Roles of melatonin and prolactin in testicular crudesence in mink (*Mustela vison*)

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Peripubertal male mink (*Mustela vison*) were treated with prolactin, melatonin or antibodies against melatonin to determine the effects of altered circulating concentrations of prolactin and melatonin throughout one season of testicular development. Treatment began on 1 October and continued until 4 March. Administration of 0.5 mg ovine prolactin day⁻¹ by minipump increased the circulating concentration of prolactin for the duration of the study and increased serum concentrations of LH. This treatment had no effect on the testosterone concentration or on testis size. Neither chronic treatment with melatonin throughout the period of crudesence nor passive immunization against melatonin for 79 days affected the circulating concentrations of prolactin, LH, testosterone or testis size. These results show clearly that, unlike in other seasonally breeding species, prolactin does not play a significant role in testis growth in the mink. Administration of melatonin to male mink in October did not affect testis growth, presumably because the melatonin signal that cues photoperiodic events had already been received. Administration of antibodies against melatonin did not affect any of the features measured, suggesting that melatonin may have neural but not peripheral effects. Further support for this view can be found in the absence of an influence of melatonin on testis growth or on the plasma concentration of testosterone.

Introduction

Melatonin and prolactin exhibit seasonal as well as circadian fluctuations in the plasma of mammals (melatonin: Goldman *et al.*, 1981; prolactin: Harris and Murphy, 1981; Agu *et al.*, 1986). The seasonal rhythms arise because synthesis and secretion of these hormones are mediated by photoperiod. In mammals, melatonin is synthesized and secreted in the dark phase of the light cycle (see review by Steinleider and Niklowitz, 1992). In mink, as in other species, the pineal gland is the source of melatonin (Ravault *et al.*, 1986; Tillet *et al.*, 1989), and nocturnal plasma melatonin concentrations are increased for less time as the photoperiod increases to more than 12 h (Allain *et al.*, 1981). Administration of melatonin to female mink prevents the vernal rise in plasma prolactin concentrations (Murphy *et al.*, 1990), and exogenous melatonin administered in July suppresses endogenous prolactin concentrations in male mink (Rose *et al.*, 1985).

In carnivores, the cycle of change in pelage (Rose *et al.*, 1984, 1985), embryonic diapause (Murphy and James, 1974) and testicular crudesence (Duby and Travis, 1972; Allain *et al.*, 1981) are influenced by photoperiod. Recent reports by

Martinet *et al.* (1992a, b) suggest that the annual pelage cycle is controlled endogenously, while the testicular cycle depends on photoperiodic change. The seasonal increase in testis volume that begins in October in the northern hemisphere is preceded by an increase in plasma concentrations of androgens (Pilbeam *et al.*, 1979). Ablation of the pineal gland (Boissin-Agasse *et al.*, 1988) or its deafferentation (Maurel *et al.*, 1990) prevent pubertal changes if performed before the beginning of testicular growth. Administration of melatonin to adult or pubertal male mink before the onset of short days (mid-July) triggers FSH and LH secretion, inhibits prolactin secretion (Martinet *et al.*, 1992a) and accelerates testicular growth (Allain *et al.*, 1981; DiGregorio and Murphy, 1987). The effects of photoperiod, transduced by melatonin, on testicular growth may be reflected in the secretion of prolactin in mink. Treatment of male mink with slow-release melatonin implants in late June causes a rapid decrease in the plasma concentrations of prolactin after mid-July to concentrations similar to those found during November (Rose *et al.*, 1985).

During long days, the duration of the nocturnal rise in serum melatonin concentrations is brief, and the concentration of prolactin is high when testis mass and the plasma concentration of testosterone are increasing in Syrian hamsters (Bartke *et al.*, 1975). Bex *et al.* (1978) reported that prolactin increases the sensitivity of the hamster testis to LH. However, this interaction was not observed in the male blue fox (Smith *et al.*, 1987). The roles of melatonin and prolactin in the onset and completion of testicular growth in mink are not well understood. The objective of this study was to examine the effects of

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Fig. 1. Experimental design. The experiment was conducted between 1 October and 4 March. Treatment protocols are shown above the calendar axis: (i) administration of a single implant containing 5 mg melatonin (MT); (ii) 84 mg ovine prolactin delivered s.c. by an Alzet osmotic minipump every 4 weeks for 110 days beginning on 1 November (day 32) (PRL); and (iii) passive immunization with rabbit serum containing an antibody against melatonin once a week for 11 weeks (MT Ab). The time course for sample collections is below the calendar axis. Blood samples were collected and the length of the left testis was measured biweekly.

altered concentrations of these two hormones on gonadal development in ranch mink.

Materials and Methods

Animals and treatments

Prepubertal male mink (*Mustela vison*) of the Pastel variety were purchased from Montgomery Fur Ranch (Wetaskiwin, Alberta). Animals were housed outdoors under an ambient photoperiod and temperature at 52° latitude. They were fed a daily ration purchased from the Montgomery Fur Ranch, consisting of fish and poultry offal, cereals, vitamins and minerals, which is a diet known to be compatible with successful reproduction. Water was provided *ad libitum*. The experimental protocol is illustrated in Fig. 1. Six mink received an implant containing 5 mg crystalline melatonin (Wildlife Laboratories, Fort Collins, CO) administered s.c. on 1 October (day 0). Previous studies (B. D. Murphy, unpublished) have shown that melatonin is released linearly for 1 year from these implants. A second group of six kit males were implanted with Alzet osmotic minipumps (Palo Alto, CA) containing 84 mg ovine prolactin (NIH AFP-8277E) on 1 November. The implant released 0.5 mg prolactin per day and was inserted s.c. in the scapular region under general anaesthesia. The pump was replaced every 4 weeks and a final replacement was inserted on 18 February. A third group of six kit males were injected with rabbit polyclonal antiserum to melatonin every 7 days until day 79. The treatment protocol, developed in a pilot study, was such that injections of 0.5 ml antiserum kg^{-1} bodymass every 7 days maintained titres of melatonin antibody detectable at 1:1000 dilution of serum. An untreated group of six kit males served as controls.

Jugular blood samples were taken after immobilization with 0.4 mg ketamine kg^{-1} (Rogarsetic, Rogar ST, Montréal, PQ) and the left testis was measured between 10:00 h and 15:00 h on 1 October and biweekly thereafter up to day 155 of the experiment. Blood was allowed to clot overnight at 4°C

and was centrifuged at 1000 *g* for 30 min to collect serum. Anti-melatonin titres were evaluated in serum from mink that had received rabbit anti-melatonin antiserum, by determining binding after incubation with 3.0×10^4 c.p.m. of [^3H]melatonin (*N*-acetyl-5-methoxytryptamine, 87 Ci mmol^{-1} ; New England Nuclear, Boston, MA) for 15 h at 4°C.

Immune response to prolactin infusion

Antibody titres against prolactin were measured in animals treated with exogenous hormone using a modification of the prolactin radioimmunoassay reported by Agu *et al.* (1986), in which the binding of ^{125}I -labelled canine prolactin to diluted serum samples was determined. Total specific binding was not greater than nonspecific binding at dilutions of serum between 1:4 and 1:100, indicating that none of the mink developed antibodies to prolactin during its administration.

Production of rabbit anti-melatonin antibody

Rabbits were immunized biweekly for 6 weeks with 0.3 mg melatonin conjugated to BSA. Antiserum diluted 1:1000 specifically bound 35% of [^3H]melatonin added in a primary binding assay consisting of 0.1 ml of antiserum diluted 1:1000, 100 μl of [^3H]melatonin at 30 000 c.p.m. and PBS to a final volume of 0.6 ml. Antibodies in the plasma of the rabbit with the highest titre of melatonin antibody were purified by dialysis and passed over a DEAE Affi-Gel Blue column (BIO-RAD, Richmond, CA). The eluate was concentrated and dialysed against PBS overnight at 4°C and stored at -20°C until administered as described above.

Radioimmunoassays

A double antibody radioimmunoassay for prolactin that had previously been validated in mink (Murphy *et al.*, 1990) was used. The intra-assay coefficient of variation calculated between duplicates ranged from 2.1 to 9.5%. The interassay coefficient

Fig. 2. Mean ($\pm$ SEM) serum concentrations of prolactin between 1 October and 4 March in male mink that were either untreated ($\square$) or infused with 0.5 mg ovine prolactin ($\circ$) day⁻¹ for 110 days beginning on day 32 (1 November). Asterisks indicate significant differences between means on the day the sample was taken ($P < 0.05$).

of variation of one sample assayed at 24 ng ml⁻¹ in four assays was 33.7%. The lower limit of sensitivity of the assay was 0.3 ng ml⁻¹.

Testosterone concentration was determined in a single radioimmunoassay in 0.1 ml of all serum samples following petroleum ether:benzene extraction using an antiserum supplied by N. C. Rawlings (NCR-89, Cook *et al.*, 1982). The tracer was [³H]dihydrotestosterone [1,2,4,5,6,7-³H(N)] (New England Nuclear, Boston, MA). Dextran-coated charcoal was used to separate bound from free radiolabelled antigen. The intra-assay coefficient of variation calculated between duplicates was 9.7%. Assays of LH in duplicate aliquots of 0.1 ml mink serum were conducted by the double antibody radioimmunoassay procedure using GDN no. 15 anti-ovine LH antibody, as described by Murphy (1979). All samples were evaluated in a single assay and the intra-assay coefficient of variation calculated between duplicates was 10.4%.

Statistical analyses

Mean $\pm$ SEM values were calculated for hormone concentrations and the length of the left testis at each sampling date. Analysis of variance for repeated measures using the general linear models procedure (SAS) was used to determine significant differences within and among treatments (Steele and Torrie, 1981). A one-way analysis of variance was used to evaluate changes in testis length. When significant values were found, individual comparisons between treatments were made using the Student–Newman–Keuls test.

Results

Effects of prolactin

Shortly after the infusion of prolactin began, the serum concentrations of this hormone increased significantly ($P < 0.01$; Fig. 2). The overall mean serum concentrations of LH were significantly higher than controls ($P < 0.05$) in animals

Table 1. Overall mean ($\pm$ SEM) serum concentrations of LH in male mink for each of the treated groups from 1 October to 4 March

Treatment	<i>n</i>	Serum LH concentration (mean $\pm$ SEM)
Control	4	3.42 $\pm$ 0.45 ^{bc}
Melatonin	5	4.43 $\pm$ 0.41 ^b
Prolactin	5	8.48 $\pm$ 1.37 ^a
Melatonin–Ab	5	2.69 $\pm$ 0.27 ^c

Values with different letters are significantly different ($P < 0.05$).

treated with prolactin (Table 1). However, serum concentrations of testosterone or the mean testis length did not differ from control values at any time throughout the experiment (Fig. 3a, b) ($P > 0.05$).

Effects of chronic melatonin

Chronic treatment with melatonin did not alter the length of the left testis on any of the sampling days of the experiment (Fig. 3a). Furthermore, exogenous melatonin had no effect on serum concentrations of LH or testosterone (Table 1; Fig. 3b). Administration of 5 mg melatonin to pubertal mink for 155 days did not affect serum concentrations of prolactin (Fig. 3c).

Passive immunization against melatonin

The change in left testis length following passive immunization against melatonin followed the trend seen in control mink. There were brief episodes when the length of the left testis was less in antibody-treated animals than in controls (days 141 and 155; $P > 0.01$; Fig. 3a). Otherwise, there were no detectable differences in testis length between the two groups.

Passive immunization appears to transiently alter serum concentrations of testosterone relative to controls (Fig. 3b). On day 29, the serum concentration of testosterone was significantly higher in antibody-treated mink compared with controls ($P > 0.001$). The serum testosterone concentration had returned to control amounts by day 42 and was not different from controls for the remainder of the experiment.

Injections of polyclonal melatonin antiserum did not alter serum concentrations of prolactin in comparison to controls (Fig. 3c). The apparent increase in serum prolactin in the antibody-treated group relative to controls seen on days 58 and 126 was not statistically significant. There appeared to be no effect of passive immunization on serum concentrations of LH compared with controls throughout the experiment (Table 1).

Discussion

In this study, prepubertal males were used so that the changes observed in testicular function would reflect pubertal development before the first breeding season. Comparison of puberty and annual recrudescence in male mink suggests that

Fig. 3. (a) Mean ($\pm$ SEM) length of the left testis measured biweekly between 1 October and 4 March in male mink that were untreated ($\square$), implanted s.c. with an implant containing 5 mg melatonin on day 0 (1 October) ($\blacksquare$), infused with 0.5 mg ovine prolactin day⁻¹ beginning on day 32 (1 November) for 110 days ($\circ$), or passively immunized against melatonin with 0.5 ml antiserum kg⁻¹ bodymass every 7 days for 11 weeks ($\bullet$). (b) Mean ($\pm$ SEM) serum concentrations of testosterone from 1 October to 4 March in male mink that were untreated ($\square$), passively immunized against melatonin with 0.5 ml antiserum kg⁻¹ bodymass every 7 days for 11 weeks ($\bullet$), implanted s.c. with an implant containing 5 mg melatonin on day 0 (1 October) ($\blacksquare$), or infused with 0.5 mg ovine prolactin day⁻¹ beginning on day 32 (1 November) for 110 days ($\circ$). (c) Mean ($\pm$ SEM) serum concentrations of prolactin from 1 October to 4 March in male pubescent mink that were untreated (control, $n = 4$; $\square$), injected with 0.5 ml melatonin antiserum kg⁻¹ bodymass every 7 days for 11 weeks ($n = 5$; $\bullet$), or implanted s.c. with an implant containing 5 mg melatonin on day 0 (1 October) ($n = 5$; $\blacksquare$).

these processes are similar. For example, the initiation of an increase in testis volume that leads to attainment of breeding capability begins in October in the northern hemisphere in both prepubertal and adult animals (Pilbeam *et al.*, 1979; Martinet *et al.*, 1992a). The development of spermatogenesis (Pelletier, 1986) and increases in circulating testosterone concentrations (Pilbeam *et al.*, 1979) follow a time course that is indistinguishable between young and adult mink. The measurement of the plasma testosterone concentration and testis length in controls in the present study demonstrated the expected pattern of changes.

The temporal relationship between maximum prolactin concentrations and the onset of testicular regression in mink (Martinet *et al.*, 1992a, b) suggests that prolactin may have an effect on the testes. A goal of the present study was to determine whether artificially increased prolactin concentrations had an effect on the process of testicular development. The results demonstrate that animals treated with minipumps releasing prolactin have circulating concentrations of prolactin up to 30 times greater than controls, with no observable effect on the testosterone concentration or on testis length. Although there was an overall increase in the serum concentration of LH, the pattern of testicular development was not altered in this group compared with that of controls. The lack of an effect of prolactin cannot be attributed to the mounting of an immune response, as no circulating antibodies against prolactin could be detected. We have previously shown that ovine prolactin administered by minipump induces luteal activation in female mink and is therefore biologically active in this species (Murphy *et al.*, 1990). We therefore conclude that prolactin has no effect on the course of testicular development in mink, once the process has been initiated.

The dissociation of the prolactin and testicular cycles is consistent with the findings of Martinet *et al.* (1992a), who reported that animals maintained on constant photoperiods displayed the annual pattern of prolactin concentrations in the absence of testicular recrudescence. Together, these findings distinguish the process of testicular development in mink from that of other seasonal breeders such as hamsters (Bartke *et al.*, 1975), in which prolactin appears to have an important role in gonadal growth.

In the study reported here neither exogenous melatonin nor peripheral neutralization of melatonin affected the course of testicular development or the patterns of circulating testosterone and prolactin concentrations. Data from the present study are consistent with the view that melatonin serves as a cue to initiate testicular crudesence, which then follows a pre-programmed course to completion. Further support for this view is provided by studies of pinealectomy in mink (Boissin-Agasse *et al.*, 1988) or denervation of the pineal gland by superior cervical ganglionectomy (Maurel *et al.*, 1990), which, if performed during the time of testicular growth, prevents gonadal development. However, denervation of the pineal gland after recrudescence has begun has little effect on the process (Maurel *et al.*, 1990). In other studies, we have shown that if mink are given melatonin implants in July, before testicular crudesence begins, the testes begin to grow some 6 weeks earlier than in control animals (DiGregorio and Murphy, 1987). Thus, the time of melatonin implantation is critical for induction of testis growth in this species.

The site and mode of action of melatonin in mink are not known. Delivery from s.c. implants results in constant, high concentrations of this hormone in the circulation (Martinet *et al.*, 1992a). If it is administered in July, when daylength is longest, it causes a rapid decline in the circulating concentration of prolactin (Rose *et al.*, 1985; Martinet *et al.*, 1992a). A neural site of action can be inferred from studies of the spotted skunk, in which the effects of melatonin on hastening the onset of testicular recrudescence are mimicked by lesions to the suprachiasmatic nucleus of the hypothalamus (Berria *et al.*, 1990). In the present study, the lack of an effect of passive immunization against melatonin implies a neural site, since the antibodies we administered were not expected to cross the blood-brain barrier. Extraneural action is suggested by the occurrence of binding sites for labelled melatonin in the pars tuberalis of sheep (Morgan *et al.*, 1989), spotted skunks (Duncan and Mead, 1992) and ferrets (Weaver and Reppert, 1990). Recent reports indicate that melatonin has a direct effect on the fetal ovine pituitary by reducing the responsiveness of pars tuberalis cells to LHRH in perfused organ cultures (Skinner *et al.*, 1993); melatonin also alters the sensitivity of the pituitary of the western spotted skunk to dopamine (Rozell and Mead, 1993).

In summary, administering prolactin or melatonin from the time of the first observable growth of the testes of the prepubertal mink has no effect on the course of gonadal crudesence. We conclude that melatonin serves as a cue to initiate development and recrudescence of the testes, and that both processes are independent of prolactin.

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