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Endogenous opioids and the control of LH secretion during the reproductive cycle in the ram induced by treatment with melatonin

G. A. LINCOLN

with the technical assistance of Norah ANDERSON and Gillian HAY

MRC Reproductive Biology Unit,
37, Chalmers Street,
Edinburgh EH3 9EW, U.K.

Summary.

To investigate the role of endogenous opioid peptides (EOP) in the inhibitory control of LH secretion in the ram, the acute effects of naloxone (opioid antagonist) on episodic LH secretion were measured in rams at different stages of a reproductive cycle induced by treatment with melatonin. Groups of SCGx rams (functionally pinealectomized) and pineal intact rams were housed under long days (16 h light : 8 h darkness) and treated with alternating 16 week periods with exogenous melatonin (continuous melatonin from silastic implant) and 16 week periods with no exogenous melatonin for 3 or 4 consecutive cycles. The LH response to naloxone (1.6 mg/kg i.v.) was measured at 2-4 week intervals on 9 occasions during one of the treatment cycles. The periodic treatment with melatonin resulted in a clearly defined cycle in the plasma concentrations of LH, FSH, testosterone and prolactin, and associated changes in size of the testes, intensity of the sexual skin flush and moulting of the pelage ; maximum size of the testes occurred 8-16 weeks after the start of each melatonin treatment. Naloxone induced an increase in plasma LH concentrations at all times but the response varied in relation to the stage of the melatonin-induced reproductive cycle. During testicular recrudescence, naloxone induced large increases in mean LH concentration (low frequency, high amplitude LH pulses), at the peak of the reproductive cycle naloxone induced smaller increases in plasma LH (high frequency, low amplitude pulses) and during testicular regression naloxone induced only minor increments in plasma LH. The results are consistent with the role of EOP in the inhibitory control of LH secretion with this system most active during the sexually active phase of the reproductive cycle.

Introduction.

Endogenous opioid peptides (EOP), particularly β-endorphin, play an inhibitory role in the control of LH secretion, apparently acting within the hypothalamus to inhibit the pulsatile release of LHRH from the median eminence (reviews : Kalra and Kalra, 1983 ; Yen *et al.*, 1985 ; Malven, 1986 ; Brooks,

Lamming and Haynes, 1986). The activity of the opioidergic neurones influencing LHRH secretion is modulated by changes in the circulating concentrations of gonadal steroids and much experimental evidence in the laboratory rat indicates that these opioid pathways are involved in the homeostatic mechanism by which gonadal steroids have a negative feedback effect on LH secretion. In the ram, the evidence that EOP influence LH secretion is based on the observations that naloxone (opioid receptor antagonist) induces an increase and morphine (opioid agonist) induces a decrease in mean LH concentrations and LH pulse frequency (Schanbacher, 1982, 1985 ; Ebling and Lincoln, 1985). The EOP mechanisms appear to change in rams related to the seasonal cycle in testicular activity since the opiate drugs have maximal effect in the sexually active phase under stimulatory short days and minimal effect in the quiescent phase under inhibitory long days (Ebling and Lincoln, 1985 ; Lincoln, Ebling and Martin, 1987). By manipulating the gonadal steroid concentrations in castrated rams it has been shown that the decline in the LH response to naloxone in the non-breeding season is not merely due to a decline in testosterone secretion from the testes but involves a change in the functional state of the hypothalamus induced by changes in photoperiod. The hypothesis proposed by these studies is that EOP mechanisms act as part of the steroid feedback control of LH secretion during the active phase of the reproductive cycle but non-opioidergic mechanisms cause the long-term suppression of LH secretion associated with the non-breeding season (Lincoln *et al.*, 1987).

The aim of the current study was to investigate the EOP regulation of pulsatile LH secretion in rams at different stages of a reproductive cycle induced by treatment with melatonin. For this a series of reproductive cycles was induced by a novel treatment protocol based on the observation that the administration of constant melatonin from a silastic implant to rams housed under inhibitory long days will cause development followed by regression of the reproductive axis (Lincoln and Ebling, 1985). The rams were treated periodically with melatonin implants and the EOP regulation of LH secretion was assessed by measuring the acute LH responses to naloxone given at different stages of one of the experimental cycles. Observations were made on groups of pineal intact and functionally pinealectomized (superior cervical ganglionectomy, SCGx) rams.

Materials and Methods.

Animals. — Ten sexually mature Soay rams (mean body weight : 33.2 $\pm$ 0.7 kg) were housed in light-proof buildings near Edinburgh (Lincoln and Short, 1980). Melatonin implants were made from Silastic sheeting (500-1 Dow Corning, Midland, MI, USA) sealed into an envelope with a total surface area of approximately 32 cm^2 containing 1 g melatonin (Sigma Chemicals, Poole, Dorset, UK). The implants were placed beneath the skin above the rib cage using a local anaesthetic. Naloxone hydrochloride (Sterling-Winthrop, Surbiton Surrey, UK) was administered at a dose of 50 mg/animal (mean dose : 1.6 mg/kg *i.v.*).

Experiment 1. — Six long-term SCGx Soay rams which had been superior cervical ganglionectomized 4 years previously (Appleton and Waites, 1955), were housed in a light-proof shed and exposed to long days (16 h light : 8 h darkness, 16L : 8D) from January 1983 to September 1986. At week 16 the rams were given the first melatonin implant which was removed at week 32. Further implants were given for 16 week periods starting at weeks 48, 80 and 112 and the animals were monitored for a further 64 weeks after the final treatment (see figs. 1 & 2).

Experiment 2. — Four pineal intact Soay rams were housed in a separate light-proof shed from April 1984 to July 1987. They were initially exposed to alternating 16 week periods of long days (16L : 8D) and short days (8L : 16D) and then held on long days (16L : 8D) for the remainder of the experiment. After 16 weeks under long days (week 48) the rams were given the first melatonin implant which was removed at week 64. Further implants were given for 16 week periods starting at week 82 and 116 and the animals were monitored for a further 50 weeks after the final treatment (see figs. 5 & 6). In both experiments, the changes in the diameter of the testes, the intensity of the sexual skin flush in the inguinal region, and moulting of the wool from the scrotum were recorded every 2 weeks (Lincoln and Davidson, 1977). The period of rutting behaviour was indicated by the overt aggressive behaviour of the rams. A blood sample was collected from all animals from the jugular vein every week. On 9 occasions during one melatonin-treatment cycle for both experiments, blood samples were collected at 15 min intervals for 4 h before and 10 min intervals for 4 h following the *i.v.* injection of naloxone (50 mg, 1.6 mg/kg). All blood samples were heparinized, and the plasma frozen at − 20 °C.

Radioimmunoassays. — The concentrations of LH, FSH, prolactin and testosterone in the blood plasma were measured by radioimmunoassay (Ebling *et al.*, 1985 ; Lincoln *et al.*, 1987).

Analysis. — An LH pulse was defined as a single LH value which exceeded the previous value by 2 times the intra-assay *c.v.* The significance of the changes in the reproductive parameters and the effect of naloxone was assessed using a split-plot ANOVA.

Results.

Experiment 1.

Long-term reproductive changes. — The treatment of the SCGx rams with melatonin induced clearly defined and synchronised cycles in all the reproductive parameters began before the end of each period of implantation, the nadir of the and full development of the testes and sexual skin colourations occurred during each period when the rams were implanted with melatonin. The decline in these parameters began before the end of each period of implantation, the nadir of the cycle occurred in the middle of the period with no treatment while the onset of recrudescence began before the next period with melatonin. Reciprocal changes occurred in the plasma concentrations of prolactin ; the high levels of prolactin which occurred during the non-implant period were associated with moulting of

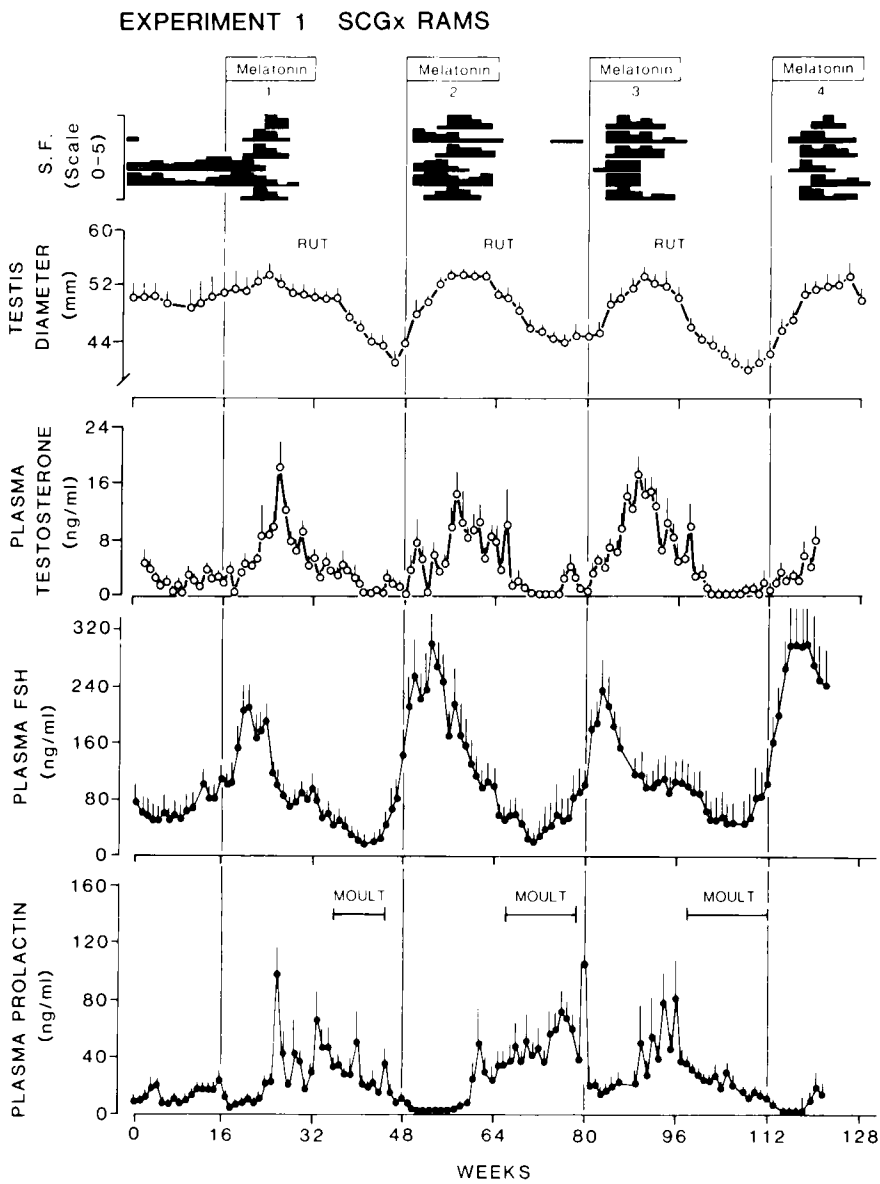

FIG. 1. — *Cyclical changes in the reproductive parameters in 6 SCGx Soay rams housed under long days* (16L : 8D) *and treated with melatonin for 16 week periods* (constant release Silastic implants) *at weeks 16, 48, 80 and 112.* Values are mean ± SEM and SF indicates sexual skin flush.

the pelage. Cyclical changes persisted for more than a year after the end of the periodic treatment with melatonin (fig. 2). The initial onset of testicular redevelopment occurred at a similar time to that during the earlier treatment cycles, however the subsequent testicular cycle was more protracted (table 1).

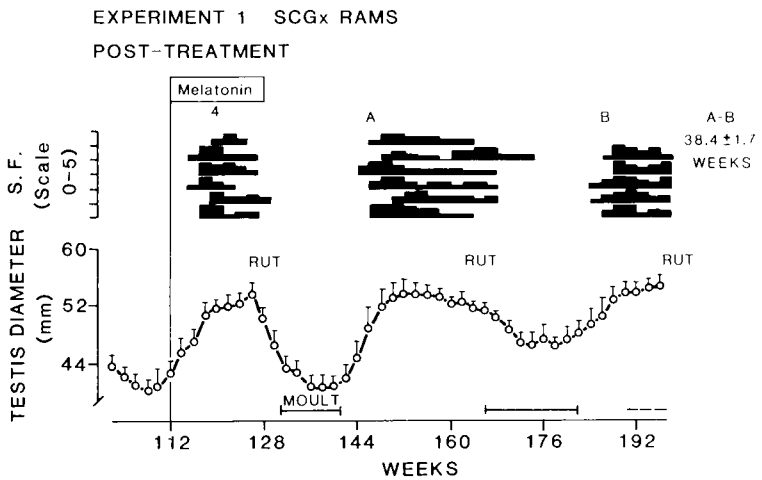

FIG. 2. — *Changes in the diameter of the testes* (mean ± SEM), *intensity of the sexual skin flush* (SF) *and timing of the moult in 6 SCGx Soay rams housed under long days* (16L : 8D) *following the periodic treatment with melatonin* (data continued from fig. 1).

TABLE 1

Duration of the reproductive cycle in a group of SCGx Soay rams (mean ± SEM, n = 6) *treated with melatonin using a 32 week protocol* (16 weeks with and 16 weeks without exogenous melatonin).

	Duration of the cycle (weeks)	
Melatonin treatment	Testis diameter (peak)	Sexual skin flush (onset)
Cycle 1 (wk 48-96)	32.8 ± 0.7	32.0 ± 0.7
Cycle 2 (wk 80-128)	32.5 ± 1.0	34.3 ± 0.6
Post-treatment		
Cycle 3 (wk 112-160)	29.2 ± 1.3	30.7 ± 1.0
Cycle 4 (wk 144-192)	38.8 ± 2.1*	38.4 ± 1.7*

* Significantly different from cycle 1, $p < 0.05$ Student's paired t test.

LH-response to naloxone. — The mean LH concentration and LH pulse frequency (based on pretreatment period) changed significantly (ANOVA, $p < 0.001$) related to the melatonin-induced reproductive cycle ; both parameters of LH secretion were at a maximum during the period of full testicular activity when the rams were implanted with melatonin (figs. 3 & 4). Naloxone (1.6 mg/kg *i.v.*) induced increases in LH secretion at all times, however the response varied significantly (ANOVA, $p < 0.01$) related to the stage of the reproductive cycle. The largest increases in mean LH concentrations occurred early in the cycle when the LH pulses were of high amplitude, while the largest increases in LH pulse frequency occurred during the period of increased testicular activity when the LH pulses were of low amplitude (figs. 3 & 4).

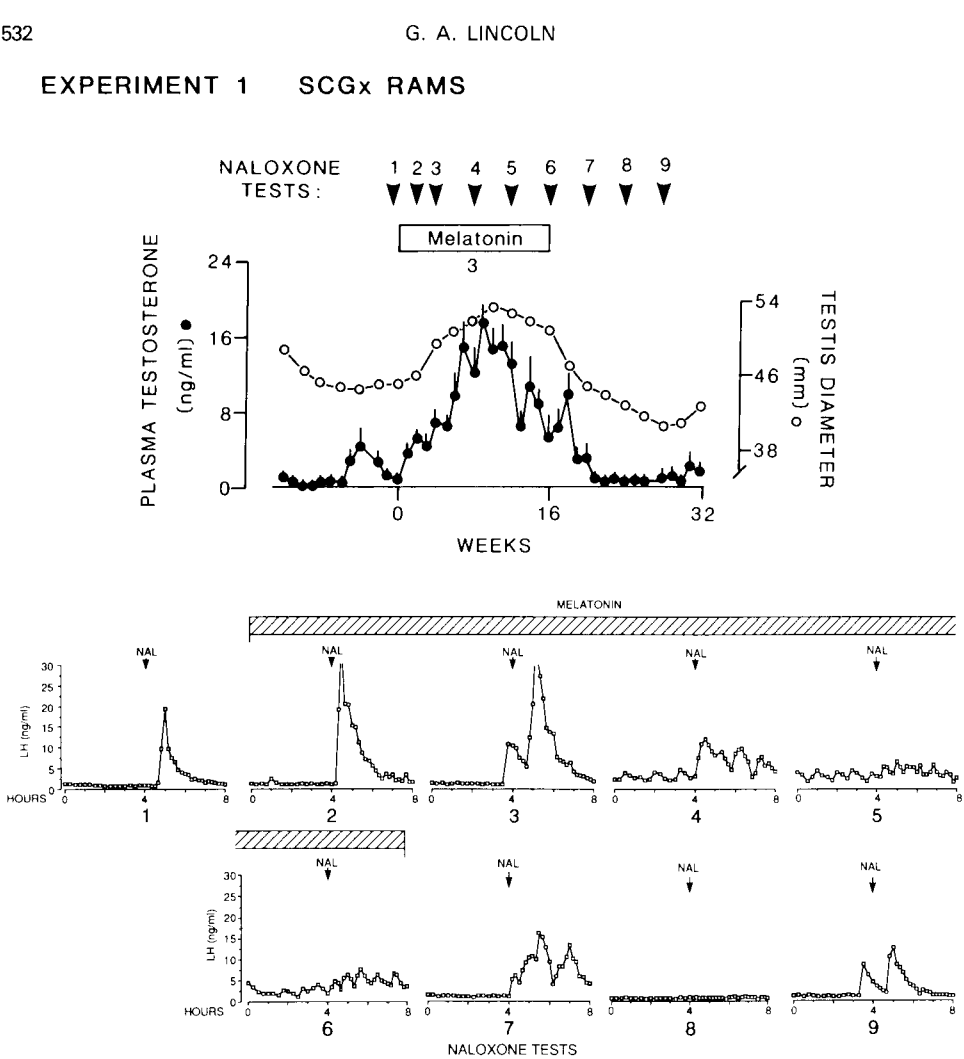

FIG. 3. — *(Top) Testicular cycle in 6 SCGx Soay rams during the third treatment with melatonin* (see fig. 1), *showing the timing of the 9 naloxone tests* (▼). *(Bottom) Changes in the plasma LH concentrations following the i.v. injection of 50 mg naloxone* (1.6 mg/kg) *in one representative SCGx Soay ram studied on 9 occasions before, during and after the treatment with melatonin* (constant release implant : hatched bar).

Experiment 2.

Long-term reproductive changes. — The exposure of the pineal intact rams to a change from long days to short days and back to long days resulted in a cyclical change in all the reproductive parameters (fig. 5). Maximum plasma concentrations of FSH and testosterone and full testicular activity occurred during the period of short days. Similar cyclical changes in these parameters occurred during the periodic treatments with melatonin, with the peak in the reproductive cycle synchronised with the period when the rams were implanted with

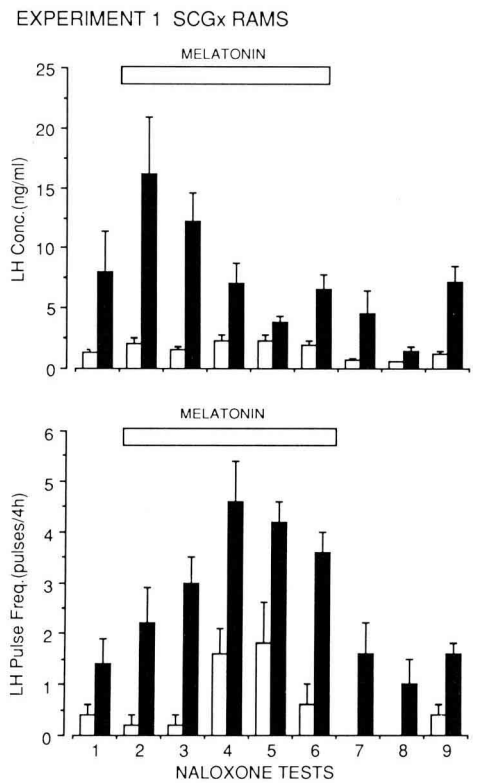

FIG. 4. — *Summary of the changes* (mean ± SEM, n = 6) *in mean plasma LH concentrations and LH pulse frequency in SCGx Soay rams treated with naloxone* (1.6 mg/kg *i.v.*) *based on 4 h pretreatment* (open histogram) *and 4 h post-treatment* (filled histogram). The rams were studied on 9 occasions before, during and after a 16 week treatment with melatonin (constant release implant).

melatonin. The temporal relationship between the different reproductive parameters was similar to that observed in the SCGx rams in the previous experiment. Cyclical changes in testicular activity persisted after the end of the melatonin treatments ; the initial onset of testicular activity was slightly delayed compared to treatment cycles (table 2, fig. 6).

LH response to naloxone. — As in the previous experiment the pattern of LH secretion and the LH response to naloxone changed significantly (ANOVA, $p < 0.01$) in relation to the melatonin-induced reproductive cycle (figs. 7 & 8). The rams were in a relatively sexually active state at the start of the study having not shown a full decline in testicular activity following the first treatment cycle with melatonin (fig. 5). A decline in mean LH concentrations and LH pulse frequency and the response to naloxone occurred during the second half of the study in parallel with the decline in testicular activity (figs. 7 & 8).

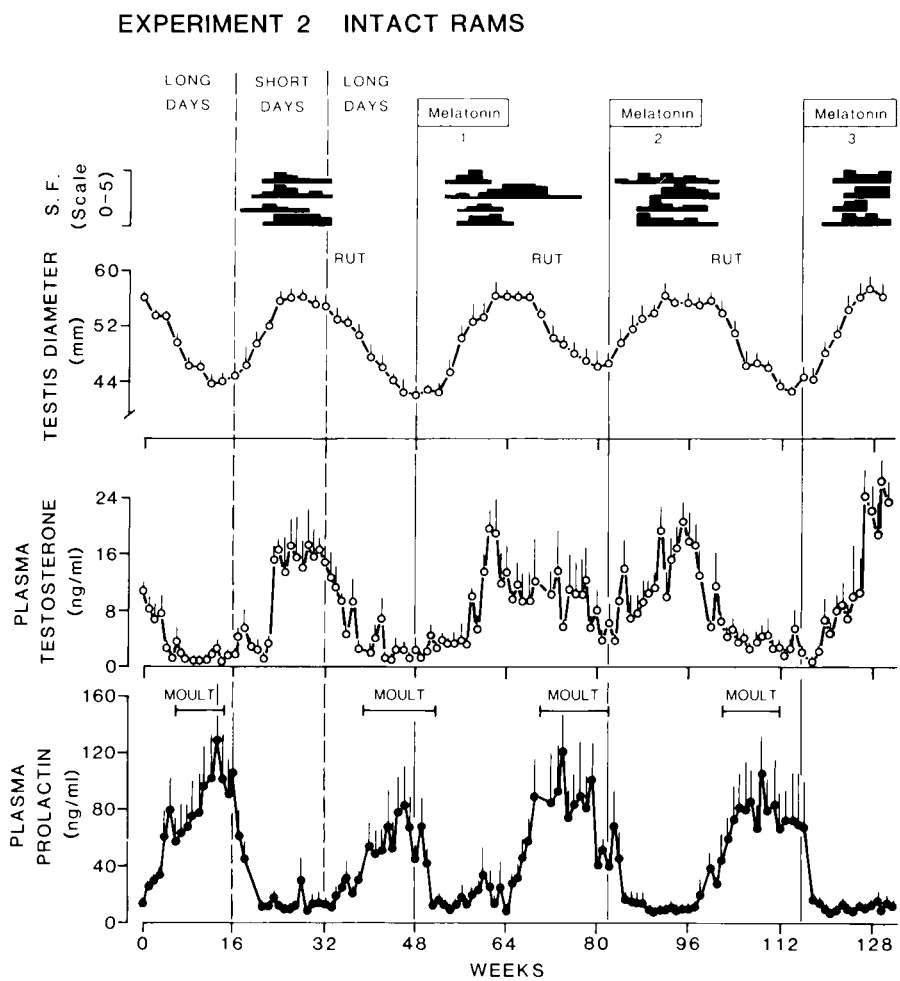

FIG. 5. — *Cyclical changes in the reproductive parameters in 4 intact Soay rams exposed to alternating 16 week periods of long days* (16L : 8D) *and short days* (8L : 16D) *and then maintained on long days and treated with melatonin for 16 week periods* (constant release implants) *at weeks 48, 82 and 116.* Values are mean ± SEM and SF indicates sexual skin flush.

TABLE 2

Duration of the reproductive cycle in a group of pineal intact Soay rams (mean ± SEM, n = 4) *treated with melatonin using a 32-34 week protocol* (16 weeks with and 16-18 weeks without exogenous melatonin).

	Duration of the cycle (weeks)	
Melatonin treatment	Testis diameter (peak)	Sexual skin flush (onset)
Cycle 1 (wk 48-98)	32.8 ± 2.0	32.5 ± 1.1
Cycle 2 (wk 82-132)	33.0 ± 2.2	34.5 ± 1.1
Post treatment		
Cycle 3 (wk 116-166)	35.3 ± 0.9*	37.5 ± 1.5*

* Significantly different from cycle 1, $p < 0.05$ Student's paired t test.

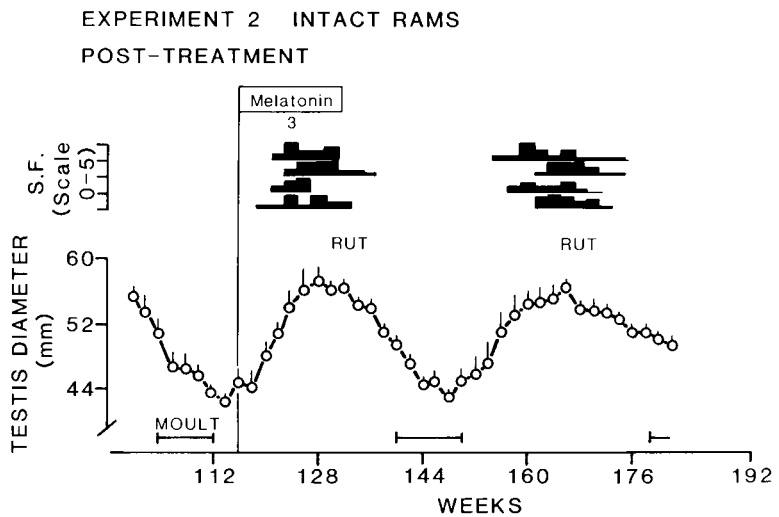

FIG. 6. — *Changes in the diameter of the testes* (mean ± SEM), *intensity of the sexual skin flush* (SF) *and timing of the moult in 4 intact Soay rams housed under long days* (16L : 8D) *following the periodic treatment with melatonin* (data continued from Fig. 5).

Discussion.

We previously demonstrated that pineal intact photo-inhibited Soay rams can be induced to show reactivation of the testicular axis by treatment with a constant release implant of melatonin ; the response is similar to that produced by exposure of rams to short days (Lincoln *et al.,* 1985). The current results extend these observations to illustrate that if the implants of melatonin are given for 16 weeks and removed for 16 weeks it is possible to repeat the procedure and induce a series of complete cycles in testicular activity every 32 weeks. There were associated changes in the plasma concentrations of LH, FSH, prolactin and testosterone similar to those induced by exposure to alternating 16 week periods of long days and short days (Lincoln and Short, 1980).

The results for the SCGx rams are of particular interest since they show that the periodic treatment with melatonin is effective in the absence of a functional pineal gland secreting a normal 24 h pattern of melatonin. In these animals gonadotrophin secretion and testicular activity were at a maximum during each melatonin-implantation period and the treatment resulted in a clearly defined cycle in all the reproductive parameters. Based on these results it is tempting to conclude that the introduction of the exogenous melatonin in each cycle had the effect of stimulating testicular recrudescence. However the results for the post-treatment period when no melatonin was given illustrate that this is not the complete explanation. During this period, cyclical changes in testicular activity continued to occur in the absence of the periodic treatment with melatonin (fig. 2). The initial reactivation of the reproductive axis occurred at the normal

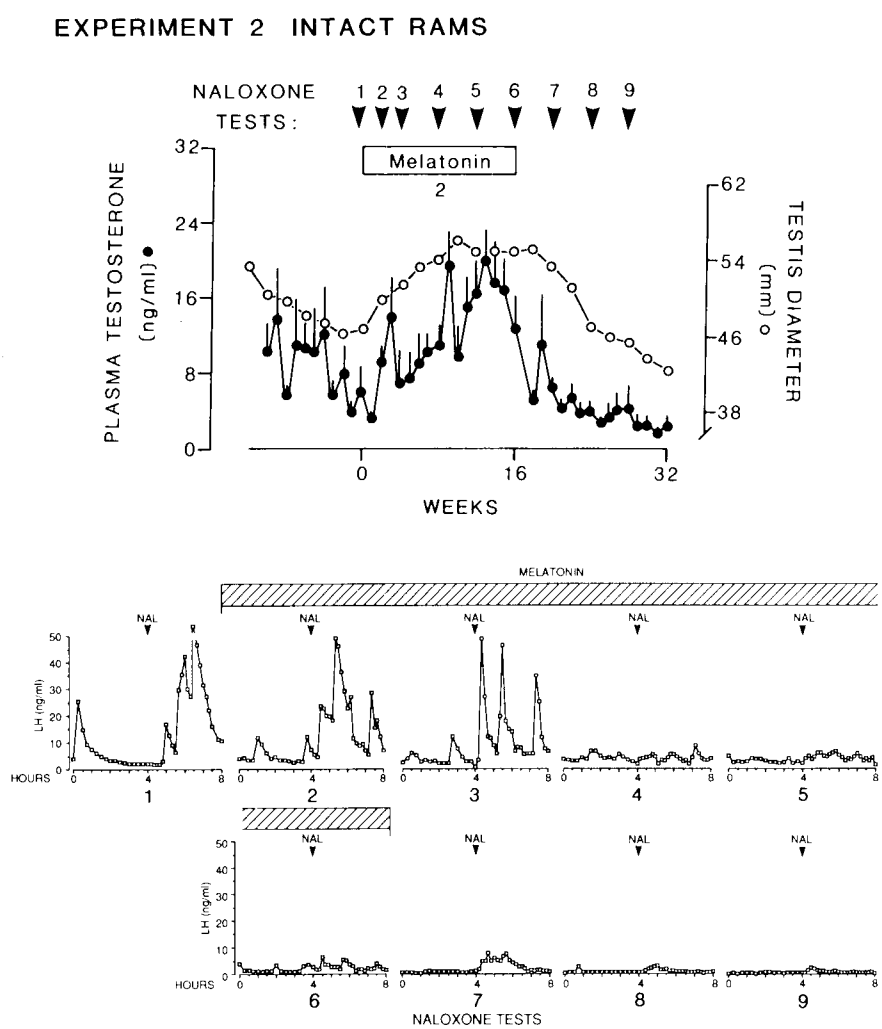

FIG. 7. — *(Top) Testicular cycle in 4 intact Soay rams during the second treatment with melatonin* (see fig. 5) *showing the timing of the 9 naloxone tests* (▼). *(Bottom) Changes in plasma LH concentrations following the i.v. injection of 50 mg naloxone* (1.6 mg/kg) *in one representative Soay ram studied on 9 occasions before, during and after the treatment with melatonin* (constant release implant : hatched bar).

time as in the treatment cycles, while the subsequent phase of testicular activity was more extended (table 1). This indicates that the principal effect of the exogenous melatonin was not to initiate testicular recrudescence but to cause the subsequent decline in testicular function, thus ending the phase of sexual activity. During the melatonin-treatment cycles, the decline in plasma FSH concentrations and the other reproductive parameters was usually evident before the end of the 16 week periods with the melatonin implant, which is consistent with the

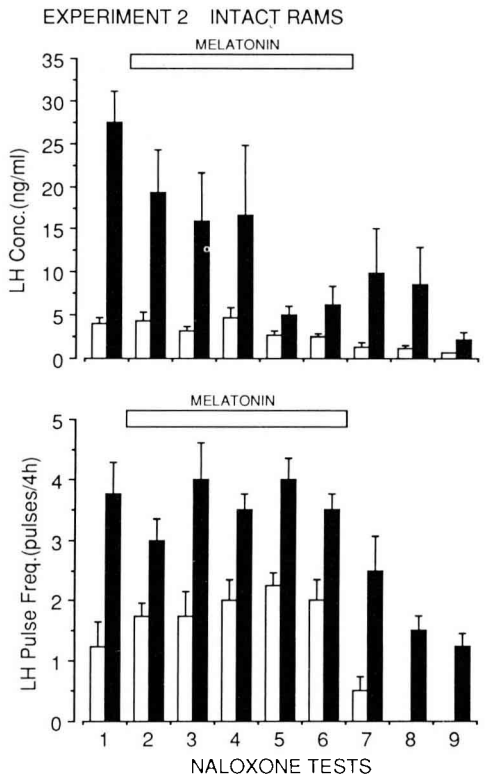

FIG. 8. — *Summary of the changes* (mean ± SEM, n = 4), *in mean plasma LH concentration and LH pulse frequency in intact Soay rams treated with naloxone* (1.6 mg/kg *i.v.*) *based on 4 h pretreatment* (open histogram) *and 4 h post-treatment* (filled histogram). The rams were studied on 9 occasions before, during and after a 16 week treatment with melatonin (constant release implant).

treatment causing the « switch off » of the pituitary-testicular axis. This inhibitory effect appeared to last for at least 8 weeks after removal of the implant as judged from the low plasma gonadotrophin levels, after which the recovery commenced spontaneously to initiate the next reproductive cycle.

The SCGx rams are thought to be unable to respond to photoperiod due to the lack of a functional pineal gland (Lincoln, 1979) ; thus the long term reproductive changes observed in these animals were due solely to the periodic treatment with melatonin. For the pineal intact rams, the situation should be different since they are competent to respond to the long day photoperiod when not being treated with exogenous melatonin. This could account for some of the differences between the intact and SCGx rams, for example the higher plasma concentrations of prolactin in the intact rams during long days. Also the intact rams showed a delay in the testicular recrudescence during the post-treatment phase when melatonin was not administered (table 2). These results indicate that

the treatment with melatonin has the additional effect in the pineal-intact rams of blocking the influence of the prevailing long-day photoperiod which is normally relayed by the endogenous secretion of melatonin.

The role of EOP in the regulation of LH secretion was investigated by measuring the LH response to naloxone at different stages of the reproductive cycle induced by melatonin. On most occasions the single *i.v.* injection of naloxone resulted in an increase in mean LH concentrations and LH pulse frequency which is consistent with an inhibitory role of EOP in the control of the pulsatile release of LHRH from the hypothalamus. The response was found to vary in relation to the reproductive cycle ; maximum responses occurred during the period of increased testicular activity after the treatment with melatonin (fig. 4). This is a somewhat similar result to that obtained in pineal intact rams in which the reproductive cycle was entrained by exposure to alternating 16 week periods of long days and short days ; the maximum LH responses to naloxone occurred during the sexually active phase of the cycle (Lincoln *et al.,* 1987).

The changes in the EOP activity within the hypothalamus may be part of the mechanism by which melatonin affects the long-term reproductive cycle in the ram. If the responses to naloxone can be used as an index of EOP activity, it is evident that this system increases in activity in parallel with the recrudescence of the testes. To what extent these apparent changes in EOP activity are the cause or the consequence of the changes associated with the reproductive cycle cannot be deduced at present. One possibility is that enhanced EOP activity occurring at the peak of the cycle causally induces the changes within the hypothalamus which leads to the the subsequent « switch off » of the hypothalamic-pituitary-testicular axis associated with the non-breeding season. Since one of the principal effects of melatonin has been shown to be the inhibition of reproductive activity, this effect may be due to the activation of the EOP mechanisms within the hypothalamus.

Colloquium on « Neuroendocrine mechanisms and light control of reproduction in domestic mammals » I.N.R.A., Nouzilly, 17-18 September 1987.

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Résumé. *Opiacés endogènes et contrôle de la sécrétion de LH au cours du cycle de reproduction induit par l'administration de mélatonine chez le Bélier.*

Pour rechercher le rôle des opiacés endogènes (EOP) sur le contrôle inhibiteur de la sécrétion de LH chez le bélier, les effets de la Naloxone (antagoniste des opiacés) sur la sécrétion pulsatile de LH ont été mesurés à différents moments du cycle de reproduction induit par un traitement de mélatonine. Des groupes de béliers SCGX (pinéalectomie fonctionnelle) et de béliers ayant leur pinéale intacte, ont été soumis à des jours longs (16L : 8D) et traités alternativement pendant des périodes de 16 semaines avec de la mélatonine exogène (relargage continu de mélatonine par implants silastic), et de 16 semaines sans mélatonine exogène, et ceci pendant 3 ou 4 cycles consécutifs. La

réponse de LH à la Naloxone (1.6 mg/kg *i.v.*) était mesurée à des intervalles de 2-4 semaines à 9 moments de l'un des cycles du traitement lumineux. La période de traitement par la mélatonine a produit un cycle bien défini des concentrations en LH, FSH, testostérone et prolactine, et les changements associés de la taille des testicules, de l'intensité de la turgescence de la peau sexuelle, et de la mue du pelage. La taille maximale des testicules s'est produite 8-16 semaines après le début de chacun des traitements par la mélatonine. La Naloxone a induit une augmentation dans les concentrations de LH à chaque intervalle, mais la réponse a varié selon le stade du cycle de reproduction induit par la mélatonine. Pendant l'augmentation de la taille des testicules, la Naloxone a provoqué une forte augmentation dans la concentration moyenne de LH (faible fréquence, grande amplitude des pulses de LH), au moment le plus élevé du cycle de reproduction la Naloxone a provoqué une augmentation plus faible de la LH plasmatique (fréquence élevée, faible amplitude des pulses), et durant la régression testiculaire la Naloxone a seulement induit de faibles augmentations de LH. Ces résultats confirment le rôle des EOP dans le contrôle inhibiteur de la sécrétion de LH, ce système étant le plus actif durant la période d'activité sexuelle du cycle de reproduction.

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