A Novel Melatonin Antagonist, *N*-(2,4-Dinitrophenyl)-5-Methoxytryptamine Neutralizes Some Effects of Melatonin in the Female Syrian Hamster (42926)

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Abstract. **In this present study we evaluated the ability of a recently synthesized melatonin antagonist, *N*-(2,4-dinitrophenyl)-5-methoxytryptamine (ML-23), to antagonize the effects of afternoon injections of melatonin on the reproductive and thyroid axes in the female Syrian hamster. Thirty-six animals were divided into four groups and treated daily for 13 weeks with an afternoon injection of melatonin (25 μg/injection) or saline diluent. ML-23 was given via the drinking water to both melatonin- and saline-treated groups. The experiment was continued until 78% of melatonin-treated animals exhibited acyclicity. The results show that ML-23 partially reversed the effects of melatonin on pituitary follicle-stimulating hormone concentrations but was without effect on the decreased pituitary and plasma prolactin concentrations induced by melatonin treatment. Furthermore, ML-23 antagonized the effects of melatonin on plasma thyroxine levels and significantly increased plasma triiodothyronine concentrations and the free triiodothyronine index when used in combination with melatonin. The decrease in ovarian weight and plasma estradiol, but not progesterone, obtained with melatonin treatment also was reversed by ML-23.**

Our data suggest that ML-23 prevents the effects of melatonin treatment on ovarian weight, pituitary follicle-stimulating hormone levels, plasma estradiol, and thyroxine concentrations in the female Syrian hamster. Since ML-23 did not prevent the effects of melatonin on pituitary weight, plasma luteinizing hormone and prolactin, and pituitary prolactin concentrations, the actions of ML-23 may involve only peripheral sites of action of melatonin. Alternatively, the dose of ML-23 may not have been optimal to prevent all of the central effects of the indoleamine. [P.S.E.B.M. 1989, Vol 191]

Melatonin, an indoleamine secreted by the pineal gland, interacts with the hypothalamic-pituitary-gonadal axis of most mammals and these effects have been widely documented, particularly in rodents (1–3). Although the action of melatonin on the endocrine system has been investigated in many different experimental protocols, its site and mechanism of action remain obscure. Prolonged exposure to reduced photoperiods (less than 12.5 hr of light/day) or to afternoon melatonin injections renders female Syrian hamsters infertile; this phenomenon is completely reversed by pinealectomy (4, 5) or superior cervical ganglionectomy (1, 2). In fact, the estrous cycles of short photoperiod or melatonin-treated animals are disrupted (6, 7), the uteri become infantile (6, 8), and the ovarian weights usually increase due to the marked proliferation of the interstitial tissue (8, 9). As far as the hormone levels are concerned, gonadotropin secretion is unexpectedly augmented in light-restricted female animals and pituitary reserve of these hormones is greater than it is in hamsters kept under a long photoperiod (10). A marked decrease in pituitary and plasma prolactin is usually observed in pineal-mediated reproductive regression in the female Syrian hamster (8, 11).

Recently, the existence of a reciprocal interaction between the pineal and the thyroid gland has been proposed (12). In this scheme, thyroid secretory products may influence the growth of the pinealocytes while pineal hormones, melatonin and 5-methoxytryptamine, negatively influence the proliferation and mean nuclear volume of thyroid follicular cells (12, 13). Additionally, short photoperiod (14) or melatonin treat-

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ment (15, 16) reduces circulating thyroxine (T_4) concentrations in female Syrian hamsters.

A novel melatonin antagonist, *N*-(2,4-dinitrophenyl)-5-methoxytryptamine, has been recently synthesized by Zisapel and Laudon (17), providing an interesting tool in elucidating the sites and modes of melatonin action. This compound has been found to be a potent antagonist of melatonin effects on dopamine release from female rat hypothalamus *in vitro* (17) and in reversing many of the effects of melatonin on reproductive physiology in this species. Since the Syrian hamster has been extensively used to examine the interrelationship between melatonin and the neuroendocrine system (17, 18), we investigated the ability of this antagonist to prevent the effects of afternoon injections of melatonin on the neuroendocrine-reproductive and thyroid axes in female Syrian hamsters.

Materials and Methods

Animals. Thirty-six female Syrian hamsters (Sasco) were housed in a temperature- and light-controlled room (22 ± 2°C; 14:10 light:dark cycle, lights on from 0600 to 2000 hr, using a cool-white fluorescent illumination). They had free access to water and standard laboratory chow and were 45 days old at the onset of the experiment.

Chemicals. Melatonin was obtained from Sigma Chemical Co., St. Louis, MO. The melatonin solution was freshly prepared daily just before the injections; it was dissolved in 50 μl of absolute ethanol prior to dilution with saline. The dose of melatonin was 25 μg/injection, administered subcutaneously in 0.1 ml of alcoholic saline at 1700 hr.

The melatonin antagonist, *N*-(2,4-dinitrophenyl)-5-methoxytryptamine (ML-23), was prepared by one of the authors (Dr. Nava Zisapel, University of Tel Aviv, Tel Aviv, Israel). The ML-23 was given via the drinking water at the dose of 0.04 mg/liter. The solution was prepared fresh every 3 days and the bottles were covered with aluminum foil to prevent light-induced damage of the drug. Each hamster ingested approximately 1–2 μg/day. All other chemicals, reagents, and solvents used in this study were obtained from standard commercial sources.

Experimental Protocol. Two groups of animals (nine each) received the ML-23 in the drinking water and received injections late in the afternoon either with melatonin or 0.9% alcoholic saline diluent. Two other groups did not receive the ML-23 but were injected either with 25 μg of melatonin or the vehicle.

In order to monitor the functional status of their reproductive system, daily vaginal cyclicity examination was performed. Syrian hamsters have highly regular 4-day estrous cycles; the cycles can be easily followed by examining the discharge from the vaginal introitus on a daily basis. Using this method, normal cycling animals have a postestrous discharge on the morning of every fourth day (19). Interruption of these 4-day discharges is indicative of suppression of reproduction (20).

The experiment was continued until 78% of the melatonin-treated animals (7 of 9 hamsters) exhibited vaginal acyclicity; at this time, 56% (5 of 9) of the hamsters treated with melatonin and ML-23 were also acyclic. Hamsters were considered acyclic when they had missed two consecutive postestrous discharges. At this point (13th week), each animal still cycling was sacrificed on the afternoon of the day of proestrus (between 1300 and 1500 hr); the acyclic animals were killed during the same time interval. Proestrus was selected as the time to kill the cycling animals because this coincides with their ovulatory hormone surges (21). The cycling hamsters would then be comparable to the acyclic animals since these animals exhibit a similar gonadotropin surge every afternoon (22).

Trunk blood samples were collected in heparinized plastic tubes and centrifuged at 3000 rpm for 30 min at 4°C and plasma was stored frozen at −20°C until the time of hormone determination. Pituitaries were dissected, weighed, and stored at −70°C until hormone determination. Ovarian, uterine, and body weights were also recorded.

Hormone Determination. Pituitary and plasma luteinizing hormone (LH) and follicle-stimulating (FSH) concentrations were determined in duplicate using kits supplied by the Hormone Distribution Program of NIADDK as previously described (23). Prolactin (PRL) was determined by a homologous hamster prolactin assay (24). T_4 and triiodothyronine (T_3) plasma levels were determined by specific radioimmunoassay methods using commercially available kits (Diagnostic Products, Los Angeles, CA) as previously described (25); estradiol and progesterone were also determined. These commercial antisera are highly specific and the assay sensitivity is 8 pg/ml for estradiol, 0.05 ng/ml for progesterone, 7 ng/dl for T_3, and 0.3 μg/dl for T_4. An inverse index of the fraction of T_3 and T_4 bound to the plasma protein was ascertained by determining the *in vitro* proportional T_3 uptake (Diagnostic Products) from plasma onto immobilized antibody. Free T_3 and T_4 indices (FT_{3I} and FT_{4I}) were obtained by calculating the product of the respective total thyronine concentration and the T_3 uptake value. Intrassay variation for all assays was less than 10%.

Statistical Analysis. Data are expressed as the mean ± SE of groups consisting of nine animals each. Only absolute weights are shown since no effects of melatonin or ML-23 on body weights were noted. Statistical evaluation was performed using a two-way ANOVA followed by a Student-Newman-Keuls test where appropriate.

Results

Effects of Melatonin and/or Antagonist on Organ Weights. After 13 weeks of treatment, melatonin alone or in combination with the antagonist caused a signifi-

cant reduction in the pituitary and uterine weights. The expected rise in the ovarian weight was observed in hamsters receiving melatonin only. The combination of melatonin plus ML-23 completely reversed the effects of melatonin on the ovary but not on pituitary and uterine weights (Fig. 1). None of the treatments significantly altered body weight.

Effects on Hormone Levels. Plasma LH and pituitary FSH increased significantly after melatonin treatment. Plasma and pituitary PRL levels were significantly lowered by melatonin treatment alone compared with the saline-treated group. The combination of melatonin plus ML-23 reversed ($P < 0.01$) only the rise in pituitary FSH levels that followed daily melatonin administration (Fig. 2).

Melatonin treatment caused a significant decrease in plasma T_4 concentrations and the free T_4 index (Fig. 3). However, the administration of melatonin and ML-23 in combination significantly increased T_3 and FT_3I when compared with treatment with either melatonin or ML-23 alone (Fig. 3).

Melatonin administration inhibited circulating plasma estradiol levels when the values were compared

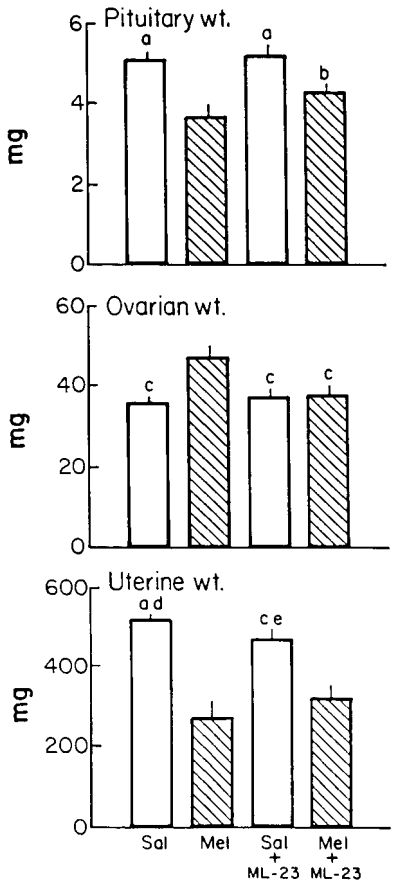

Figure 1. Pituitary, ovarian, and uterine weights of female hamsters treated for 13 weeks with afternoon injections of melatonin (Mel) or saline diluent (Sal); two groups of hamsters also received ML-23 in the drinking water. Mean ± SE are indicated. a, $P < 0.005$ vs Mel; b, $P < 0.05$ vs Sal; c, $P < 0.025$ vs Mel; d, $P < 0.005$ vs Mel + ML-23; e, $P < 0.01$ vs Mel + ML-23.

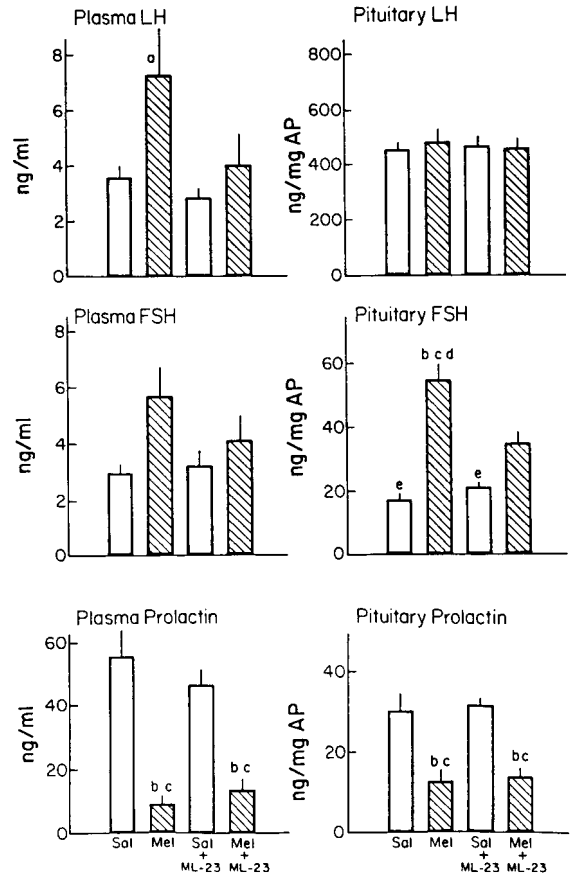

Figure 2. Plasma and pituitary concentrations of LH, FSH, and prolactin for female hamsters treated for 13 weeks with afternoon injections of melatonin (Mel) or saline (Sal) diluent; two groups of hamsters also received ML-23 in the drinking water. Mean ± SE are indicated. a, $P < 0.05$ vs Sal; b, $P < 0.001$ vs Sal; c, $P < 0.001$ vs Sal + ML-23; d, $P < 0.001$ vs Mel + ML-23; e, $P < 0.025$ vs Mel + ML-23.

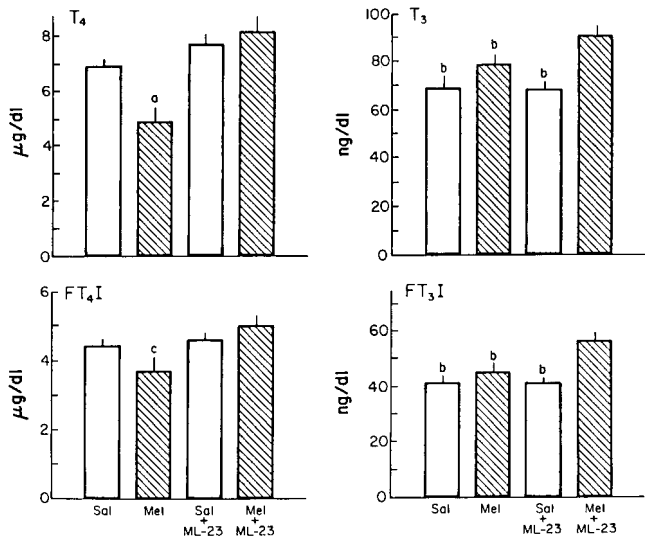

Figure 3. Plasma concentrations of T_4 and T_3 and their free indices (FT_4I, FT_3I) in female hamsters treated with melatonin (Mel) or saline diluent (Sal); two groups of hamsters also received ML-23 in their drinking water for 13 weeks. Mean ± SE are indicated. a, $P < 0.05$ vs Sal; b, $P < 0.025$ vs Mel + ML-23; c, $P < 0.05$ vs Mel + ML-23.

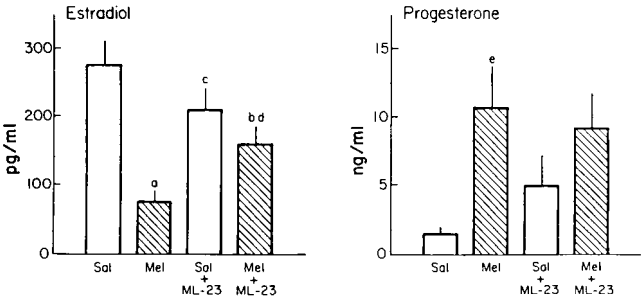

Figure 4. Plasma concentrations of estradiol and progesterone for female hamsters treated with melatonin (Mel) or saline diluent (Sal); two groups of hamsters received ML-23 in their drinking water for 13 weeks. Mean ± SE are indicated. a, $P < 0.001$ vs Sal; b, $P < 0.025$ vs Sal; c, $P < 0.005$ vs Mel; d, $P < 0.05$ vs Mel; e, $P < 0.01$ vs Sal.

with the values of the saline-treated group. Circulating progesterone concentrations were significantly increased after melatonin treatment (Fig. 4). The combination of melatonin plus ML-23 partially reversed the effects of melatonin on estradiol concentrations. Progesterone levels were not affected by the combination treatment of melatonin and ML-23 (Fig. 4).

Discussion

The data presented in this article confirm that late afternoon melatonin administration to the female Syrian hamster is able to alter the neuroendocrine-reproductive function of this animal affecting central and peripheral hormone levels. The net effect of the altered hormonal milieu is enlarged, nonfunctional ovaries, infantile uteri, and vaginal acylicity. ML-23 inhibited the rise in ovarian weights and partially inhibited the fall in estradiol concentrations. However, this partial effect on estradiol concentration was not sufficient to elevate uterine weight to within the normal range for cycling animals. It may be that the action of estradiol in the uterus was prevented by melatonin (26).

As in a number of other earlier studies (2, 10), the hormone that exhibited the most consistent change in the current report was PRL. The reduction in pituitary and plasma PRL levels seems to be of a great importance in ensuring ovarian atrophy in female hamsters and this may be related to the maintenance of the LH and FSH receptors at the gonadal level as has been documented in the male hamster (27).

The marked rise in pituitary FSH concentrations observed in our study is a common feature of hypothalamo-pituitary-gonadal suppression induced by melatonin treatment in the female Syrian hamster (1). As far as plasma LH and FSH levels are concerned, the increase in both gonadotropin concentrations in the melatonin-treated group is in agreement with the earlier documented daily afternoon surge of these two hormones in the acyclic animal; cycling animals have a surge on the afternoon of proestrus (21, 22, 28) and in this experiment was apparently partially missed. ML-23 was unable to reverse the effects of melatonin on pituitary and plasma LH and PRL, but it did significantly raise plasma estradiol levels above those of the melatonin-treated group. Additionally, ML-23 partially prevented the rise in pituitary FSH due to melatonin treatment alone. Most of these data are well in agreement with those of Laudon *et al.* (29) obtained in the rat and may suggest a largely peripheral action for this drug.

As observed in previous studies (14, 15, 25), melatonin treatment alone caused a significant decrease in T_4. Previous studies in female hamsters have also shown a depression in T_3 concentrations but this was not confirmed in the present experiment. Since melatonin has direct metabolic effects on the thyroid gland of rats (30) and the mean nuclear volume of the hamster thyroid, it might be involved in the control of not only the thyroid function but also thyroid growth. As previously mentioned, the data in this experiment, taken along with those from the literature (14, 15, 29, 30), suggest that the action exerted by melatonin on the thyroid is complex and may involve an inhibitory effect on the metabolism of the gland.

ML-23 administered with melatonin reversed the effects of melatonin on plasma T_4. The fact that T_3 plasma concentrations and FT_3I were significantly increased by the combination of melatonin + ML-23, but not by either of the two treatments alone, suggests that these compounds may act synergistically to increase either the conversion rate of T_4 to T_3 and/or the bioavailability and/or degradation of T_3. Precedence for such an effect was shown recently (31). In that experiment, melatonin and 5-methoxytryptamine when administered together elevated circulating T_3 levels in female hamsters (31). 5-Methoxytryptamine is also a pineal indole and resembles ML-23.

We demonstrated for the first time herein that the melatonin antagonist ML-23 is able to reverse the effects of melatonin on ovarian weight, pituitary FSH, T_4, FT_4I, and estradiol in the female Syrian hamster. The bulk of evidence obtained in this study suggests that its action is at a peripheral site. However, ML-23 was able to partially reverse the effect of melatonin on pituitary FSH, thereby indicating some central role for the antagonist as well.

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