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Sex Difference in Serum Luteinizing Hormone Postgonadectomy in the Rat

Role of γ-Aminobutyric Acid-ergic Inhibition

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In adult male rats, serum luteinizing hormone (LH) rises within a few hours of castration. By contrast, in adult female rats, serum LH does not increase reliably until 4–6 d after ovariectomy. The release of gonadotropin-releasing hormone (GnRH) declines in female rats postovariectomy, suggesting an increase in inhibition of the release of GnRH. We investigated whether differences in γ-aminobutyric acid (GABA)-ergic transmission, which inhibits GnRH release, accounts for the sex difference in the response of serum LH to gonadectomy. We examined the effects of GABA-A receptor antagonist bicuculline methiodide (BMI), GABA-B receptor antagonist phaclofen, and transaminase inhibitor aminooxyacetic acid (AOAA), injected subcutaneously, on the postgonadectomy rise in LH. AOAA prevented the postcastration rise in male rats ($p < 0.05$). Female rats treated with BMI, phaclofen, or both BMI and phaclofen ($p < 0.05$) showed a significant increase in LH postovariectomy. BMI had no effect in male rats. GnRH antagonist blocked the BMI-induced increase in serum LH. We conclude that the delay in the rise of serum LH in female rats postovariectomy is at least partly owing to GABAergic inhibition of the release of GnRH.

Key Words: γ-Aminobutyric acid; gonadotropin-releasing hormone; luteinizing hormone; gonadectomy; bicuculline; phaclofen.

Introduction

The hypothalamic-pituitary-gonadal axis in the rat is moderated by a negative feedback loop. Evidence for this process is a rise in serum luteinizing hormone (LH) following removal of the gonads. In the male rat, this increase is immediate, occurring within 12 h. In the female rat, serum LH rises significantly and reliably only after 4–6 d *(1)*. The sex difference in serum LH during the first 24 h is not owing to a difference in LH pulse frequency and amplitude but to a difference in the baseline of LH release *(2)*. In the female rat, the baseline increases 2–8 d after ovariectomy *(3,4)*. In addition to serum LH, gonadotropin-releasing hormone (GnRH) receptor and its mRNA increase more rapidly in male rats *(5,6)*. The latter finding suggests an increase in the sensitivity of the pituitary in the male rat. However, in both male and female rats, pituitary sensitivity to GnRH declines postgonadectomy in vitro and is not significantly changed when measured in vivo *(7,8)*.

We have previously demonstrated that the slower rise of serum LH in the female than in the male rat postgonadectomy is owing to a decrease in GnRH secretion *(9)*. GnRH pulse frequency decreases by 48 h postovariectomy and remains low *(10–12)*. In the male rat, GnRH pulse frequency and/or amplitude have been reported to increase moderately or remain unchanged postcastration *(9,13–15)* for periods ranging from 1 d to 1 yr. These studies suggest that the release of GnRH is inhibited in the female rat but not in the male rat postgonadectomy.

A possible inhibitory regulator of GnRH is γ-aminobutyric acid (GABA). In male rats, there is a decrease in GABA accumulation or turnover 2 d postcastration *(16–18)*, which is reversible by testosterone *(16,19)*. In the female rat, an increase in GABA accumulation, at 9 d postovariectomy, is prevented by administration of estrogen *(20,21)*. At longer intervals, 3 to 4 wk postovariectomy, GABA levels decline *(22,23)*.

We hypothesized that an early increase in GABA inhibition of the release of GnRH postovariectomy may prevent an increase in the release of LH in the female rat and that a decrease in GABA inhibition of GnRH release postcastration results in an increase in the release of LH in the male rat. We tested this hypothesis in male and female rats 12 h postgonadectomy, measuring serum LH after treatment with the GABA-A antagonist bicuculline methiodide (BMI), the GABA-B antagonist phaclofen, and the GABA-transaminase inhibitor aminooxyacetic acid (AOAA).

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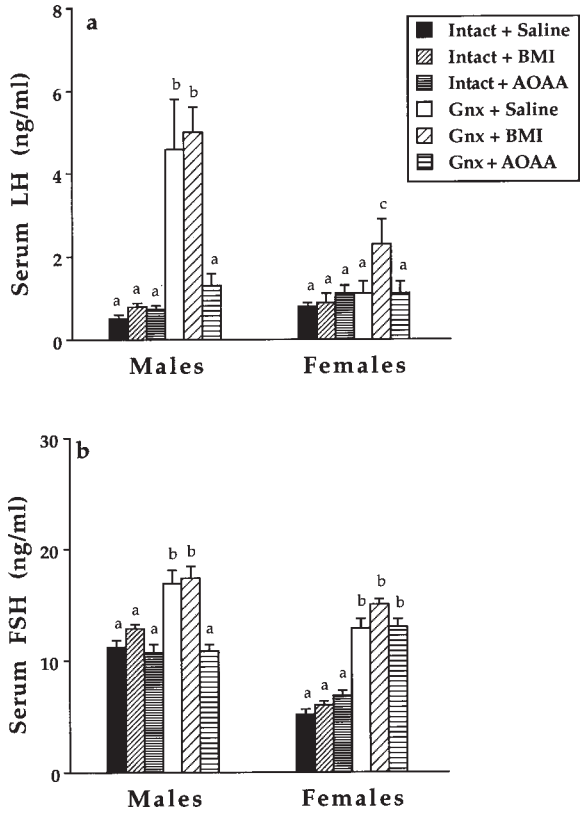

Fig. 1. Effects of BMI or AOAA on serum LH **(A)** and FSH **(B)** in intact and 12-h gonadectomized (Gnx) male and female rats. Groups that do not share a letter (a,b,c) are significantly different from each other ($p < 0.05$). Values are mean ± SEM. Number of rats in each group is as follows: Intact + Saline, $n = 10$; Intact + BMI, $n = 3$ males and females; Intact + AOAA, $n = 4$ males and females; Gnx + Saline,$n = 9$; Gnx + BMI, $n = 9$; Gnx + AOAA, $n = 9$. BMI increased serum LH in female rats postovariectomy compared to control groups. AOAA suppressed the rise in serum LH and FSH in male rats post-castration.

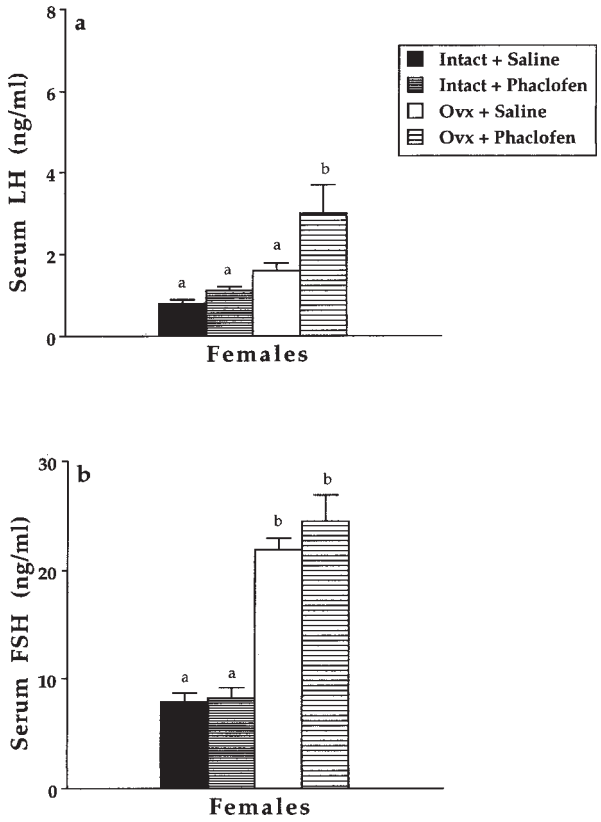

Fig. 2. Effects of phaclofen on serum LH **(A)** and FSH **(B)** in intact and 12-h Ovx female rats. Groups that do not share a letter (a,b) are significantly different from each other ($p < 0.01$). Values are mean ± SEM; $n = 6$ for all groups. Phaclofen increased serum LH in female rats postovariectomy compared to control groups.

Results

Serum LH increased significantly in the male rats by 12 h postcastration (Fig. 1A), reaching levels more than four times higher than serum LH levels in the female rats. AOAA suppressed the rise in serum LH ($p < 0.01$) seen in castrated male rats (Fig. 1A). AOAA had no effect on serum LH of intact male rats or intact or ovariectomized (Ovx) female rats. Ovx rats treated with BMI (Fig. 1A) or phaclofen (Fig. 2A) had significantly higher serum LH (BMI, $p < 0.05$; phaclofen, $p < 0.01$) than intact rats treated with BMI or phaclofen, or intact or Ovx rats treated with saline. BMI treatment had no effect on serum LH in the male rats (Fig. 1A). The GnRH antagonist WY45,760-1 suppressed serum LH in Ovx rats to metestrous levels (Fig. 3A). WY45,760-1 also prevented the increase in serum LH induced by BMI ($p < 0.01$). Serum LH of rats treated with both BMI and phaclofen was significantly increased above serum LH of saline-treated Ovx rats ($p < 0.05$) (Fig. 4A). However, serum LH of the rats treated with both BMI and phaclofen was significantly less than the serum LH of the rats treated with phaclofen alone ($p < 0.05$) (Fig. 4A). We also tested a second GABA-A antagonist, bicuculline, which, in contrast to BMI, crosses the blood-brain barrier *(24)*. It had no effect on serum LH in either intact or Ovx male or female rats (data not shown).

Serum FSH increased in both male and female rats postgonadectomy (Fig. 1B). This increase was affected modestly by some treatments. AOAA prevented the rise in serum FSH in castrated male rats ($p < 0.05$) (Fig. 1B). As expected, WY45,760-1 suppressed the increase in FSH in Ovx female rats (Fig. 3B).

Discussion

The present data suggest that the decline in the release of GnRH postovariectomy in female rats is owing to GABA inhibition of GnRH. Blockade of either the GABA-A or GABA-B receptor results in an increase in serum LH. Our hypothesis is supported by data showing that GABA accumulation in the hypothalamus in the first week after ovariectomy is higher in untreated rats compared to that in Ovx rats treated with estrogen *(21)*. This may reflect either greater GABA synthesis or lower GABA uptake *(25)*

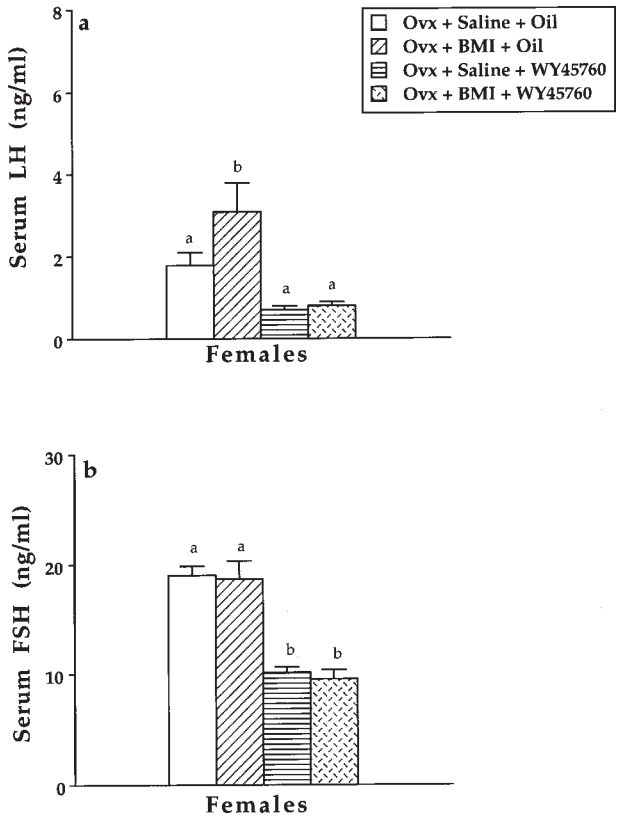

Fig. 3. Effect of GnRH antagonist WY45,760-1 on serum LH **(A)** and FSH **(B)** in saline and BMI-treated Ovx female rats. Groups that do not share a letter (a,b) are significantly different from each other ($p < 0.01$). Values are mean ± SEM; $n = 6$ for all groups. There was an increase in serum LH in BMI-treated compared with saline-treated Ovx female rats. WY45,760-1 suppressed the increase in serum LH seen in BMI-treated rats. WY45,760-1 also suppressed the increase in FSH seen in both saline- and BMI-treated rats.

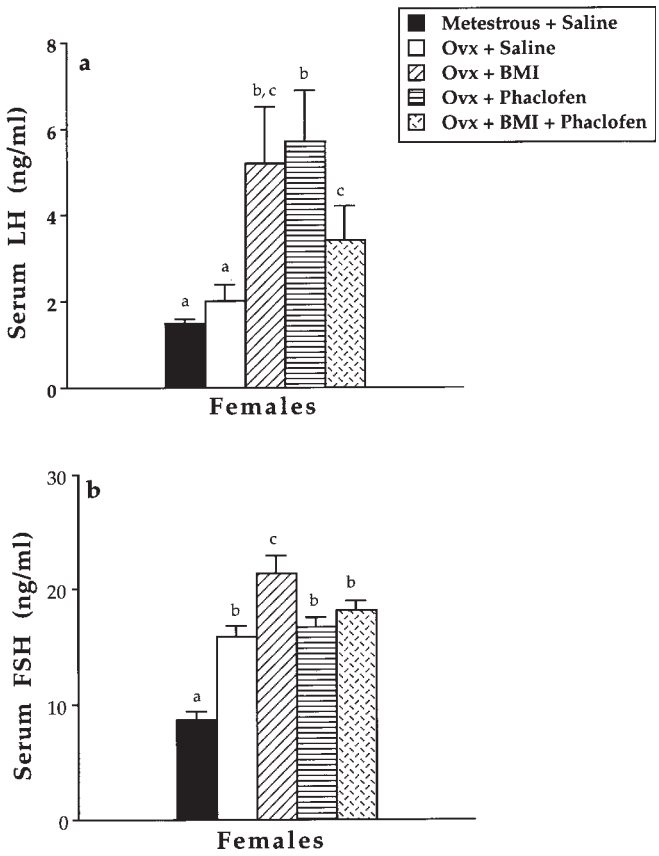

Fig. 4. Effect of BMI and phaclofen combined on serum LH **(A)** and FSH **(B)** in female rats postovariectomy (Ovx). Values are mean ± SEM; $n = 6$ for all groups. Groups that do not share a letter (a,b,c) are significantly different from each other ($p < 0.05$). BMI, phaclofen, and a combination all increased serum LH over saline-treated Ovx rats. Serum LH levels of rats treated with both BMI and phaclofen were significantly less than those treated with phaclofen alone. BMI increased serum FSH over saline-, phaclofen- and BMI + phaclofen–treated Ovx rats.

initially after ovariectomy. The decrease in accumulation *(23)* and release *(22)* and the increase in GABA uptake *(25)* weeks later suggest a slow response of tonic GABA inhibition of GnRH to the loss of gonadal steroids, although this has not been measured directly. An alternative explanation for the increase or maintenance of GABA inhibition postovariectomy is an increase in GABA-A or GABA-B receptors in the hypothalamus *(26)*. Neither the blockade of GABA-A nor GABA-B receptors resulted in serum LH levels equivalent to those of the castrated male rat at the same time point, and there was no further increase in serum LH when both receptors were blocked at the same time. These results, along with the data that blockade of either GABA receptor has no effect on control metestrous rats, suggest that the release of tonic GABA inhibition *(23,27)* is not enough to increase serum LH to levels seen in the castrated male rat.

Our results support the hypothesis that a decrease in GABA postcastration may permit the rapid rise in LH in the male rat. The data from our study support those of Donoso et al. *(20)*, who found that an increase in GABA, induced by γ-vinyl-GABA injected into the preoptic area (POA) and the locus coeruleus, prevented the postcastration rise in LH. Interestingly, the blockade of GABA-A receptors had no effect on serum LH in male rats. This suggests that GABA-A receptors are not involved in the tonic inhibition of LH secretion or that the release of tonic inhibition is not enough to increase serum LH in intact rats. An increase in a stimulating input to the GnRH neuron may also be necessary.

Because AOAA also suppressed the rise in follicle-stimulating hormone (FSH) postgonadectomy in the male rat, but not in the female rat, the rise in FSH in the male rat may be more dependent on changes induced by the loss of steroid hormones than the rise in FSH in the female rat *(28)*. The rise in FSH in the female rat is dependent on a reduction in circulating inhibin; an antiserum to the α-subunit of inhibin increases serum FSH in female but not male rats *(29)*. That the increase in serum FSH in the female rat postovariectomy is somewhat independent of GnRH is also suggested by the effects of the GnRH antagonist; the increase

in FSH was only partially suppressed by WY45,760-1, in agreement with earlier data in long-term Ovx rats *(30)*.

In our studies, we have demonstrated that in the female rat both the "natural" and BMI-induced increases in serum LH postgonadectomy are completely suppressed by the GnRH antagonist. This shows that GnRH stimulation is necessary for the BMI-induced increase in serum LH. However, it does not prove that the antagonist is acting on GnRH neurons. Because our effects were seen after SC injection, and neither BMI nor phaclofen cross the blood-brain barrier *(24,31)*, the action of the antagonists could have been either at the level of the median eminence or the arcuate nucleus of the hypothalamus, or directly on the pituitary. Several lines of evidence suggest that the effects of endogenous GABA on serum LH are mediated centrally through GnRH. GABA neurons synapse with GnRH neurons *(32)* and GABA receptors are present on GnRH neurons *(33)*. Infusion of GABA, or GABA agonists, into the POA or arcuate nucleus in vivo decreases GnRH mRNA *(34,35)*, serum LH *(36)*, and LH pulse frequency *(37)*. Central infusion of the GABA-A antagonist bicuculline advances the LH surge *(38)*. Alternatively, or additionally, the actions of the GABA antagonists could have been on the pituitary. GABA receptors are present in the anterior pituitary *(39)*, and GABA-A receptor agonists can stimulate the release of LH from cultured pituitary cells *(40)*. There is some evidence that GABA may act by altering the sensitivity of the pituitary to GnRH stimulation in vitro *(41)*. However, GABA agonists do not affect GnRH stimulation of LH in vivo *(42,43)*.

Interestingly, another GABA-A antagonist, bicuculline, had no effect on serum LH in either male or female rats. Comparisons of the potency of BMI and bicuculline are difficult to make because one crosses the blood-brain barrier and one does not. However, bicuculline, at the same dose, affects other central nervous system responses such as learning and memory *(44,45)*. Perhaps bicuculline affects the hypothalamic-pituitary-gonadal axis more generally than BMI. GABA is important for maintaining GnRH pulsatility at the level of the POA *(46)*. Bicuculline injected subcutaneously, in contrast to BMI, may reach GnRH cell bodies in the POA, disrupting GnRH pulsatility in addition to affecting the release of GnRH.

The work of other investigators suggests an important role for GABA as an intermediary in estrogen-negative feedback on gonadotropins. GABA neurons in the POA contain estrogen receptors *(47),* and estrogen increases the concentration of GABA in the POA under negative feedback conditions *(23)*. In female rats, a decrease in GABAergic activity precedes the LH surge *(20,48)*. GABA agonists and catabolic inhibitors block the estrogen/progesterone-induced surge *(20,49)* and increase GnRH mRNA in the estrogen/progesterone-primed rat *(50)*. Studies have also demonstrated estrogen-induced changes in glutamic acid decarboxylase, the rate-limiting enzyme in the synthesis of GABA. GAD67 mRNA in the mPOA decreases at 3:00 PM *(37)* and decreases with estrogen treatment of Ovx rats *(51)*.

In summary, our data suggest that GABA plays a role in the sexually differentiated response of LH and GnRH to the loss of gonadal steroid–negative feedback. We hypothesize that the withdrawal of gonadal steroids results in a rapid decrease in GABA inhibition in the male rat, and an increase or maintenance of GABA inhibition of LH and GnRH in the female rat, in the first few days postgonadectomy. In the male rat, a rapid increase in the release of GnRH is followed closely by an increase in the release of LH. In the female rat, a decline in the release of GnRH prevents an immediate rise in serum LH in response to an acute drop in gonadal steroids. This response may help maintain the estrous cycle in the female rat. A rapid increase in LH in response to the abrupt fall in serum estradiol that occurs on the day of proestrus of each cycle could be deleterious to follicular development.

Materials and Methods

Animals

Fifty-five- to sixty-d-old male and female Sprague-Dawley Crl:CD(SD)BR-CD rats were obtained from Charles River Laboratories and maintained under a 14 h light:10 h dark cycle, with lights on at 5:00 AM. Standard rat chow and tap water were provided ad libitum. Estrous cyclicity in female rats was monitored by daily inspection of vaginal cytology. Female rats were used on metestrus after they exhibited at least two consecutive 4-d cycles. Male rats were used no earlier than 1 wk after arrival. Bilateral gonadectomies were performed under Metofane anesthesia between 8:00 and 10:00 AM. Male rats were castrated transcrotally. Rats were killed by decapitation between 8:00 and10:00 PM and trunk blood was collected for hormone assay.

Drugs

BMI and bicuculline are GABA-A receptor blockers. BMI does not cross the blood-brain barrier *(24)* but bicuculline does. BMI was dissolved in saline and administered subcutaneously at a dose of 1 mg/kg. This dose was based on the work of Moguilevsky et al. *(52)*, who found that BMI injected peripherally decreased serum LH in prepubertal rats and increased serum LH in peripubertal rats. Bicuculline was dissolved first in 0.1 *N* HCl, brought to pH 4.0 with 0.1 *N* NaOH, and brought to a final volume with saline. Two subconvulsive doses, 1 and 2 mg/kg, were tried (ED_{50} = 4.1 mg/kg) *(53)*. BMI was purchased from Sigma (St. Louis, MO) and bicuculline was purchased from Research Biochemicals (Natick, MA).

Phaclofen blocks GABA-B receptors and does not cross the blood brain barrier *(31)*. It was dissolved in 0.1 *N* HCl, neutralized with 0.1 *N* NaOH, and brought to a final volume with saline. The dose used, 1 mg/kg, was based on the work

by Shafizadeh et al. *(54)*, who found that phaclofen decreased antinociception induced by baclofen, a GABA-B receptor agonist. Phaclofen was purchased from Research Biochemicals. No behavioral effects of BMI, bicuculline, or phaclofen were observed in these animals.

AOAA inhibits GABA-transaminase, thereby increasing GABA levels and GABAergic inhibition *(55)*. AOAA was dissolved in saline and used at a dose of 25 mg/kg. The dose of AOAA was based on the range of doses used by Kang et al. *(50)* to block the LH surge and increase GnRH mRNA levels, and by Masotto and Negro-Vilar *(42)* to inhibit naloxone-stimulated increases in LH. We performed a small dose-response study in our laboratory to determine the most effective dose and injection interval (results not shown). AOAA was purchased from Sigma. Animals treated with AOAA were lethargic and less responsive than control animals or animals treated with the GABA antagonists.

WY45,760-1 antagonizes GnRH action by occupying the GnRH receptor. The dose of WY used, 100 μg in 250 μL of sesame oil per rat, has been used in our laboratory previously. It suppresses bioactive serum LH and FSH, and immunoreactive LH, without acutely affecting pituitary content of either gonadotropin *(56)*.

Hormone Assays

Serum gonadotropins were determined by double antibody radioimmunoassay (RIA). Serum LH was measured with an ovine-rat RIA using the NIH LH S25 as standard and S10 LH antibody. Serum FSH was measured using the rat-rat NIDDK assay with FSH RP-2 as standard and S11 FSH antibody. In experiment 1, serum LH and FSH were assayed in three different RIAs. Intraassay coefficients of variation were 9.1, 18.7, and 13.3% for LH; and 3.9, 8.4, and 13% for FSH. The interassay CV was 45.5% for LH and 57.4% for FSH. Serum from experiments 2 and 3 was assayed together. The intraassay CV was 5.1% for LH and 14.2% for FSH. In experiment 4, the intraassay CV was 27.7% for LH and 11.3% for FSH.

Statistics

Data from all experiments were analyzed using the statistical software package CRISP (Crunch Software, Oakland, CA). Analysis of variance, followed by Scheffe's and Newman-Keuls multiple comparison post-hoc tests, was used to determine significant differences in serum hormone levels owing to treatment.

Protocols

Experiment 1: Effects of AOAA and BMI on Release of LH

Male and female rats were divided into six experimental groups. Three groups were left intact, and three groups were gonadectomized between 8:00 and 9:00 AM. Females were used on metestrus. Immediately after surgery, rats were injected with BMI (1 mg/kg), AOAA (25 mg), or saline ($n = 9$ each sex, each group). Intact rats were also divided into three groups and injected with BMI ($n = 4$ females, 3 males), AOAA ($n = 5$ females, 4 males), or saline ($n = 9$ both). Injections of BMI and saline were repeated every 2 h for 12 h. Injections of AOAA were repeated once, 6 h after the first one.

Experiment 2: Effects of Phaclofen on Serum LH

Metestrous female rats were divided into six experimental groups ($n = 6$). Two groups were left intact, and two groups were ovariectomized between 9:00 and 10:00 AM. Immediately after surgery, rats were injected with phaclofen (1 mg/kg) or saline. Intact rats were also divided into two groups and injected. Injections were repeated every 2 h for 12 h.

Experiment 3: Effects GnRH Antagonist on BMI-Induced Release of LH

Metestrous female rats were divided into four experimental groups ($n = 6$). All rats were gonadectomized between 9:00 and 10:00 AM. Two groups were injected with BMI (1 mg/kg) immediately after surgery, and the other two groups received saline. One BMI-treated group and one control group were then injected with WY45,760-1 (100 μg). The other two groups received an injection of oil. Thereafter BMI and saline were injected every 2 h for 12 h. The WY45,760-1 and oil injections were given only once.

Experiment 4: Combined Effects of BMI and Phaclofen on the Release of LH in Female Rats

Metestrous female rats were divided into five experimental groups ($n = 6$). One group was left intact, and four groups were Ovx between 8:00 and 9:00 AM. Immediately after surgery, rats were injected with saline, BMI (1 mg/kg), phaclofen (1 mg/kg), or both BMI and phaclofen (1 mg/kg each drug). Intact rats were injected with saline. Injections were repeated every 2 h for 12 h.

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