EXPERIMENTAL STUDY

5-HT₁ and 5-HT₂ receptor activation reduces N-methyl-D-aspartate (NMDA)-stimulated LH secretion in prepubertal male and female rats

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Abstract

Objective: Excitatory amino acids and serotonin are involved in the control of gonadotropin secretion. The actions of these neurotransmitters are interconnected and recently we have reported that 5-HT₁ and 5-HT₂ receptor agonists blunted ($\pm$)- α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-stimulated GH secretion in prepubertal rats. The present experiments were carried out to analyze the effects of activation of different 5-hydroxytryptamine (5-HT) receptor subtypes on gonadotropin secretion and their role in the N-methyl-D-aspartate (NMDA)-stimulated LH release.

Design and methods: We analyzed the gonadotropin secretion after manipulation of serotonergic and aminoacidergic systems and their interactions in 5-, 16- and 23-day-old male and female rats. To this end, serum LH and FSH concentrations were measured in rats treated with 5-hydroxytryptophan methyl ester (5-HTP) (a precursor of 5-HT synthesis) plus Fluoxetine (Fx, a blocker of 5-HT reuptake), D,L-p-chlorophenyl-alanine methyl ester (PCPA, a blocker of 5-HT synthesis), R-(+)-8-hydroxydipropylaminotetralin hydrobromide (8-OH-DPAT, an agonist of 5-HT_{1A} receptors), ($\pm$)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) and α -methyl-5-hydroxytryptamine (α -Me-5-HT, agonists of 5-HT₂ receptors), and 1-Phenylbiguanide (1-PHE an agonist of 5-HT₃ receptors). In addition, the effects of 8-OH-DPAT and DOI on NMDA-stimulated LH secretion were analyzed.

Results: Neither the activation nor blockade of the serotonergic system modified LH secretion. Basal gonadotropin secretion remained unchanged in 23-day-old male and female rats after activation of 5-HT_{1A}, 5-HT₂ and 5-HT₃ receptors. The stimulatory effect of NMDA on LH secretion was blocked in both sexes after activation of the serotonergic system, through specific 5-HT₁ and 5-HT₂ receptor agonists.

Conclusions: Activation of serotonergic receptors decreased the stimulatory effect of NMDA on LH secretion in prepubertal male and female rats.

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Introduction

The hypothalamic control of gonadotropin secretion is exerted through the pulsatile release of luteinizing hormone-releasing hormone (LHRH), which in turn is regulated by many neurotransmitters and neuropeptides such as norepinephrine (1), endogenous opioids (2), gamma-aminobutyric acid (GABA) (3), nitric oxide (4), galanin (5), excitatory amino acids (EAAs) (6) and serotonin (7).

Excitatory amino acids (glutamic and aspartic acids) control LHRH secretion through different receptor subtypes. Activation of N-methyl-D-aspartate (NMDA) and Kainate (KA) receptors increases luteinizing hormone (LH) secretion (8–10), whereas ($\pm$)- α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors probably do not participate in the regulation of LHRH/LH secretion (11, 12).

The effect of serotonin in the control of gonadotropin secretion depends on the experimental model used, and the age and sex of the animal. In prepubertal rats, serotonin stimulates LH secretion in females but not in males (13), while follicle-stimulating hormone (FSH) secretion after 5-hydroxytryptophan methyl ester (5-HTP) administration increases only in males (14).

Physiological interactions between different neurotransmitters involved in the control of LHRH/LH secretion have been described. For example, bicuculline (antagonist of GABA_A receptors) and phaclofen (antagonist of GABA_B receptors) blocked glutamic acid-evoked LHRH secretion by arcuate nucleus–median eminence preparations (15). Similarly, the stimulatory effect of muscimol (agonist of GABA_A receptors) on LH secretion was blocked by MK-801 (antagonist of NMDA receptors) (16).

Recently, we have described that activation of 5-HT₁ and 5-HT₂ serotonergic receptors blunted the AMPA-stimulated GH secretion in prepubertal male rats (17), and that NMDA receptors, but not AMPA receptors, are needed for the stimulatory effect of GABA on GH secretion (18). In the present experiments, we aimed to obtain information about (a) the role of different 5-hydroxytryptamine (5-HT) receptor subtypes in the control of gonadotropin secretion in different stages of prepubertal development and (b) the interactions between AMPA and NMDA with different serotonergic receptors in the control of LH secretion.

Materials and methods

Animals

Wistar male and female rats born in our laboratory were kept under controlled conditions of light (12 h light:12 h darkness, lights on at 0700 h) and temperature (22 °C), with free access to pelleted food (Pacsa Sanders, Seville, Spain) and tap water. On day 1 of life, each dam was left with eight pups. The pups were separated from their mothers immediately before starting treatments and they were kept warm by a heating source next to their cage.

Drugs

The following drugs were used:

AMPA (Research Biochemical Inc. (RBI), MA, USA), an agonist of AMPA receptors, was dissolved initially in a few drops of dimethylsulfoxide and thereafter in saline up to the working concentration. A dose of 2.5 mg/kg was i.p. injected 15 min prior to decapitation. The protocol (doses and timing) for drug administration was selected on the basis of previous studies showing changes in LH secretion in prepubertal ovariectomized female rats (12).

NMDA (RBI), an agonist of NMDA receptors, was dissolved in saline. A dose of 15 mg/kg was i.p. injected 15 min prior to decapitation. The protocol for drug administration was selected on the basis of previous studies showing an increase in LH secretion (19–21).

Fx hydrochloride (RBI), a selective serotonin reuptake inhibitor, was dissolved in saline. A dose of 10 mg/kg was i.p. injected 45 min before administration of AMPA.

5-HTP (Sigma, Barcelona, Spain), a precursor of serotonin synthesis, was dissolved in saline. A dose of 100 mg/kg was i.p. injected 45 min before administration of AMPA. The protocol for Fx plus 5-HTP administration was selected on the basis of previous studies showing an increase in prolactin (PRL) secretion (11, 22).

PCPA (D,L-*p*-chlorophenyl-alanine methyl ester) (Sigma), an inhibitor of serotonin synthesis, was dissolved in saline containing 0.1% ascorbic acid. A dose

of 250 mg/kg was s.c. injected 72 h before administration of NMDA. The protocol for drug administration was selected on the basis of previous studies showing a reduction in serotonin hypothalamic concentration (23).

8-OH-DPAT (R-(+)-8-hydroxydipropylaminotetralin hydrobromide) (RBI), an agonist of 5-HT_{1A} receptors, was dissolved in saline. Doses of 5 or 10 mg/kg were i.p. injected 45 min before administration of AMPA. The protocol for drug administration was selected on the basis of previous studies showing an increase in LH secretion (24).

DOI ((±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride) (RBI), an agonist of 5-HT₂ receptors, was dissolved in saline. Doses of 20 or 30 mg/kg were i.p. injected 45 min before administration of AMPA. The protocol for drug administration was selected on the basis of previous studies showing changes in LH secretion (25).

α-Me-5-HT maleate (α-methyl-5-hydroxytryptamine maleate) (RBI), an agonist of 5-HT₂ receptors, was dissolved in saline. A dose of 30 mg/kg was i.p. injected 45 min before administration of AMPA. The protocol for drug administration was selected on the basis of previous studies showing changes in LH secretion (25).

1-PHE (RBI), an agonist of 5-HT₃ receptors, was dissolved in saline. A dose of 10 mg/kg was i.p. injected 45 min before administration of AMPA. The protocol for drug administration was selected on the basis of previous studies showing a decrease in PRL secretion (22).

Experimental designs

Experiment 1 In order to determine the effects of inhibition of the serotonergic system on basal gonadotropin levels and NMDA-stimulated LH secretion, 20-day-old male and female rats were s.c. injected with vehicle or PCPA (250 mg/kg). On day 23, animals were injected with NMDA (15 mg/kg) or vehicle. Animals were decapitated 15 min after NMDA injection. Each group consisted of eight to ten animals.

Experiment 2 In order to determine the effects of activation of the serotonergic system on gonadotropin secretion in AMPA-treated animals, 23-day-old male rats were i.p. injected with vehicle or Fx (10 mg/kg) plus 5-HTP (100 mg/kg) 45 min before AMPA (2.5 mg/kg) or vehicle administration. Animals were decapitated 15 min after AMPA injection. Each group consisted of eight to ten animals.

Experiment 3 To analyze the effects of selective activation of serotonergic receptor subtypes on gonadotropin secretion, 16- and 23-day-old male rats were i.p. injected with vehicle, 8-OH-DPAT (5 or 10 mg/kg), DOI (20 or 30 mg/kg), α-Me-5-HT maleate (30 mg/kg) or 1-PHE (10 mg/kg) 60 min before decapitation. Each group consisted of eight to ten animals.

Experiment 4 Previous experiments evidenced that agonists of different receptors subtypes selectively inhibited FSH secretion in 16-day-old male rats. To confirm the effects of different agonists in females, 16- and 23-day-old female rats were injected i.p. with vehicle, 8-OH-DPAT (5 mg/kg) or DOI (20 mg/kg) 60 min before decapitation. Each group consisted of eight to ten animals.

Experiment 5 In order to determine the cross-talk between serotonergic and EAA pathways in the control of gonadotropin secretion, 5-, 16- and 23-day-old male and female rats were i.p. injected with vehicle, 8-OH-DPAT (5 mg/kg) or DOI (20 mg/kg) 45 min before administration of vehicle, NMDA (15 mg/kg) or AMPA (2.5 mg/kg). Animals were decapitated 15 min after NMDA or AMPA injection. Each group consisted of eight to ten animals.

All experiments were approved by the Cordoba University ethical committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals.

Hormone determinations

After centrifugation (1600 *g* at 4 °C for 20 min), serum was collected, frozen and stored at –20 °C until use. The concentrations of LH and FSH were measured in 10–25 µl using a double antibody RIA method using a kit supplied by NIH (Bethesda, MD, USA). Rat-LH-I-9 and Rat-FSH-I-8 were labeled with ¹²⁵I by the Chloramine T method (26) and hormone concentrations were expressed using reference preparation (RP) LH-RP-S3 and FSH-RP-S2 as standards. Intra- and interassay variations were 8% and 10% respectively. The sensitivity of the assay was 20 and 7.5 pg/tube, respectively, for LH and FSH. All samples of each experiment were measured in the same assay.

Statistics

Values are expressed as means ± S.E.M. Differences between groups were analyzed using one- or two-way ANOVA followed by Tukey's test.

Table 1 Serum LH and FSH concentration (ng/ml) in 23-day-old male and female rats treated with PCPA (250 mg/kg) s.c. 72 h before NMDA (15 mg/kg) i.p. administration. Animals were decapitated 15 min after NMDA injection. Values are given as means ± S.E.M. (*n* = 8–10 animals per group).

Treatment	Males		Females	
	LH	FSH	LH	FSH
Vehicle + vehicle	0.15 ± 0.03	7.14 ± 0.6	0.13 ± 0.009	11.80 ± 0.8
Vehicle + NMDA	1.15 ± 0.27**	7.87 ± 0.7	2.39 ± 0.56**	10.90 ± 1.0
PCPA + vehicle	0.12 ± 0.02	5.15 ± 0.6**	0.14 ± 0.04	6.87 ± 0.4**
PCPA + NMDA	1.50 ± 0.13**	8.20 ± 0.7	4.37 ± 0.75**, a	11.08 ± 1.15

** *P* < 0.01 vs vehicle + vehicle-injected group. a, *P* < 0.01 vehicle + NMDA vs PCPA + NMDA-injected group (ANOVA followed Tukey's test).

Results

Experiment 1

Blockade of serotonin synthesis after PCPA administration decreased serum FSH concentrations in 23-day-old male and female rats, whereas LH concentrations remained unchanged. The stimulatory effect of NMDA on LH secretion in 23-day-old female rats was increased after treatment with PCPA (Table 1).

Experiment 2

Neither the activation of the serotonergic system (with Fx and 5-HTP) nor AMPA receptor activation affected serum LH and FSH concentrations in 23-day-old male rats. The combined administration of 5-HTP and Fx with AMPA was also ineffective (Table 2).

Experiment 3

In male rats (16 or 23 days old), serum LH concentrations remained unaffected after injection of 8-OH-DPAT (5 or 10 mg/kg), DOI (20 or 30 mg/kg), α-Me-HT (30 mg/kg) or 1-PHE (10 mg/kg). In contrast, serum FSH concentrations were inhibited on day 16 by 8-OH-DPAT, α-Me-5-HT and 1-PHE (Table 3).

Experiment 4

In 16-day-old female rats, 8-OH-DPAT, but not DOI, significantly increased serum LH and FSH concentrations. This effect disappeared on day 23 (Table 4).

Experiment 5

Males The stimulatory effect of NMDA on LH secretion was observed on all days studied (Fig. 1). The effects of NMDA were reduced by 8-OH-DPAT on days 5, 16 and 23, the reduction being statistically significant on days 5 and 23. DOI also blocked the NMDA-stimulated LH secretion; this effect was not observed on day 5 (Fig. 1).

Females The stimulatory effect of NMDA on LH secretion was observed on all days studied (Fig. 2). The effect of NMDA was significantly reduced by

Table 2 Serum LH and FSH concentrations in 23-day-old male rats treated with Fluoxetine (Fx) + 5-HTP (10 and 100 mg/kg respectively) 45 min before administration of AMPA (2.5 mg/kg). Animals were decapitated 15 min after AMPA injection. Values are given as means $\pm$ S.E.M. ($n = 8-10$ animals per group).

Treatment	LH (ng/ml)	FSH (ng/ml)
Vehicle + vehicle	0.04 $\pm$ 0.005	1.95 $\pm$ 0.16
Vehicle + AMPA	0.14 $\pm$ 0.05	2.48 $\pm$ 0.39
Fx + 5-HTP + vehicle	0.16 $\pm$ 0.06	2.11 $\pm$ 0.27
Fx + 5-HTP + AMPA	0.07 $\pm$ 0.01	2.52 $\pm$ 0.20

8-OH-DPAT on days 5, 16 and 23, whereas DOI only blocked significantly the NMDA-stimulated LH secretion on day 16 (Fig. 2).

Serum LH levels did not change in 16- and 23-day-old male and female rats injected with AMPA alone or AMPA plus 8-OH-DPAT, DOI, α -Me-HT or 1-PHE (data not shown).

Discussion

The results from this study indicate that activation of the serotonergic system with 5-HTP plus Fx did not affect gonadotropin secretion in 23-day-old males, which agrees with previous data obtained in 16-day-old males (23). PCPA selectively decreased FSH secretion in 23-day-old males and females. This effect is in keeping with the increase in gonadotropin

secretion observed in 16-day-old female rats after administration of 5-HTP or 8-OH-DPAT (24).

In recent years, different subtypes of 5-HT receptors have been identified in the brain and subtype-specific agonists and antagonists have been developed. At least seven families of 5-HT receptors have been so far reported (27–29). To advance in the pharmacological characterization of the serotonin receptor subtypes involved in the control of gonadotropin secretion as well as in the effects of NMDA and AMPA on gonadotropin release, we used selective agonists of 5-HT₁, 5-HT₂ and 5-HT₃ receptor subtypes in male and female rats. Data obtained confirmed the sexual differences in the control of gonadotropins by the serotonergic system (13, 14), since: (1) activation of different 5-HT subtypes did not affect LH secretion in either 16- or 23-day-old males (Table 3), whereas 8-OH-DPAT stimulated LH in 16-day-old females (Table 4); and (2) FSH secretion was inhibited by 8-OH-DPAT in 16-day-old males (Table 3) and increased in females (Table 4).

Serum FSH levels were reduced selectively in 16-day-old males after activation of 5-HT_{1A}, 5-HT₂ and 5-HT₃ receptors (with 8-OH-DPAT, α -Me-5-HT and 1-PHE respectively), whereas LH remained unchanged. Concerning these selective actions on FSH secretion, it is noticeable that in prepubertal female rats phaclofen (an antagonist of GABA_B receptors) selectively stimulated FSH secretion without changes in LH release (21).

Regarding the mechanism(s) involved in such a FSH-specific inhibitory action, the possibility that activation

Table 3 Effects of 5-HT₁, 5-HT₂ and 5-HT₃ agonists on gonadotropin secretion in prepubertal male rats. Animals were decapitated 60 min after injections. Values are given as means $\pm$ S.E.M. ($n = 8-10$ animals per group).

Treatment	16 days		23 days	
	LH	FSH	LH	FSH
Vehicle	0.10 $\pm$ 0.03	7.72 $\pm$ 1.11	0.11 $\pm$ 0.02	5.64 $\pm$ 0.81
8-OH-DPAT (5 mg/kg)	0.47 $\pm$ 0.16	4.19 $\pm$ 0.29*	0.14 $\pm$ 0.01	4.67 $\pm$ 0.51
8-OH-DPAT (10 mg/kg)	0.15 $\pm$ 0.01	4.47 $\pm$ 0.43*	0.13 $\pm$ 0.03	6.40 $\pm$ 0.52
DOI (20 mg/kg)	0.12 $\pm$ 0.03	5.67 $\pm$ 0.50	ND	ND
DOI (30 mg/kg)	0.04 $\pm$ 0.02	6.19 $\pm$ 0.82	0.15 $\pm$ 0.02	4.34 $\pm$ 1.13
α -Me-HT (10 mg/kg)	0.14 $\pm$ 0.02	4.88 $\pm$ 0.49*	0.25 $\pm$ 0.06	5.32 $\pm$ 0.62
1-PHE (10 mg/kg)	0.11 $\pm$ 0.02	3.76 $\pm$ 0.55**	0.06 $\pm$ 0.02	4.21 $\pm$ 0.34

* $P < 0.05$; ** $P < 0.01$ vs vehicle-injected group (ANOVA followed by Tukey's test). ND, not determined. 1-PHE, 1-Phenylbiguanide.

Table 4 Effects of 5-HT₁ and 5-HT₂ agonists on gonadotropin secretion in prepubertal female rats. Animals were decapitated 60 min after injections. Values are given as means $\pm$ S.E.M. ($n = 8-10$ animals per group).

Treatment	16 days		23 days	
	LH	FSH	LH	FSH
Vehicle	0.16 $\pm$ 0.01	22.6 $\pm$ 1.2	0.11 $\pm$ 0.007	4.47 $\pm$ 0.45
8-OH-DPAT (5 mg/kg)	0.54 $\pm$ 0.09**	31.0 $\pm$ 2.2**	0.10 $\pm$ 0.009	5.77 $\pm$ 0.51
DOI (20 mg/kg)	0.23 $\pm$ 0.02	26.7 $\pm$ 1.6	0.12 $\pm$ 0.01	5.25 $\pm$ 0.41

** $P < 0.01$ vs vehicle-injected group (ANOVA followed by Tukey's test).

Figure 1 Serum LH concentrations, at different age-points, in male rats injected with vehicle, NMDA (15 mg/kg) alone or in combination with 8-OH-DPAT (5 mg/kg) or DOI (20 mg/kg). Values are expressed as means $\pm$ S.E.M. ($n = 8-10$ animals per group). ** $P \leq 0.01$ vs vehicle-injected group; a, $P \leq 0.01$ vs group injected with NMDA alone (ANOVA followed by Tukey's test).

Figure 2 Serum LH concentration, at different age-points, in female rats injected with vehicle, NMDA (15 mg/kg) alone or in combination with 8-OH-DPAT (5 mg/kg) or DOI (20 mg/kg). Values are expressed as means $\pm$ S.E.M. ($n = 8-10$ animals per group). ** $P < 0.01$ vs vehicle-injected group; a, $P < 0.01$ vs group injected with NMDA alone (ANOVA followed by Tukey's test).

of 5-HT_{1A}, 5-HT₂ and 5-HT₃ receptors reduced LHRH secretion is difficult to reconcile with the absence of concomitant changes in serum LH concentrations. Different studies have repeatedly proposed the existence of a selective FSH-releasing factor (30, 31) to explain dissociation in the control of both gonadotropins. It can not be ruled out that this specific FSH-releasing factor might be specifically controlled by the serotonergic system. Alternatively, it should be considered that the selective release of both gonadotropins is related to the frequency and amplitude of LHRH pulses. Pituitary stimulation with low-frequency pulses of LHRH selectively stimulates FSH secretion (32, 33); it is possible that activation of 5-HT_{1A}, 5-HT₂ and 5-HT₃ receptors selectively reduced the frequency of LHRH pulses.

It has been reported previously that the role of serotonergic pathways in the neuroendocrine control changes during sexual maturation and the stimulatory effect of serotonin on gonadotropin secretion in prepubertal rats younger than 20 days of age disappears after this age (34–36). Similar changes have been described for the GABAergic control of gonadotropin secretion (37, 38). It is possible that the inhibitory effect on FSH release, observed only on day 16 of age, after activation of 5-HT_{1A}, 5-HT₂ and 5-HT₃ receptors is limited to specific periods of development, as is the case for the serotonergic control of GH secretion (17).

In addition, the present experiments were designed to analyze the interactions between NMDA, AMPA and 5-HT₁, 5-HT₂ and 5-HT₃ receptors in the control of LH secretion, using specific agonists of these

receptors. One of the major findings of the present study is that, as is the case for GH release (17), the stimulatory effect of NMDA on LH secretion can be blocked by coactivation of the serotonergic system, through specific 5-HT₁ and 5-HT₂ receptor-mediated pathways. In this sense it has been described (39) that the serotonergic system via 5-HT₁, but not 5-HT₂, receptors inhibits LH release in ovariectomized rats primed with steroids. The inhibitory effect of 8-OH-DPAT and DOI on NMDA-stimulated LH secretion (Figs 1 and 2) agrees with the potentiation of LH response to NMDA stimulation after blockade of serotonin synthesis with PCPA (Table 1). The inhibitory effect of 8-OH-DPAT was observed in both sexes at all ages studied, whereas the inhibitory effect of DOI appeared after day 5, which suggests a different temporal coordination between NMDA and serotonergic receptors subtypes.

The mechanism whereby NMDA stimulates gonadotropin secretion involves an increase in LHRH release (6), through pathways that are not clearly understood at present. Only a 5% of hypothalamic LHRH neurons express NMDA receptor mRNA (40, 41), which suggests that NMDA stimulates LHRH release through interneurons, such as those releasing nitric oxide (42). Unfortunately, the location of different serotonin receptor subtypes in hypothalamic neurons has not been elucidated, making it difficult to explain the pathway whereby NMDA-induced LH secretion was blunted by activation of 5-HT₁ and 5-HT₂ receptors. One possibility is that 5-HT₁ and 5-HT₂ receptors are present in axonic terminals of LHRH neurons and their activation inhibited the release of LHRH. Another possibility is that NMDA receptors involved in the neuroregulation of LHRH secretion could be regulated by serotonin receptors. In this sense, it is noticeable that 5-HT acting through 5-HT_{1A} and 5-HT_{1B} receptors depressed excitatory postsynaptic potentials, mediated mainly by NMDA and AMPA receptors, in entorhinal cortex (43, 44), hippocampal slices (45) and nucleus accumbens (46). If a similar effect occurs in hypothalamic neurons, the modulation by serotonergic receptors of gonadotropin responses to NMDA could be explained.

In conclusion, our results indicate that although basal LH secretion is not under the control of the serotonergic system, NMDA-elicited LH release was blunted by activation of 5-HT₁ and 5-HT₂ receptors in prepubertal male and female rats.

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