, 1992). Exogenous melatonin administration at doses resulting in increased concentrations of melatonin for at least 8 h, increased LH concentrations and LH pulse amplitude during the follicular, but not the luteal, phase of the menstrual cycle (Cagnacci *et al.*, 1995). Studies in rats indicate that melatonin might be important in the timing of the LH surge (that is, in the afternoon of the day of pro-oestrus) (Chiba *et al.*, 1994), although high doses of melatonin given over the critical

period can block the LH surge (Ying and Greep, 1973; Rivest *et al.*, 1985). These opposing effects may be explained by the age at which the observations were made; in general it appears that melatonin is inhibitory in young fertile subjects and has a stimulatory effect with increasing age.

It has been suggested that melatonin may also be involved in ageing (Reiter, 1992). Melatonin is produced and secreted into the blood in a circadian rhythm; maximum production occurs during the dark phase of the day. The 24 h rhythm of melatonin production is very robust in young animals, but this circadian rhythm deteriorates with age. Thus, in old animals, the amount of melatonin secreted is lower than in young individuals and the administration of melatonin may be beneficial in delaying age-related degenerative conditions (Reiter, 1994). Long-term melatonin treatment in rats can postpone the age-related decrease in survival rates, circulating sex steroids and ¹²⁵I-labelled melatonin binding sites in the brain (Oaknin-Bendahan *et al.*, 1995).

Alterations in LH surges become apparent in middle-aged rats (Harney *et al.*, 1996). Lambalk *et al.* (1997) postulated that post-menopausal ageing has a major suppressive effect on the function of pituitary gonadotrophes. In addition, the study of Arias *et al.* (1996) supports the hypothesis that a

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decrease in the excitatory inputs to LHRH neurones may be involved directly in the reduction of the activity of the hypothalamic–pituitary–ovarian axis observed during ageing. However, it is not known to what extent the hypothalamus and pituitary are involved in this process. The aim of the present study was to investigate the possible role of melatonin in the regulation of the reproductive system in ageing female rats at both the hypothalamus and the pituitary.

Materials and Methods

A total of 30 control female Wistar rats (11-month-old) from our colony were used. Animals were housed under a 12 h light: 12 h dark photoperiod (lights on at 08:00 h) at a temperature of approximately 23°C. Standard rat chow and tap water were available *ad libitum*. Vaginal smears were taken from animals between 10:00 h and 11:30 h for 15 days to select rats showing irregular duration (that is > 5 days) of the oestrous cycle. In these animals the duration of the oestrous cycle ranged from 6 to >10 days. None of the female rats studied showed conditions such as persistent oestrus or recurrent pseudopregnancy. Vaginal smears were also taken from the animals after melatonin treatment.

Plasma concentrations of gonadotrophins, prolactin and oestradiol throughout the oestrous cycle before and after melatonin treatment

After the first experiment involving vaginal smears was completed, blood samples at different stages of the oestrous cycle were randomly obtained from all female rats by jugular venepuncture under ether anaesthesia. In this first stage of the experiment, the rats were considered as the control group. Blood samples (1 ml) were collected between 11:00 h and 12:00 h for dioestrus ($n = 28$), pro-oestrus a.m. ($n = 28$), oestrus ($n = 27$) and metoestrus ($n = 22$) and between 17:00 h and 17:30 h for pro-oestrus p.m. ($n = 28$). After this stage of the experiment was completed, the animals were treated with melatonin and were considered as the experimental group. Melatonin (M-5250, Sigma Chemical Co, St Louis, MO) was dissolved in a small volume of absolute ethanol and diluted in 0.9% (w/v) NaCl. A dose of 150 µg melatonin per 100 g body weight was administered s.c. once a day at the end of the light phase (18:30–19:00 h) for 2 months to 11-months-old female rats showing irregular duration of the oestrous cycle. After the melatonin treatment, blood samples were obtained at different stages of the oestrous cycle: dioestrus ($n = 17$), pro-oestrus a.m. ($n = 13$), oestrus ($n = 13$), metoestrus ($n = 13$) and pro-oestrus p.m. ($n = 12$). Melatonin treatment was continued until blood sample collections were completed. Blood sampling was carried out using the same procedure described above. The duration of the oestrous cycle after melatonin treatment was between 4 and 9 days. The blood samples were centrifuged and plasma was stored at –20°C until assays for LH, FSH, prolactin and oestradiol were conducted.

Plasma concentrations of gonadotrophins on the day of pro-oestrus

A different group of 11- to 15-month-old control rats ($n = 50$) showing irregular duration of the oestrous cycle was used to study the effects of melatonin treatment on the timing of LH and FSH surges on the day of pro-oestrus. The pro-oestrous stage of the oestrous cycle was confirmed by vaginal smears. Serial blood samples (1 ml) were taken at 07:00 h ($n = 8$), 10:00 h ($n = 7$), 14:00 h ($n = 10$), 17:00 h ($n = 20$), 21:00 h ($n = 9$) and 01:00 h ($n = 10$) using the procedure described earlier. After this stage of the experiment was completed, the animals were treated with melatonin (150 µg per 100 g body weight) for 2 months, after which serial blood samples were taken at the same time points (07:00 h ($n = 6$), 10:00 h ($n = 5$), 14:00 h ($n = 6$), 17:00 h ($n = 18$), 21:00 h ($n = 8$), 01:00 h ($n = 5$)) using the procedure described earlier. Blood samples were processed as in the first experiment.

Pituitary responsiveness to LHRH

The effect of chronic administration of melatonin on pituitary gonadotrophins and prolactin responsiveness to LHRH was tested in 12-month-old female rats showing irregular duration of the oestrous cycle. Animals were randomly assigned to two groups: control ($n = 12$) and melatonin treated (150 µg per 100 g body weight) ($n = 21$). The control group received 10% ethanol–90% saline. Animals were treated for 1 month, after which the first dioestrus was detected by vaginal smears performed at 16:00 h. After detection of the first dioestrus, an LHRH test was performed between 16:00 h and 18:00 h. Pituitary responsiveness to a bolus injection of LHRH was tested by taking a basal sample and subsequently injecting animals i.v. with LHRH (50 ng LHRH per 100 g body weight) (Sigma) diluted in saline. Injected volumes ranged from 0.05 to 0.1 ml. Additional blood samples were obtained at 15, 30, 60 and 90 min after the LHRH injection. Blood samples (1 ml) were obtained by jugular venepuncture under slight ether anaesthesia. Blood samples were centrifuged immediately after each time point, and each rat received its own red blood cells suspended in 0.9% saline to avoid anaemia. Plasma was stored at –20°C until assayed for LH, FSH and prolactin.

Radioimmunoassay of gonadotrophins and prolactin

Plasma LH, FSH and prolactin concentrations were measured by specific radioimmunoassays using second antibody-facilitated separation and reagents provided by the National Hormone and Pituitary Program (NIADDK, Bethesda, MD). The assays were validated as described by Tresguerres *et al.* (1981). LH concentrations were expressed as pg ml^{–1} in terms of NIADDK rat LH-RP-3. The sensitivity of the assay was 20 pg LH ml^{–1}. The final dilution of anti-rat LH serum was 1:100 000. The intra-assay coefficient of variation for the LH assay was 6%. FSH concentrations were expressed in pg FSH-RP-3 ml^{–1} and the sensitivity of the assay was 12.5 ng FSH ml^{–1}. The final dilution of anti-rat FSH

serum was 1:2000. The intra-assay coefficient of variation for the FSH assay was 9.5%. Prolactin concentrations were expressed as pg rat prolactin RP-3 ml⁻¹ and the sensitivity of the assay was 5 µg l⁻¹. The final dilution of anti-rat prolactin-S-9 serum was 1:5000. The intra-assay coefficient of variation for the prolactin assay was 7%. All samples were run in the same assay for each hormone to avoid interassay variations.

Radioimmunoassay of oestradiol

Plasma oestradiol was measured using an ¹²⁵I-labelled radioimmunoassay kit using commercial ImmunoChem™-coated tubes according to the manufacturer's instructions (ICN Pharmaceuticals, Inc., Costa Mesa, CA). The minimum amount of oestradiol significantly different from 0 was 10 pg ml⁻¹. The maximum binding of the assay was 28.6% and the intra-assay coefficient of variation was 5.5%.

Statistical analysis

Data from each age group were adjusted to a normal distribution test before they were used in the statistical analysis. A 99% accuracy to normal distribution was required. Statistical analysis was performed using the SIGMA statistical program (Copyright Horus Hardware, 1986). Results were

expressed as mean ± SEM. Comparisons among groups of data for hormones were determined by one-way ANOVA; individual comparisons were then made by Newman-Keuls multi-range test. *P* values < 0.05 were considered significant.

Results

Hormone concentrations throughout the oestrous cycle

LH concentrations were low at all stages of the oestrous cycle except in the afternoon of the day of pro-oestrus when an LH surge was observed (Fig. 1). After 11-months-old rats showing irregular duration of the oestrous cycle were treated with melatonin for 2 months, no differences were observed in LH secretion at dioestrus, pro-oestrus p.m., oestrus and metoestrus compared with control animals. However, the LH surge in the afternoon of the day of pro-oestrus was significantly greater (*P* < 0.01) in the melatonin treated group compared with the controls. FSH concentrations were similar in all stages of the oestrous cycle in control rats. After melatonin treatment, the FSH surge in the afternoon of the day of pro-oestrus was significantly greater (*P* < 0.01) compared with that in the control animals. A significant increase in FSH concentrations (*P* < 0.05) in melatonin treated rats compared with control rats was also observed at pro-oestrus a.m. and oestrus. Melatonin administration for 2 months to 11-month-

Fig. 1. Concentrations of plasma LH (a), FSH (b), prolactin (c) and oestradiol (d) in all stages of the oestrous cycle in control (□) and melatonin treated (■) 11- to 13-month-old female rats. Rats were treated with 150 µg melatonin per 100 g body weight for 2 months. **P* < 0.01, ***P* < 0.05 versus control group.

old rats produced increased prolactin concentrations in pro-oestrus (a.m. and p.m.) and oestrus; this increase was significantly higher ($P < 0.01$) in the afternoon of the day of pro-oestrus compared with the control group. Oestradiol concentrations were higher at dioestrus and in the morning of the day of pro-oestrus in rats that received melatonin treatment, although the increase was only significantly higher ($P < 0.05$) in the morning of the day of pro-oestrus compared with the control group. In the afternoon of the day of pro-oestrus, as well as at oestrus and metoestrus, oestradiol concentrations were similar in both groups.

Hormone concentrations throughout pro-oestrus

Plasma LH and FSH concentrations on the day of pro-oestrus in 11- to 15-month-old female rats are shown (Fig. 2). LH concentrations in the control and treatment group were similar from 07:00 h to 14:00 h, but a significant increase ($P < 0.01$) in LH was observed at 17:00 h in the melatonin-treated animals compared with the controls. FSH concentrations during the day of pro-oestrus in control rats

Fig. 2. Concentrations of plasma LH (a) and FSH (b) throughout pro-oestrus in control (—◆—) and melatonin treated (---■---) 11- to 15-month-old rats. Rats were treated with 150 µg melatonin per 100 g body weight for 2 months. * $P < 0.01$, ** $P < 0.05$ versus control group.

showing irregular duration of the oestrous cycle increased progressively from 07:00 h to 01:00 h, reaching the highest values at 17:00 h and 01:00 h. Melatonin treatment only affected the concentration of FSH at 17:00 h, when significantly increased concentrations were observed ($P < 0.01$) compared with controls.

Pituitary responsiveness to LHRH

The effects of the administration of 50 ng LHRH to 12-month-old female rats are shown (Fig. 3). A positive response in LH secretion was observed 15 min after injection of LHRH, and this was maintained for 90 min in both the control and the melatonin treated groups. The magnitude of the response was higher in the melatonin treated group compared with the controls, as significantly higher ($P < 0.05$) LH concentrations were observed 30 and 60 min after LHRH.

Fig. 3. Concentrations of plasma LH (a), FSH (b) and prolactin (c) at dioestrus after injection of 50 ng LHRH per 100 g body weight in control (□) and melatonin treated (■) 12-month-old female rats. Rats were treated with 150 µg melatonin per 100 g body weight for 1 month.

** $P < 0.05$ versus control group.

In the control group, FSH secretion showed no response to LHRH. However, a positive response was observed in melatonin-treated rats showing irregular duration of the oestrous cycle: at 15, 30, 60 and 90 min after LHRH and FSH concentrations were significantly ($P < 0.05$) higher in these animals compared with the controls. In melatonin treated rats at 90 min after injection of LHRH, FSH concentrations decreased to basal concentrations, but remained significantly higher ($P < 0.05$) than in the control group. Prolactin secretion in both groups studied showed a negative response to LHRH and reached the lowest concentration 30 min after injection of LHRH. No significant differences were observed between the two groups at any time after administration of LHRH.

The percentage distribution of the duration of the oestrous cycle in 11- to 13-month-old female rats, before melatonin treatment, were as follows: 4–5 days, 0%; 6–10 days, 23.08%; > 10 days, 84.61%. After melatonin treatment for 2 months the distribution was as follows: 4–5 days, 38.46%; 6–10 days, 23.08%; > 10 days, 38.46%.

Discussion

Cyclical release of pituitary gonadotrophins and prolactin in female rats is regulated by interactive neuroendocrine mechanisms (Sarkar *et al.*, 1974; Debeljuk *et al.*, 1977). The results of the present study showed increased plasma LH concentrations only on the afternoon of the day of pro-oestrus in control and melatonin treated rats, as was reported in young female rats with a 4 day oestrous cycle (Sarkar *et al.*, 1974; Parnet *et al.*, 1990). Other studies indicated that the timing of melatonin treatment is critical to produce an inhibitory effect on the LH secretion during pro-oestrus in young cyclic rats (Cardinali, 1974; Sorrentino, 1975; Rivest *et al.*, 1985). Ying and Greep (1973) found that inhibition of LH secretion was observed when melatonin was administered between 14:00 h and 16:00 h on the day of pro-oestrus. Chronic melatonin administration delayed sexual maturation of female rats and pro-oestrous surges of gonadotrophins and oestradiol occurred at a reduced frequency (Rivest *et al.*, 1985). However, in the present study on middle-aged female rats, significantly increased LH concentrations were observed in the afternoon of the day of pro-oestrus. A facilitatory role of melatonin on the synchronization of the LH surge was observed in pinealectomized rats. After melatonin treatment in dioestrus during the critical period before the lights were switched off, the individual differences in the timing of the LH surge in pinealectomized rats were reduced (Chiba *et al.*, 1994). The results of the present study indicate that melatonin administration for 2 months also enhanced the amount of LH released during the surge and may have improved synchronization, since significantly higher LH and FSH concentrations after melatonin treatment were observed only at 17:00 h, while in the control middle-aged animals increased concentrations were observed over a long period of time. Although, the possible physiological implications of the results are important, the mechanisms involved in this process await further study. Harney *et al.* (1996) reported that the suprachiasmatic nucleus influences the rhythm of

gonadotrophin secretion by gonadotrophin releasing hormone (LHRH) neurones; melatonin receptors have been described in the suprachiasmatic nucleus (Vanecek *et al.*, 1987). The results of the present study indicate that melatonin treatment in middle-aged rats, in which melatonin secretion is decreased (Pang *et al.*, 1984), may improve LH as well as FSH secretion through a possible effect on the suprachiasmatic nucleus.

Several studies have indicated that melatonin regulates prolactin secretion (Bispink *et al.*, 1990; Okatani and Sagara, 1993). Administration of melatonin resulted in a marked and rapid decrease of previously high plasma prolactin concentrations observed in pituitary-grafted rats (Esquifino *et al.*, 1989). In the present experiments in which prolactin concentrations were low, melatonin restored the prolactin peak values during the afternoon of the day of pro-oestrus in middle-aged rats. It is known that the pattern of prolactin secretion during the oestrous cycle in rats is characterized by low concentrations except for a brief period on the afternoon and evening of the day of pro-oestrus (Neill, 1970; Ojeda *et al.*, 1976; Anselmo-Franci *et al.*, 1997). The results of the present study indicate that melatonin has opposing effects depending on whether prolactin concentrations are initially high or low.

Surges in the secretion of LH, FSH and prolactin are preceded and stimulated by the increasing concentrations of follicular oestrogen (Neill *et al.*, 1971; Parnet *et al.*, 1990). The results of the present study indicate that melatonin treatment in middle-aged rats had a positive influence on this mechanism; oestradiol concentrations were increased on the morning of pro-oestrus, which preceded the gonadotrophin and prolactin surges observed in the afternoon of pro-oestrus. The possibility that melatonin acts directly on the ovary must be considered since melatonin has been detected in human follicular fluid (Brzezinski *et al.*, 1987), and specific melatonin receptors (Cohen *et al.*, 1978) are present in hamster, rat and human ovaries. In addition, direct effects of melatonin on ovarian steroidogenesis *in vitro* have been reported (Macphee *et al.*, 1975).

The results of the present study showed a stimulatory effect of melatonin on the gonadotrophin response to LHRH, since treated rats showed greater LH secretion and amplitude of FSH surge than did control rats. Cagnacci *et al.* (1995) reported a stimulatory effect of melatonin on the gonadotrophin response to physiological or submaximal doses of LHRH during the follicular phase of the menstrual cycle in women. How melatonin augments this pituitary response to LHRH remains unknown. The presence of specific melatonin receptors in the pars tuberalis in rodents (Vanecek *et al.*, 1987; Weaver and Reppert, 1989) and humans (Weaver *et al.*, 1993) supports a direct effect of melatonin on pituitary cells. However, the effect of melatonin on the hypothalamic nuclei mentioned above, which directly or indirectly influences the pituitary response to LHRH, cannot be disregarded. Among the factors involved in the regulation of prolactin secretion, LHRH may be inhibitory (Esquifino *et al.*, 1989) or stimulatory (Asch *et al.*, 1985; Debeljuk *et al.*, 1985). The results of the present study indicate an inhibitory role of LHRH, since plasma prolactin concentrations decreased 30 min after LHRH injection. Several studies have indicated that melatonin regulates prolactin secretion

(Bispink *et al.*, 1990; Okatani and Sagara, 1993). The similarity in the pituitary response to LHRH observed in both groups of middle-aged rats in the present study indicates that exogenous melatonin does not stimulate the lactotroph response to LHRH during dioestrus.

Aschheim (1976) reported that in 12 month-old Wistar rats, 60% showed normal oestrous cycles, 20% showed persistent oestrus and 20% showed repetitive pseudo-pregnancies, which is indicative of reduced activity of the hypothalamic-pituitary-ovarian axis during ageing (Arias *et al.*, 1996; Fujimoto *et al.*, 1996). It is well documented that melatonin administration to young female rats suppresses the functional activity of the ovary (Tamarkin *et al.*, 1976). However, the results of the present study indicate that melatonin treatment during ageing has an opposite role on gonadotrophins and prolactin, since oestrous cyclicity was improved. Whatever the mechanism, these results indicate a facilitatory role of melatonin on the hypothalamic-pituitary-ovarian axis in middle-aged rats showing irregular duration of the oestrous cycle.

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