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ORIGINAL ARTICLE

Effect of melatonin on in vitro gonadotropins and prolactin release from pituitary LHRH stimulated, and median eminence and on ovarian response to hCG in middle-aged female rats

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Abstract

The possible influence of exogenous melatonin upon the reproductive axis of middle-aged (15-19-month-old) female rats showing irregular estrous cycle length was analyzed. In vitro pituitary and median eminence (ME) luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin release and responsiveness to luteinizing hormone releasing hormone (LHRH) were investigated in control (n=12) and melatonin (150 μg/100 g BW) treated (n=16) rats. In vitro ovarian steroidogenic activity and its response to hCG were also evaluated. Basal secretion, first incubation after one hour (I_1), second incubation after 2 hours (I_2) and after two hours plus LHRH (10^{-7}) (I_{2+LHRH}) or hCG (20 UI) (I_{2+hCG}) were studied. Melatonin administration to middle-aged rats showed a different effect upon in vitro LH release than upon FSH since only lower FSH secretion rates were found. In vivo melatonin administration reduced in vitro LH release after LHRH stimulation and this was impaired to pituitary LH content, indicating that in vivo melatonin administration alters the mechanism of LH release but does not affect the LH synthesis. Neither melatonin nor LHRH affected hemipituitary in vitro prolactin release. In vivo melatonin administration again showed a different effect upon LH release than upon FSH from ME, showing decreasing FSH secretion rates. Similarly, melatonin did not affect prolactin release or ME content. Melatonin reduced estradiol release from hCG stimulated ovaries of middle-aged rats. We concluded that melatonin may have a physiological role in middle-aged rats since decreased in vitro basal FSH release and blunted LH and estradiol increase after stimuli.

Abbrevations and Units

BW	body weight
FSH	follicle stimulating hormone
g	grams
h	hour
hCG	human chorionic gonadotropin
I_1	incubation 1
I_2	incubation 2
I_{2+hCG}	human chorionic gonadotropin stimulating incubation 2
I_{2+LHRH}	luteinizing hormone releasing hormone stimulated incubation 2
IECL	irregular estrus cycle length
IU	international units
KR	Krebs ringer
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
ME	median eminence
MEL	melatonin
ml	millilitre
mRNA	messenger ribonucleic acid
ng	nano grams
pg	pico grams
PRL	prolactin
RIA	radioimmunoanalysis
µ g	micro grams

Introduction

It is known that melatonin, the main hormone of the pineal gland, acts on the neuroendocrine-reproductive axis, exerting an anti-gonadotropic effect [1, 2]. However, in some experimental conditions, melatonin has been shown to induce stimulatory hypophyseal-gonadal axis [3].

Luteinizing hormone release hormone (LHRH) is the physiologic stimulatory neuropeptide that regulates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) [4]. LHRH increased in vitro LH and FSH release in female rats [5]. There is substantial evidence suggesting that melatonin affects the gonadotropin response to LHRH. Exposure of pituitary tissue from Golden hamsters to melatonin during perfusion caused a decrease in the basal secretion of LH but did not affect the LH or FSH response to GnRH release regardless of the time of pituitary removal [6]. Melatonin had no effect on pituitary response to LHRH and inhibited prolactin secretion during a 48h culture period [7].

Regulation of prolactin secretion involves multiple factors, inhibitory as well stimulatory [8]; these factors include LHRH, which was shown to induce inhibitory [9] or stimulatory effects [10] and melatonin [11, 12].

In the other hand, melatonin was found in preovulatory follicles indicating that it may affect ovarian steroidogenesis [13]. Other authors [14] postulated that melatonin, through its direct action on the ovaries, may increase the output of progesterone and therefore facilitate the initiation of cyclicity. Melatonin may also be involved in the aging process, as the rhythm of melatonin production is robust in young animals, but this cycle deteriorates during aging [15].

It is well known, that there are significant changes in the pituitary response to LHRH [16]. In aging rats with impairment of the estrous cycle or persistent estrous, both LH release and LH contents from pituitaries were significantly lower as compared to pituitaries from young rats. A decrease in the excitatory inputs to LHRH neurons could be directly involved in the reduction of the hypothalamic-pituitary-ovarian axis observed during aging [17].

We have studied the possibility that in middle-aged rats (15-19-month-old) in vivo administration of melatonin modifies the in vitro pituitary LH, FSH and prolactin release and response to LHRH. Having in mind that LH containing neurons within the hypothalamus as well as hypothalamic prolactin-like substances have been described [18, 19], we have also studied the influence of melatonin upon median eminence (ME) gonadotropins and prolactin secretion rate. The effects of human chorionic gonadotropin (hCG) on the ovarian steroidogenic activity of rats treated with melatonin also have been studied.

Materials and methods

Animals

Middle-aged (15-19 month-old) female Wistar rats were used. Animals were housed in a 12h light/dark cycle (lights on at 08.00h) and at room temperature of approximately 23ºC, with standard rat chow and tap water ad libitum. In order to select rats showing irregular estrous cycle lengths (IECL), vaginal smears were checked for a 15-day period, between 10.00-11.30. None of the female rats under study showed repetitive 4-5 day estrous cycles, nor aging estrous cycle conditions such as persistent estrous or repetitive pseudopregnancy. Animals were divided into two groups: control (n=12) and melatonin treated (n=16).

Melatonin treatment

Melatonin (Sigma Chemical Co, St Louis) was dissolved in a small volume of 100% ethanol and diluted in 0.9% ClNa up to a dose of 150 μg/100 g BW. Melatonin treatment was administered daily for a

month at the end of the light phase 90 min before the lights went off. Control group received vehicle. Then animals were killed by decapitation after treatment regardless of the stage phase of the estrous cycle.

Pituitary incubations

Anterior pituitaries were removed freed from the neural lobe and pituitary halves and placed in incubation tubes containing 1 ml of Krebs-Ringer (KR) medium to examine LH, FSH and prolactin release. They were incubated in a Haake SWD 20 shaking bath at 37°C shaking at 60 cycles/minute and were gassed with 95% O_2: 5% CO_2. After 30 minutes, the medium was removed and discarded, and 1 ml of fresh medium was added. One hour later the medium (I_1) was removed and were frozen for measurement of the basal rate hormones secretion; then it was replaced with 1 ml of new KR medium (I_2) or 1 ml of fresh KR plus LHRH (10^{-7}M) ($I_{2\text{-LHRH}}$). One hour later, both incubation media were removed and frozen (-20°C), until measurement of the hormones. Hemipituitary tissues were then homogenized and also frozen (-70°C), for hormonal assay.

Median eminence incubations

Median eminences were removed in situ, from medio-basal-hypothalus immediately dissected out and used for in vitro studies to examine LH, FSH, and prolactin release during two hours, following the above mentioned experimental protocol. Basal rate medium (I_1) and second incubation medium after two hours of incubation (I_2), were frozen (-20°C) until hormone determinations. Median eminences were homogenized and frozen (-70°C) for posterior hormone determinations.

Ovary incubations

Ovaries were removed, immediately dissected and used for in vitro studies, to examine the effect of human chorionic gonadotropin (hCG) (Physex Leo, 500) on the release of 17β-estradiol. Ovaries were placed in incubation tubes as described above. After the first incubation medium (I_1) was aspirated and frozen, 1 ml of fresh KR (I_2) or 1 ml of fresh KR containing 20 U.I. of hCG ($I_{2\text{-HCG}}$) was added to the incubation tubes. One hour later, both incubations media (I_2 and $I_{2\text{-HCG}}$) were removed and frozen (-20°C). Ovarian tissues were homogenized and frozen (-70°C) until 17β-estradiol determination.

Radioimmunoassay of gonadotropins and prolactin

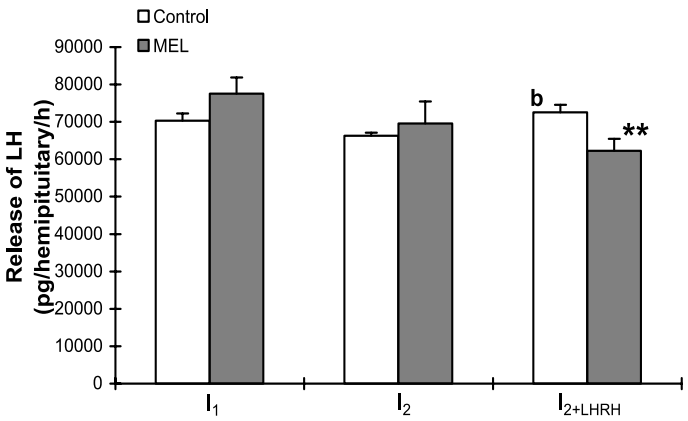
LH, FSH and prolactin levels were measured by specific RIAs employing second antibody-facilitated separation and reagents kindly donated by the National Institute of Health (NIADDK, Bethesda, MD). The methods have been described in detail in previous reports [9, 20, 21]. Values of pituitary LH and FSH release were expressed as pg/hemipituitary/h, values of pituitary prolactin secretion were expressed as ng/hemipituitary/h in Fig. 1; pituitary LH and FSH content as pg/homogenized hemipituitary, pituitary prolactin content as ng/homogenized hemipituitary in Fig. 2; ME, LH and FSH secretion and content as pg/ME/h, ME prolactin secretion and content as ng/ME/h in Fig. 3.

Radioimmunoassay of 17β-estradiol

Release of estradiol was measured by ^{125}I RIA Kit using commercial ImmunoChem™ Coated Tube, according to the manufacturer's instructions (ICN Pharmaceuticals, Inc.). The minimum amount of 17 β-estradiol significantly different from zero was 10 pg/ml. Values were expressed as pg/ovary. All samples were measured with kits belonging to the same catalog's reference. The assay binding was 28.60%.

Statistical analysis

Data of each age group were adjusted to a normal distribution test before being used in the statistical analysis. A 99 percentage of 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 of hormones were determined by one-way analysis of variance (ANOVA); individual comparisons at each incubation period between both groups studied and for each group at the different incubation periods were then made by Newman-Keuls multirange test. Significant differences were quoted by *:$p<0.01$; **:$p<0.05$ for comparisons of two groups and b:$p<0.05$ represents significant differences between incuation periods.



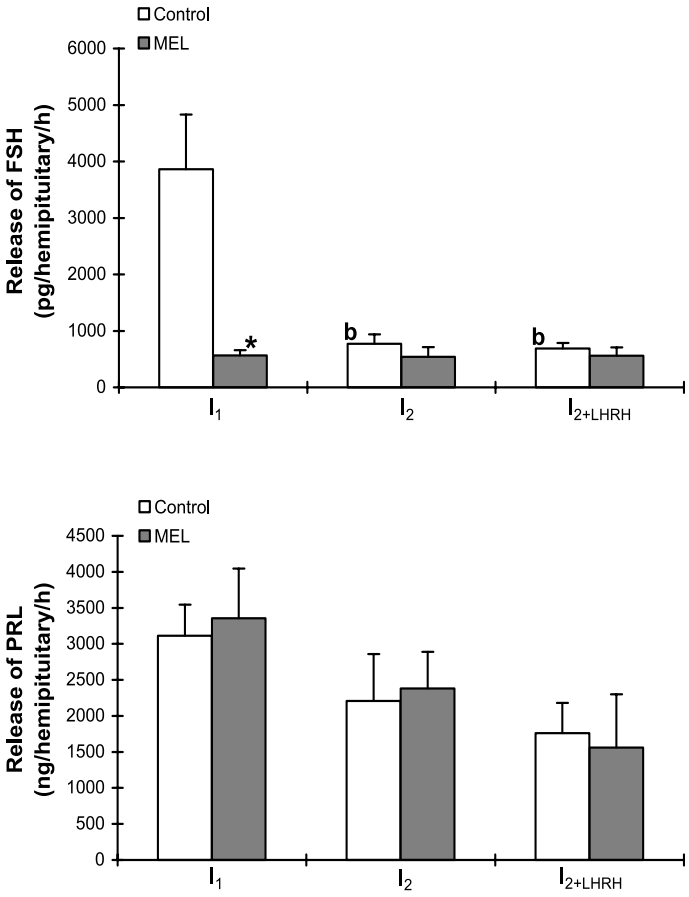


Fig.1.
In vitro LH, FSH and prolactin release and response to LHRH from hemipituitaries of control (N= 12) and melatonin treated (150µg/100gB.W.) (N= 16) female middle-aged rats. (I_1- basal rate. I_2- 2nd incubation hour. I_{2+LHRH}- 2nd incubation hour plus LHRH). Results are expressed as the mean ± SEM.
LH; I_{2+LHRH} ******: p<0.05 vs. control group, **b**: p<0.05 vs. I_2.
FSH; I_1 *****: p<0.01 vs. control group, I_2 **b**: p<0.05 vs. I_1, I_{2+LHRH} **b**: p<0.05 vs. I_1.

Shows in vitro LH, FSH and prolactin release from pituitaries of control and melatonin treated (150 μg/100 g BW) rats with IECL, at basal rate (I_1) as well as in the second non-stimulated incubation period (I_2). No significant differences were found for LH release at both I_1 and I_2 incubation periods from hemipituitaries of control and melatonin treated rats. However, LH release was significantly decreased (p<0.05) in melatonin treated rats as compared to control IECL rats in response to LHRH addition to the incubation medium. In control rats LH release after LHRH addition (I_{2+LHRH}) was significantly increased (p<0.05) as compared to LH release (I_2). FSH release at basal rate from control rats was significantly higher (p<0.01) than from pituitaries of melatonin treated rats. The FSH released from control rats was similarly decreased (p<0.05) in the second incubation period regardless of whether LHRH was added or not to the incubation media. No significant suppression of prolactin release from the hemipituitaries from either control and melatonin treated group or at each incubation period studied was found.

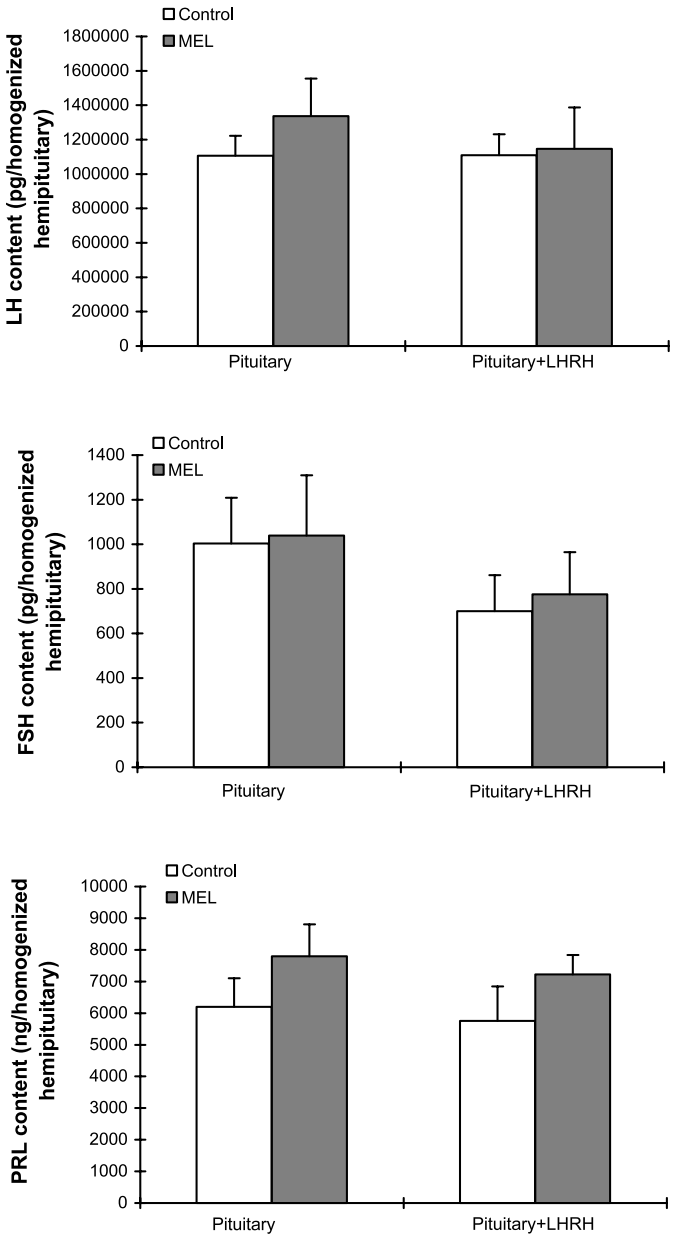


Fig.2. In vitro LH, FSH and prolactin pituitary contents before and after LHRH addition. Groups and sample sizes are the same as those shown in Fig.1. Results are expressed as the mean ± SEM.

LH, FSH and prolactin content from homogenized hemipituitaries of middle-aged (15-19 months of age) control and MEL-treated (150 μg/100 g BW) rats. No differences in pituitary hormones' content in both groups studied non-stimulated as well as in LHRH-stimulated pituitaries were found. MEL-treatment did not affect pituitary hormones' content.

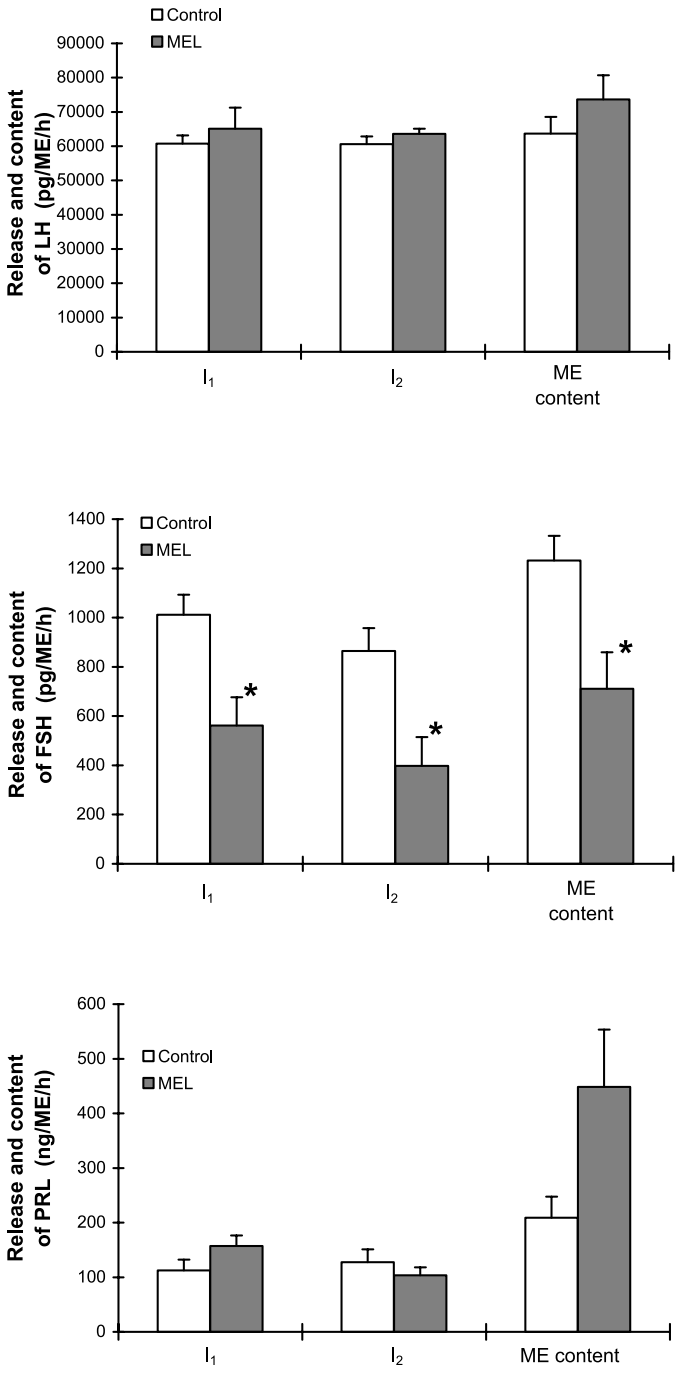


Fig.3. In vitro LH, FSH and prolactin release from median eminence (ME) and ME hormone contents. Groups and sample sizes are the same as those shown in Fig.1. Results are expressed as the mean ± SEM.
FSH; I_1, I_2 and ME content *: p<0.01 vs. control group.

Shows in vitro LH, FSH and prolactin, release and content from ME for the same groups mentioned above. Release of LH from ME in either control or melatonin treated groups showed similar values at each incubation period studied (I_1 and I_2). No significant differences in LH median eminence content in both groups studied were found. Melatonin treatment significantly reduced (p<0.01) FSH release and content from ME. ME prolactin release showed similar values and no differences were found between groups studied nor between both incubation periods (I_1 and I_2).

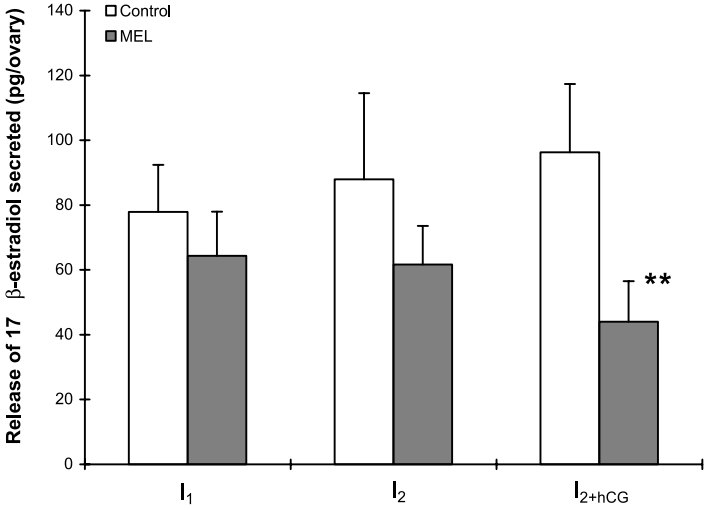


Fig.4. In vitro ovarian17β-estradiol release and response to hCG. Groups and sample sizes are the same as those shown in Fig.1. Results are expressed as the mean ± SEM. I_{2+hCG} **: p<0.05 vs. control group.

Shows 17β-estradiol release from the ovaries of the same groups of 15-19 month-old rats. In the control group, ovarian estradiol release was similar at basal rate as compared with second incubation period with or without hCG addition. In vivo melatonin significantly reduced (p<0.05) estradiol release after hCG addition (I_{2+hCG}).

Discussion

The results of this investigation show that in vivo melatonin treatment plays a different effect on in vitro pituitary FSH release than upon LH. Reduced FSH secretion at second incubation periods, regardless of whether LHRH was added to incubation media, were observed in control group which may represent a functional defect of the pituitary gland

since the initial basal secretion rate showed significantly higher values. However higher doses (100 and 200 ng LHRH/100 g BW) resulted in a more brisk FSH response in young rats [22]. LHRH also stimulated in vitro LH and FSH release in young rats [5].

A different effect of LHRH was observed on in vitro LH and FSH release which suggests that in middle-aged rats LHRH acts in a different manner upon LH than on FSH as it was previously found in cycling female rats [23, 24], which suggests that the main regulator of the FSH release could be a peptide other than the LHRH. In this way, a separate hypothalamic control of FSH and LH release has been suggested [25, 26]. Results obtained in the pituitary FSH content confirmed those obtained in FSH release from hemipituitaries of both control and melatonin treated rats, with or without LHRH addition, since similar concentrations were observed.

In vivo melatonin administration reduced LH release after LHRH stimulation and this was umpired to pituitary LH content in melatonin treated middle-aged rats. These results indicate that in vivo melatonin treatment alters the mechanisms of LH release from the pituitary but does not affect the LH synthesis.

There is evidence indicating that melatonin regulates prolactin secretion [11, 12], however, in our study prolactin release was not affected by in vivo melatonin administration or by LHRH addition to the incubation medium in both groups studied. It is known that LHRH can stimulate or inhibit prolactin release in different experimental circumstances [21, 27-29]. An important mechanism which can participate in the lack of effect of LHRH on prolactin secretion observed in our middle-aged female rats could be the hypothalamic dopaminergic system, which seems to become hypofunctional with aging and its activation requires stronger stimuli in the aged rather than in younger animals [30]. Results in prolactin pituitary release are in accordance with results on prolactin content, since no differences were found between both groups studied, before or after LHRH addition, when prolactin pituitary content was determined.

In vitro basal LH secretion rates from ME were unaffected by in vivo melatonin administration, but FSH release from ME was reduced after in vivo melatonin treatment to middle-aged rats. There is evidence to indicate that hormones secreted by the adenopituitary can diffuse into the hypophysial sinusoids and be transported back to the stalk and the ME, there to affect the activity of neurons in the hypothalamus [31]. Indeed, there is evidence to suggest the existence of LH-containing neurons within the hypothalamus [18, 19]. In the in vitro studies reported here, concentrations of LH released from ME match values to those released from the pituitary. Other in vitro studies showed that whereas there are some similarities between LH release from the pituitary and from hypothalamic tissues, differences about agents known to modulate pituitary LH release had no effect on the release of LH from hypothalamic tissues, which suggest that hypothalamic LH does not simply serve as a supplemental source of LH, whether acting as a neuromodulator within the brain [32].

From the ME prolactin results reported here they indicate that no differences exist in the releasing mechanisms between both control and melatonin treated groups. However, prolactin released from ME was lower than prolactin released from the pituitary. This discrepancy can be justified from the point of view that differences in hypothalamic prolactin-like substances had been detected as compared to pituitary prolactin. Larger molecular size of hypothalamic prolactin-like substances were reported [19], which are regulated independently of pituitary prolactin and circulating serum prolactin levels.

In vitro estradiol production was not increased by hCG stimulation. In addition, it was reported [33] that hCG stimulation of rabbit ovarian follicles was blocked by melatonin. These data may indicate that the stimulatory mechanisms necessary to induce estradiol release by hCG are affected by aging since it is known that hCG stimulates estrogen production by the ovary [33]. However, after melatonin treatment it was significantly reduced as compared to the control group. These data are consistent with those previously observed in in vitro studies in rats [34], in sheep [14], and in humans [13], in which melatonin was ineffective in stimulating estradiol production. It was previously described that melatonin is involved in the regulation of both progesterone and estradiol synthesis by granulosa cells, but the response to melatonin depends on the degree of cell differentiation [35].

In conclusion, our results show that in control middle-aged female rats melatonin may have a physiological role since decreased in vitro FSH release and blunted the LH and estradiol responsiveness to stimulatory agents.

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