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Melatonin Acts in the Brain to Mediate Seasonal Steroid Inhibition of Luteinizing Hormone Secretion in the White-Footed Mouse (*Peromyscus leucopus*) (42750)

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Abstract. A melatonin-induced supersensitivity of the gonadotropin-secretory system to the negative feedback action of sex steroids is thought to be important to the timing of seasonal reproduction. However, little is known concerning this action of melatonin. In the present study the antigonadal action of melatonin in the anterior hypothalamus (AH) of the white-footed mouse, *Peromyscus leucopus,* was used to examine the neuroendocrine mechanism whereby melatonin enhances the sensitivity to sex steroid negative feedback. Mice received a melatonin-containing pellet in the AH for 14 weeks, at which time they were castrated and treated sc with a Silastic testosterone (T) capsule for 3 weeks. At the time of castration, weight of the testes and the concentration of T in the blood of mice with a melatonin pellet were greatly reduced compared to mice with a blank (melatonin-free) implant in the AH ($P < 0.01$). In mice treated with melatonin the physiological dose of T significantly reduced the concentrations of LH in blood and pituitary ($P < 0.05$). This dose of T, however, had little effect on LH in mice with a blank pellet in the AH. Melatonin in the AH markedly increased the content of gonadotropin-releasing hormone (GnRH) in the mediobasal hypothalamus ($P < 0.05$) in mice treated with T; however, there was little effect of melatonin and/or T in any other region examined. Melatonin and T had little effect on the contents of immunoreactive β-endorphin (B-EP) in the hypothalamus, but T alone increased the content of B-EP in the preoptic area. These results are evidence that melatonin and T act in concert to induce the reproductively-quiescent state by suppressing secretion of GnRH from the hypothalamus.

The annual reproductive cycle in photoperiodic animals is regulated by seasonal changes in circulating concentrations of gonadotropins. The neuroendocrine basis of this process is not well understood, but there is evidence from studies on sheep and hamsters that both steroid-dependent and steroid-independent mechanisms are involved (1–3). The negative feedback action of sex steroids on secretion of gonadotropins has received particular attention in view of the findings that gonadectomized animals treated with sex steroid exhibit dramatic changes in the concentration of LH in blood throughout the year, with the nadir occurring during the nonbreeding season (1–4). Photoperiod is thought to be important to the seasonal inhibition of gonadotropin secretion because exposure to inhibitory day-length induces a supersensitivity to the negative feedback action of sex steroids on secretion of LH (3, 4). This effect of photoperiod, in turn, probably is mediated by the pineal indolamine melatonin, because in hamsters (5) and ewes (6) supersensitivity to sex steroid negative feedback is induced by treatment with melatonin.

The neuroendocrine mechanism whereby melatonin mediates the effects of photoperiod in the seasonal regulation of gonadotropin secretion is not clear. However, studies in the white-footed mouse (*Peromyscus leucopus*) have provided evidence that melatonin acts in the rostral hypothalamus to mediate the effects of short photoperiod on reproductive state (7, 8). These observations lend credence to the view that melatonin may alter the activity of neuronal system(s) that regulate secretion of gonadotropins (9, 10). Possibly, melatonin may exert its inhibitory action on the secretion of gonadotropins by enhancing the sensitivity of these neuronal systems to the negative feedback effect of sex steroids. In the present study, the pronounced antigonadal action of melatonin in the anterior hypothalamus (AH) of *P. leucopus* (8) was used to determine if melatonin acts in the brain to potentiate the negative feedback action of testosterone (T) on secretion of LH. Moreover, in view of the suspected roles of the GnRH and β-endorphin (B-EP) neuronal systems in the regulation of seasonal breeding (9), the contents of these neuropeptides in the hypothalamus were

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measured to determine whether these systems may be part of the neuroendocrine pathway for melatonin's antigonadal action.

Materials and Methods. *Animals.* Male *P. leucopus,* offspring of a genetically heterogeneous stock derived from mice trapped in central Connecticut, were laboratory reared under long photoperiod (16L:8D, lights on at 08.00 hr). Food (Wayne Lab Blox) and water were supplied ad libitum. The mice were used for experimentation when they were 8 weeks of age. At this age mice raised under 16L:8D are sexually mature. Pineal-intact mice were used in this study, because the antigonadal effect of an intracranial melatonin implant in pinealectomized *P. leucopus* is similar to that in pineal-intact mice (7).

Implants: (a) Intracranial melatonin pellets. Continuous-release intracranial melatonin pellets were prepared as described previously (11). The dose of melatonin released from the pellets (approximately 200 ng/day) does not affect reproduction when administered sc, and is 50 times less than that required to induce gonadal regression in *P. leucopus* when administered by peripheral injection (12). Under Chloropent anesthesia (Fort Dodge Laboratories, Fort Dodge, IA) a unilateral melatonin-containing or melatonin-free (blank) beeswax pellet was placed stereotaxically in the rostral AH, adjacent to the caudal preoptic area (POA; AP = 0.5; L = 0.2, H = 5.4, using the confluence of bregma and sagittal sutures as zero point and head level).

(b) Subcutaneous testosterone capsules. Continuous-release T capsules were prepared by packing 6 mm of a T:cholesterol (Sigma) mixture into a 12-mm length of Silastic tubing (i.d. 0.058 inches, o.d. 0.077 in; Dow Corning Midland, MI). Ends of the tubing were sealed with silicone adhesive (Duro, Cleveland, OH). Before use the capsules were soaked for 2 days in saline at 37°C and 70% ethanol for 5 min. A capsule containing 1% T inserted through the skin in the interscapular region maintained a concentration of T in blood similar to that in intact mice during the gonadally regressed state (0.2–0.5 ng/ml).

Tissue extraction. For extraction of neuropeptides, the brain was removed in a cold room (4°C) and a 2-mm-thick horizontal slice containing the hypothalamus and POA was made on a vibratome (Lancer, Ted Pella, Inc., Tustin, CA). The POA, AH, and mediobasal hypothalamus [MBH; containing the median eminence (ME); Fig. 1] were dissected under a microscope using microscissors and were homogenized in 3 ml of extraction mixture [methanol/formic acid (9/1, v/v). Recoveries of GnRH and B-EP, assessed by the addition of synthetic peptides to samples of cerebral cortex, averaged 89% for both peptides. The neuropeptides were concentrated ventromedially in the tissue blocks, and the data are expressed as content (rather than concentration) to reduce errors arising from any inconsistencies in lateral and/or horizontal limits to the dissections (10; unpublished observations).

Radioimmunoassays: (a) Luteinizing hormone. The concentration of LH in plasma and pituitary was measured by the established heterologous RIA of Niswender *et al.* (13), using GDN 15 antiserum to ovine LH and NIADDK RP2 as reference standard. Serial dilutions of serum or pituitary extract produced displacement curves that were parallel to that of the rat LH standard preparation. Pituitary content of LH was measured by manual homogenization of the whole gland in a 1.5-ml glass microhomogenizer in 1.0 ml 0.01 *M* phosphate-buffered saline. The homogenate was centrifuged at 1000*g,* and the supernatant was stored at −20°C. Intra- and interassay coefficients of variation were 9 and 12%, respectively. The lower limit of sensitivity was 0.2 ng/ml.

(b) Testosterone. The concentration of T

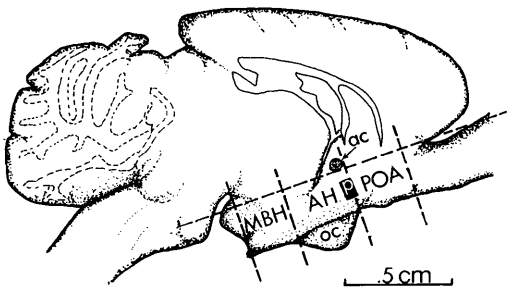

FIG. 1. Diagrammatic parasagittal section of the brain of *P. leucopus* showing the dissection scheme and placement of the intracranial implant. ac, anterior commissure; AH, anterior hypothalamus; MBH, mediobasal hypothalamus; oc, optic chiasma; P, pellet; POA, preoptic area.

in plasma was measured using the validated RIA of Lee *et al.* (14) after extraction with diethyl ether. The intra- and interassay coefficients of variation both were 13%.

(c) Gonadotropin-releasing hormone. The content of GnRH in the hypothalamus was measured by RIA using the Chen-Ramirez CRR11B73 antiserum to GnRH at a final concentration of 1:175,000 (15). The GnRH (Calbiochem, San Diego, CA) used for standard and for iodination was iodinated using Iodogen (Pierce Chemicals, Rockford, IL), and the iodinated peptide was purified on a Sephadex QAE (Q 25 129; Sigma) column eluted with phosphate-buffered saline (pH 8.9). Parallel displacement curves were obtained with serial dilutions of extracts from various brain areas. The intra- and interassay coefficients of variation were 3 and 13%, respectively, and the limit of sensitivity was 0.2 pg. The mass of GnRH in various hypothalamic areas was 0.5–3.0 ng.

(d) β-Endorphin. The content of B-EP in the hypothalamus was measured by RIA using a method similar to that of Roberts *et al.* (16). The antiserum raised against human B-EP (Milab, Accurate Chemical & Scientific Corp. Westbury, NY; batch R-776212) had cross-reactivities of 100% to human B-EP, 69% to human α-endorphin, and <1% to human β-lipotropin. RIA analysis of HPLC fractions of *P. leucopus* brain extract revealed a single immunoreactive peak that coincided with that for human B-EP. Serial dilutions of extracts from various hypothalamic areas produced displacement curves that were parallel to those of human endorphin. The human B-EP (Calbiochem) used for standards and iodination was iodinated using Iodogen, and the iodinated B-EP was purified by adsorption onto an octodecylsilane column (Sep-Pak, Waters Associates, Milford, MA). Iodinated peptide was eluted from the column by increasing concentrations of methanol in 0.1% trifluoroacetic acid and stored at −20°C. Antibody-bound and free B-EP were separated by 30 min incubation with a 0.5% solution of charcoal (Norit A, Sigma; 4°C at pH 7.1) coated with 0.2% dextran, followed by centrifugation at 1000*g*. Radioactivity in the charcoal-adsorbed fraction was measured on a gamma counter for 3 min. The intra- and interassay coefficients of variation (expressed as a percentage of the sample mean of 10 replicate samples in each assay) were 7 and 13%, respectively, and the limit of sensitivity was 30 pg.

Experimental protocol. Mice maintained under long photoperiod (16L:8D) were placed in one of two treatment groups: mice of the first group ($n = 10$) received a melatonin-containing pellet in the AH. Mice of the second group ($n = 10$) received a blank (melatonin-free) pellet in the AH to determine the effect of the implantation procedure on reproductive state. Fourteen weeks after pellet implantation, mice of both groups were anesthetized, and 100 μl of blood for RIA of T was collected via puncture of the orbital sinus. The mice then were castrated and received a subcutaneous implant that contained ($n = 5$) or did not contain ($n = 5$) a 1% mixture of T. Testes weight and the concentration of T in blood were used to confirm the antigonadal effect of the intracranial melatonin implants. After 3 weeks of treatment with steroid, the animals were killed by rapid decapitation at 0900 hr and blood and brain tissues were harvested for hormone analyses. It is important to note that the animals were castrated after 14 weeks of treatment with melatonin, rather than at the beginning of the experiment, in order to maintain a normal (endogenous) secretion of T throughout the course of gonadal regression.

Statistics. Differences in continuously varying measurements were tested using an ANOVA. In cases where a significant treatment effect was obtained, comparison of the means was made using the Tukey HSD procedure. The level for statistical significance was $P < 0.05$.

Results. *Reproductive state.* At the time of castration (after 14 weeks of treatment with a melatonin-containing or a blank pellet in the AH) melatonin had caused a 56% reduction in paired testes weight, relative to the blank controls (201 ± 30 mg vs 461 ± 25 mg; $P < 0.001$). Gonadal regression was evident in all of the mice treated with melatonin, and was accompanied by a significant decrease in the circulating concentration of T, relative to the controls (0.3 ± 0.1 ng/ml vs 1.6 ± 0.4 ng/ml; $P < 0.001$).

Secretion of LH. For castrated mice with a melatonin implant in the AH, treatment

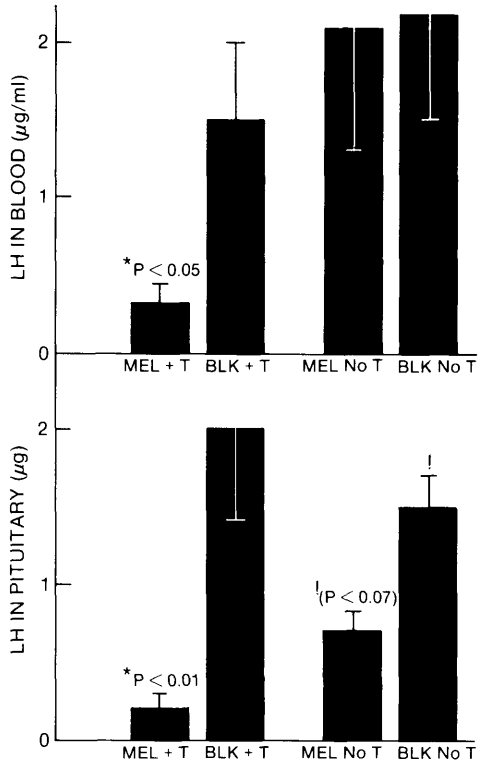

FIG. 2. Effect of a melatonin pellet in the anterior hypothalamus and testosterone on LH in blood and pituitary of castrated mice. Bars are means ± SEM. * For LH in blood and pituitary, the melatonin (Mel) + testosterone (T) group was significantly less than all other groups (probability values shown above bars). ! in the lower panel denotes the nearly significant effect of melatonin alone on the mass of LH in whole pituitary. BLK, blank implant in the anterior hypothalamus.

with T for 3 weeks significantly reduced the concentration of LH in blood (Fig. 2; $P < 0.05$). The concentration of LH in blood was not affected by separate treatments with either melatonin or T alone. Melatonin and T markedly reduced the content of LH in the pituitary gland (Fig. 2; $P < 0.01$). The content of LH also tended to be lower in mice treated with melatonin alone ($P < 0.07$).

Contents of neuropeptides. For mice with a melatonin pellet in the AH, T caused a significant increase in the content of GnRH within the MBH (Table 1). There was little effect of this treatment on the contents of GnRH in the AH or POA, and treatments with either melatonin or T alone had little effect on GnRH content in any region examined. The content of radioimmunoassayable B-EP in the hypothalamus was not affected by melatonin treatment; however, there was a T-induced increase in B-EP in the POA in mice not treated with melatonin. This effect of T also was apparent in mice with a melatonin implant, but did not reach the level of statistical significance.

Discussion. The timing of seasonal reproduction in hamsters (3) and sheep (4) involves a melatonin-induced increase in sensitivity to the negative feedback action of sex steroids on secretion of gonadotropins. The present study is the first to demonstrate that melatonin may induce the gonadally quiescent state by acting in the brain to enhance sensitivity to sex steroid feedback. In male *P. leucopus,* a melatonin pellet in the AH potentiated the inhibitory action of a dose of T that had little effect in the absence of exogenous melatonin. The steroid-dependent decrease in concentration of LH in the blood by the intracranial melatonin implant was similar to that in observed in gonadectomized Syrian hamsters (5) and ewes (6), after sc administration of melatonin. Melatonin also may have a steroid-independent mode

TABLE I. EFFECT OF MELATONIN AND TESTOSTERONE ON CONTENTS OF GONADOTROPIN-RELEASING HORMONE (GnRH) AND β-ENDORPHIN (B-EP) IN THE HYPOTHALAMUS*

	GnRH (ng/region)			B-EP (ng/region)		
Treatment	POA	AH	MBH	POA	AH	MBH
Mel + T	0.4 ± 0.2^{a}	0.5 ± 0.2^{a}	1.6 ± 0.4^{a}	87 ± 11^{a}	67 ± 9^{a}	44 ± 10^{a}
Mel no T	0.5 ± 0.3^{a}	0.6 ± 0.2^{a}	$0.7 \pm 0.3^{a,b}$	$60 \pm 14^{a,b}$	75 ± 12^{a}	63 ± 7^{a}
Blk + T	0.1 ± 0.1^{a}	0.7 ± 0.4^{a}	0.3 ± 0.1^{b}	91 ± 4^{a}	69 ± 12^{a}	57 ± 11^{a}
Blk no T	0.3 ± 0.2^{a}	0.6 ± 0.1^{a}	$0.9 \pm 0.5^{a,b}$	41 ± 4^{b}	58 ± 6^{a}	53 ± 5^{a}

* Values are means ± S.E.M. Within a region means with different superscripts are significantly different ($p < 0.05$).

of action, because the content of LH in the pituitary in castrated *P. leucopus* without a T implant was decreased 50% by a melatonin pellet. A steroid-independent effect of short photoperiod on aminergic activity in the hypothalamus has been observed in the male Syrian hamster (17).

Knowledge of the site and mechanism for melatonin's inductive effect on sex steroid negative feedback is essential to understanding the neuroendocrine basis of seasonal reproduction. However, the target for melatonin still is uncertain, and there is evidence that melatonin may act at a variety of sites to regulate reproductive processes (18–20). The hypothalamus probably is a prominent site for the action of melatonin in *P. leucopus,* because placement of a melatonin-containing pellet (8, 9) or microimplant (7) in the AH-POA region of this mouse causes dramatic regression of the gonads, and potentiates the inhibitory action of T on secretion of LH (the present study). In the Syrian hamster selective neurotoxic lesions of the anterior hypothalamic nuclei abolish the antigonadal response to short photoperiod (21) and microinjection of melatonin into this region affects the activity of AH-POA neurons (22).

The demonstration of a hypothalamic target for melatonin is of significance with regard to the finding that the contents of GnRH in the hypothalamus of *P. leucopus* are increased by a melatonin pellet implanted subcutaneously (23) or in the AH (9, the present study). In the Syrian hamster, exposure to short photoperiod generally has been found to increase hypothalamic GnRH (24–27), although some authors have not observed this (16). On the basis of these observations, it is probable that the GnRH neurons are affected by the photoperiod–melatonin signal. It is hypothesized that the increased content of GnRH in the MBH during the gonadally regressed state reflects continued synthesis and reduced secretion of GnRH. This idea is strengthened by the recent finding that seasonal inhibition of LH by sex steroids in the ewe is associated with a blockade of release of GnRH into hypothalamo–hypophyseal blood (28).

The B-EP neuronal system also may play a major role in the regulation of seasonal breeding. Short photoperiod-induced gonadal regression in the Syrian hamster (16) and *P. leucopus* (20) is accompanied by a nocturnal increase in the contents of B-EP in the AH-POA and a loss of the stimulatory effect of naloxone on secretion of LH (16). In the present study treatment with melatonin and T had little effect on hypothalamic B-EP; however, the mice were killed in the morning (0900 hr), and the increase in hypothalamic B-EP associated with gonadal regression in *P. leucopus* and Syrian hamster is apparent during the night (2100 hr), but not in the morning (0900 hr). Although time of sampling may have precluded observation of an effect of hormone treatment on B-EP, results from the present study corroborate previous observations that change in reproductive state is not associated with a morning alteration in activity of the hypothalamic B-EP system.

In summary, results from the present study provide the first evidence that melatonin may act in the brain to induce supersensitivity of the LH secreting system to inhibition by sex steroid. This action may involve a suppression in the release of GnRH from the hypothalamus, as indicated by the increased contents of GnRH in the MBH in mice treated with melatonin and T. Melatonin also may act independently of sex steroids to reduce the contents of LH in the pituitary gland. The neurochemical basis for these actions of melatonin remains to be determined.

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