Selective role of neuropeptide Y receptor subtype Y2 in the control of gonadotropin secretion in the rat


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IT IS KNOWN THAT THE INITIATION OF PUBERTY and the integrity of reproductive function are physiologically coupled to nutritional status. A large body of evidence accumulated over the last several years (14, 23, 24, 40, 42) indicates that feeding behavior and activity of the gonadotropic axis are linked and that the pancreatic polypeptide family may be pivotal in this relationship. The pancreatic polypeptide family consists of a series of related peptides composed of 36 amino acids termed neuropeptide Y (NPY), peptide YY (PYY), and pancreatic peptide (PP). NPY is located predominantly within neurons of the central and sympathetic nervous systems (1, 10, 28); PYY (70% similar to NPY) is secreted by the gastrointestinal tract and is converted into the PYY3-36 form by the enzyme dipeptidyl peptidase IV, which is widely distributed in numerous tissues and in plasma (17); and finally, PP is secreted by cells within the endocrine and exocrine pancreas. All of these peptides carry out diverse biological actions through interaction with different receptor subtypes, namely Y receptors, that are members of the seven-transmembrane domain G protein-coupled receptors associated with inhibition of adenylate cyclase.

In both humans and rats, five Y receptor subtypes (Y1–Y5) have been characterized on the basis of different pharmacological profiles and/or cloning (6, 15, 31). The Y1 receptor subtype requires the full molecule of NPY and PYY for its activation and is selectively activated by [Leu31,Pro34] homologues, although it has lower affinity for COOH-terminal fragments such as NPY3-36 and PYY3-36 (10). The Y2 receptor is preferentially activated by COOH-terminal NPY and PYY fragments and is activated to a lower extent by the [Leu31,Pro34] homologues (13). The Y3 receptor is preferentially activated by NPY rather than PYY, whereas the Y4 receptor is activated by PP and PYY but not NPY derivatives (31). Finally, the Y5 receptor subtype is activated by PP, NPY, PYY, and PYY3-36 (31). Regarding the location in the brain of different Y receptor subtypes, the Y2 receptors are the predominant Y receptor subtype in the brain, whereas the Y4 subtype represents only a very small proportion of the total population of Y receptors. The Y2 receptor is associated with suppression of the release of transmitters such as glutamate, noradrenalin, and NPY (9, 11, 27, 30), and it is involved in NPY’s effects on food intake, gastrointestinal motility, cardiovascular regulation, and neuronal excitability (8, 22, 34). A number of brain regions contained the Y3 receptor subtype, including various hypothalamic nuclei, the midline thalamus, parts of the amygdala, the hippocampus, and some midbrain and brain stem nuclei. Together with the Y1 receptor subtype, the Y5 receptor has been implicated in mediating the orexigenic effect of NPY and in regulating food intake and body weight (12, 16, 19, 20).

Although the role of different Y receptor subtypes in the control of food intake was clarified years ago (Y1 and Y5 are appetite-stimulating receptors and Y2 is an appetite-inhibiting receptor) (3, 16, 45), their participation in the control of reproductive function remained poorly defined, probably because data about the effects of different pancreatic polypeptide family members on the control of the hypothalamic-pituitary-gonadal axis are controversial. Thus, for NPY (preferential Y1 and Y5 agonist), discrepant stimulatory or inhibitory effects on sexual maturation and reproduction have been observed de-
pending upon species, steroid environment (26), the site of NPY administration into the brain (37), and a chronic (18, 38, 39) vs. acute (32) pattern of infusion. Likewise, PYY\textsubscript{3-36} (preferential Y\textsubscript{2} and Y\textsubscript{5} agonist) has been reported to be stimulatory or inhibitory or to have no effect on gonadotropin-releasing hormone (GnRH) and gonadotropin secretion depending upon the age, sex, steroid environment, and in vivo vs. in vitro studies (14, 40, 43). PP (preferential Y\textsubscript{4} agonist) has been reported to increase or not affect gonadotropin secretion depending upon the species and steroidoidal environment (21, 43, 44).

In previous experiments carried out in prepubertal and adult rats (14, 40) we have shown that PYY\textsubscript{3-36} controlled GnRH and gonadotropin secretion, and we demonstrated that its effects are modulated by nutritional status. The expression of the genes encoding Y\textsubscript{2} and Y\textsubscript{5} receptors in the hypothalamus and pituitary (14, 40) opens up the possibility that the described effects of PYY\textsubscript{3-36} were mediated by Y\textsubscript{2} or Y\textsubscript{5} receptors or the net balance between both of them. To clarify the participation of the Y\textsubscript{2} receptor subtype in the control of gonadotropin secretion, BIIE 0246 (agonist of Y\textsubscript{2} receptors) and PYY\textsubscript{13-36} (agonist of Y\textsubscript{2} receptors) were tested, either in vivo or in vitro, in male rats at different ages and with different steroidoidal background. In addition, since fasting significantly impairs reproductive function (4, 5, 7, 40), and given that the effects of PYY\textsubscript{3-36} were potentiated during fasting (40), we also analyzed the actions of PYY\textsubscript{13-36} and BIIE 0246 in male rats subjected to fasting.

**MATERIALS AND METHODS**

**Animals and Drugs**

Male Wistar rats born in our laboratory were kept under controlled conditions of light (12:12-h light-dark cycle, lights on at 0700) and temperature (22°C) with free access to pelleted food (Pascu Sanders, Sevilla, Spain) and tap water. Experiments were carried out in prepubertal (25 days) and adult animals. NPY (agonist of Y\textsubscript{1}, Y\textsubscript{2}, and Y\textsubscript{5} receptors), PYY\textsubscript{13-36} (agonist of Y\textsubscript{2} receptors), and BIIE 0246 (agonist of Y\textsubscript{2} receptors) were purchased from Bachem (Barcelona, Spain). NPY and PYY\textsubscript{13-36} were dissolved in saline or DMEM immediately before use, and BIIE 0246 was dissolved in 15% DMSO.

**Experimental Designs**

Experimental procedures were approved by the Córdoba University Ethics Committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals. The number of animals per experimental group was 10–14 in the in vivo experiments, and each experimental group consisted of 10–12 samples in the in vitro studies. Adult animals were used at 90–110 days of age. Experiments were carried out between 1000 and 1200. Special caution was taken to avoid any stressing influences upon the experimental animals (all of the animals were handled daily for 1 wk before the experiment, they were humanely killed by the same person, and the different drugs were injected at random).

In the initial set of experiments, we analyzed the effects of central injection of PYY\textsubscript{13-36} on gonadotropin secretion in intact and orchidectomized prepubertal and adult males. We also evaluated the physiological relevance of Y\textsubscript{2} receptors.

**Experiment 1: effects of NPY and PYY\textsubscript{13-36} on gonadotropin secretion in prepubertal males.** This experiment was designed to comparatively analyze the effects of acute administration of NPY and PYY\textsubscript{13-36} on gonadotropin secretion in intact and orchidectomized animals. Male rats (day 18 postpartum) were orchidectomized or sham orchidectomized and were implanted 3 days later, under light ether anesthesia, with intracerebroventricular cannulae into the lateral cerebral ventricle. The cannulae were lowered to a depth of 3 mm beneath the surface of the skull; the insert point was 1 mm posterior and 1.2 mm lateral to bregma (33, 41). On day 25 postpartum (1 wk after orchidectomy or sham orchidectomy), NPY (0.1, 1.0, or 3.0 nmol•rat\textsuperscript{-1}•10\textsuperscript{-3} µmol\textsuperscript{-1}), PYY\textsubscript{3-36} (0.1 or 1.0 nmol•rat\textsuperscript{-1}•10\textsuperscript{-3} µmol\textsuperscript{-1}), or vehicle was intracerebroventricularly injected and the animals humanely killed by decapitation 15 min after the injections. Blood samples were taken to analyze LH and FSH levels.

**Experiment 2: effects of activation of Y\textsubscript{2} receptors on gonadotropin secretion in adult males.** To analyze whether the effects of activation of Y\textsubscript{2} receptors change with age and/or are modulated by testicular secretion, adult males were orchidectomized or sham orchidectomized and were implanted 3 days later as described above with an intracerebroventricular cannulae into the lateral cerebral ventricle. One week after orchidectomy or sham orchidectomy the animals were intracerebroventricularly injected with PYY\textsubscript{13-36} (0.1, 1.0, or 3.0 nmol•rat\textsuperscript{-1}•10\textsuperscript{-3} µmol\textsuperscript{-1}) or vehicle, and blood samples were collected by jugular venipuncture after light ether anesthesia before and 15, 30, and 60 min after the injections. Blood samples were collected to analyze LH and FSH levels.

**Experiment 3: effects of activation of Y\textsubscript{2} receptors on gonadotropin secretion in adult fasted males.** It is well known (4, 5, 7, 40) that gonadotropin secretion is profoundly depressed after fasting. Since in a previous study (40) we demonstrated that the effects of PYY\textsubscript{3-36} on gonadotropin secretion are modulated by fasting, we analyzed the effects of PYY\textsubscript{13-36} in fasted male rats. Adult males fed ad libitum or submitted to a 4-day period of total food restriction were intracerebroventricularly injected with PYY\textsubscript{13-36} (0.1, 1.0, or 3.0 nmol•rat\textsuperscript{-1}•10\textsuperscript{-3} µmol\textsuperscript{-1}) or vehicle through a cannula that was implanted as described above. Blood samples were obtained by jugular venipuncture after light ether anesthesia before and 15, 30, and 60 min after the injections.

**Experiment 4: effects of antagonization of Y\textsubscript{2} receptors on gonadotropin secretion.** Data obtained in previous experiments showed that PYY\textsubscript{13-36} induced a significant inhibition of gonadotropin secretion in different experimental conditions. To analyze the functional relevance of this finding, adult male rats fed ad libitum, either orchidectomized or sham orchidectomized 1 wk earlier, and intact males subjected to 4-day fasting were implanted, as described previously, with intracerebroventricular canulae and injected with BIIE 0246 (5 nmol•rat\textsuperscript{-1}•10\textsuperscript{-3} µmol\textsuperscript{-1}) or vehicle. Blood samples were obtained by jugular venipuncture after light ether anesthesia before and 15, 30, and 60 min after the injection. The dose of antagonist was selected on the basis of previous experiments showing that higher doses (10 and 20 nmol/rat) induced similar gonadotropin responses but clear-cut signs of locomotor hyperactivity (our unpublished data).

In addition, the following set of experiments was carried out to analyze the possible targets involved in the inhibitory effect of PYY\textsubscript{13-36} (Y\textsubscript{2} receptor activation) on gonadotropin secretion.

**Experiment 5: effects of activation of Y\textsubscript{2} receptors on GnRH secretion by hypothalamic explants from adult male rats.** To analyze a potential primary action of PYY\textsubscript{13-36} upon hypothalamic GnRH secretion, a static incubation system was used. Adult male rats were humanely killed by decapitation, and the hypothalami (excised by a horizontal cut of ~2 mm depth with the following limits: 1 mm anterior from the optic chiasm, the posterior border of the mammillary bodies, and the hypothalamic fissures) were rapidly dissected out. Tissue samples were subsequently incubated in 250 µL of DMEM in a Dubnoff shaker incubator under an atmosphere of 95% O\textsubscript{2}-5% CO\textsubscript{2} at 37.5°C. After a 30-min preincubation the medium was removed, and the hypothalami were challenged for 60 min with PYY\textsubscript{13-36} (10\textsuperscript{-6} M), 56 nM KCl, or medium alone. At the end of incubation period, medium samples were boiled for 30 min to inactivate endogenous

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protease activity and were stored at −80°C until they were used for hormone measurements.

**Experiment 6: effects of activation of Y₂ receptors on pituitary basal and GnRH-stimulated gonadotropin secretion.** To analyze a potential primary action of PYY₁₃-₃₆ at the pituitary level in the regulation of gonadotropin secretion, adult male rats were humanely killed by decapitation, and the pituitaries were immediately dissected, the posterior lobe was discarded, and the pituitaries were halved. The hemipituitaries were placed in glass scintillation vials (1 hemipituitary/vial) in a Dubnoff shaker at 37°C in an atmosphere of 95% O₂-5% CO₂. Each vial contained 1 ml of DMEM. After preincubation for 60 min, the medium was replaced by fresh medium alone or medium containing PYY₁₃-₃₆ (10⁻¹⁰, 10⁻⁸, and 10⁻⁶ M), GnRH (10⁻⁷ M), or GnRH (10⁻⁷ M) plus PYY₁₃-₃₆ (10⁻¹⁰, 10⁻⁸, and 10⁻⁶ M). Samples of media were obtained after 60 and 120 min of incubation.

**Hormone Assays**

Serum and medium concentrations of LH and FSH were measured using a double-antibody method and radioimmunoassay kits kindly supplied by the National Institutes of Health (Dr. A. F. Parlow; National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Torrance, CA). Rat LH-I-9 and FSH-I-9 were labeled with¹²⁵I using the Iodo-Gen method, following the instructions of the manufacturer (Pierce, Rockford, IL), and hormone concentrations were expressed using LH-RP-3 and FSH-RP-2 as standards. All samples were measured in duplicate, and all samples from each experiment were measured in the same assay. Intra-assay coefficients of variation were <8%, and the sensitivities of the assays were 20 and 7.5 pg/50 μl for LH and FSH, respectively.

GnRH concentrations in the incubation medium were measured in 100-μl aliquots using a commercial RIA kit from Peninsula Laboratories (Bachem Group, San Carlos, CA), following the instructions of the manufacturer. The sensitivity of the assay was 1 pg/tube. All samples were measured in the same assay.

**Presentation of Data and Statistics**

Values are expressed as means ± SE. When appropriate, integrated LH and FSH secretor responses were calculated as the area under the curve, using the trapezoidal rule. Differences between groups were analyzed using the unpaired Student’s t-test or ANOVA followed by Student-Newman-Keuls multiple-range test (SigmaStat 2.0; Jandel, San Rafael, CA). *P ≤ 0.05 was considered significant.

**RESULTS**

**Effects of Central Administration of NPY and PYY₁₃-₃₆ on Gonadotropin Secretion in Prepubertal Males**

In intact and orchidectomized prepubertal male rats, intracerebroventricular administration of vehicle or different doses of neuropeptide Y (NPY). Values are expressed as means ± SE (for 10–12 animals/group). **P ≤ 0.01 vs. corresponding vehicle-injected group; *P ≤ 0.01 vs. corresponding intact group (ANOVA followed by Student-Newman-Keuls multiple-range test).
Effects of Central Administration of PYY13-36 on Gonadotropin Secretion in Adult Intact and Orchidectomized Males Fed Ad Libitum

In intact adult male rats, intracerebroventricular administration of PYY13-36 significantly decreased LH secretion at doses of 1 and 3 nmol (Fig. 3, top). This effect was evident 15 min after injection and remained significant for ≥60 min. After orchidectomy, the inhibitory effect of PYY13-36 on LH secretion was evident only with the 3-nmol dose (Fig. 3, bottom).

Central intracerebroventricular administration of PYY13-36 significantly decreased FSH secretion in intact (Fig. 4, top) and orchidectomized males (Fig. 4, bottom). In intact rats, the effect was statistically significant 15 min after injection of 1 and 3 nmol and remained evident for ≥60 min, whereas only the highest dose was effective in orchidectomized rats.

Effects of Central Administration of PYY13-36 on Gonadotropin Secretion in Adult Fasted Males

In males submitted to a 4-day fasting period, a significant decrease in body weight was observed (273 ± 4.8 vs. 320 ± 5.6 g in controls). Serum gonadotropin secretion was reduced in fasted rats (LH: 0.17 ± 0.04 vs. 1.16 ± 0.08 ng/ml in controls; FSH: 5.41 ± 1.0 vs. 9.12 ± 0.63 ng/ml in controls). Administration of PYY13-36 induced further reductions in gonadotropin secretion (Fig. 5), which was more evident for FSH, probably due to the extremely low levels of LH after fasting.

Effects of Central Administration of BIIE 0246 on Gonadotropin Secretion in Adult Fed and Fasted Males

Central intracerebroventricular administration of a single dose of 5 nmol of Y2 receptor antagonist had no significant effect on circulating LH levels at any of the time points studied either in adult males fed ad libitum or in males after 4-day fasting (Fig. 6, top and bottom). A lack of effect on FSH was also observed (data not shown). In contrast, the antagonist led to a significant increase in serum LH concentrations 15 and 30 min after injection to orchidectomized animals (Fig. 6, middle). An increase in FSH secretion was also observed at these time points in orchidectomized animals (data not shown).
Effects of PYY 13-36 on Hypothalamic GnRH Secretion

The amount of GnRH released by the hypothalami of adult intact males over 30 min significantly increased after depolarization with KCl (28.04 ± 2.07 vs. 22.51 ± 1.47 pg/hypothalamus in controls) and decreased in the presence of 10^{-6} M PYY_{13-36} (17.95 ± 1.72 vs. 22.51 ± 1.47 pg/hypothalamus in controls). The statistical analysis indicated that differences between groups were significant (F = 8.14, P ≤ 0.002).

Effects of PYY_{13-36} on Basal and GnRH-Stimulated Gonadotropin Secretion

Concentrations of LH in the medium after 60 and 120 min of incubation were unaffected by PYY_{13-36} at the doses of 10^{-10}, 10^{-8}, and 10^{-6} M. The concentration of FSH was decreased after 60 min of incubation with PYY_{13-36} at the dose of 10^{-6} M (Fig. 7, top). GnRH significantly stimulated the release of both gonadotropins, an effect that was potentiated by PYY_{13-36} with all of the doses tested (Fig. 7, bottom).

DISCUSSION

During the last decade, our knowledge on the signals involved in the cooperative control of energy balance and reproduction has significantly increased. Leptin, ghrelin, and PYY_{3-36} play an important role in the control of the hypothalamic-pituitary-gonadal axis (2, 14, 40, 48). The expression of the genes encoding Y_{2} and Y_{5} receptors in hypothalamus and pituitary (14, 40) opens the possibility that the described effects of PYY_{3-36} were mediated by Y_{2} or Y_{5} receptors or...
by the net balance between the effects mediated by each. To clarify this possibility, we took advantage of the use of PYY13-36, a selective Y2 agonist. Indeed, this peptide analog has been widely used in studies aimed to decipher the roles of Y2 receptors in different physiological processes, such as arterial vasoconstriction (50), vago-vagal reflex circuitry (8), or exocrine pancreatic secretion (49). Yet to our knowledge, no studies on the gonadotropic effects of such Y2 receptor agonists had been reported previously.

The major findings of the present study can be summarized as follows: 1) acute administration of different doses of NPY to intact and orchidectomized prepubertal males stimulated LH and FSH secretion, whereas, in contrast, PYY13-36 was inhibitory; 2) PYY13-36 significantly decreased gonadotropin secretion in intact, orchidectomized, and fasted adult males; 3) tonic activation of Y2 receptors seems to take place after removal of testicular secretion, when Y2 receptor antagonization elicits further increases in serum gonadotropins, a phenomenon that is not observed in intact males regardless of their feeding status; and 4) PYY13-36 inhibited GnRH release by hypothalamic explants ex vivo but potentiated GnRH-stimulated gonadotropin release directly at the pituitary level.

Data from experiments in prepubertal male rats showed that a single intracerebroventricular injection of NPY significantly enhanced gonadotropin secretion, which suggests the possible involvement of NPY in puberty onset, a role that was previously indicated in females (32, 46). In contrast, intracerebroventricular administration of PYY3-36 (14) or PYY13-36 (present data) induced a clear inhibition of LH secretion. Overall, these results are evidence that, in prepubertal males, the inhibitory effect of PYY13-36 on LH secretion is mediated by activation of Y2 receptors. In addition, our present data in orchidectomized rats demonstrate that neither testicular inputs nor prevailing LH and FSH levels appear to modify gonadotropin responses to NPY or PYY13-36 before puberty.

As was the case in prepubertal animals, activation of Y2 receptors after administration of 3 nmol/rat icv of PYY13-36 induced a significant reduction in LH and FSH secretion in adult male rats in a diversity of experimental conditions (intact and in orchidectomized males, as well as in fasted animals). Since previous data showed that PYY13-36 significantly enhanced gonadotropin secretion in control and fasted adult male rats (40), our present results suggest that such stimulatory effects of PYY3-36 were not mediated via Y2 receptors. In addition, our current data demonstrate that, at the adult age, testicular inputs modulated the inhibitory actions of Y2 receptor activation as central injection of 1 nmol of PYY13-36 to intact males decreased both LH and FSH secretion, whereas it was ineffective in orchidectomized animals.

To analyze the impact of blockade of endogenous Y2 receptors upon gonadotropin secretion, experiments assessing the effects of central administration of BIIE 0246, a selective Y2 receptor antagonist, were conducted in adult male rats. The lack of effects on gonadotropin levels observed in intact male rats either fed ad libitum or subjected to fasting suggests that, in these conditions, Y2 receptors are not constitutively activated. In contrast, after orchidectomy, antagonization of Y2 receptors further increased LH and FSH secretion. These opposite findings might present evidence that, after removal of testicular inhibitory inputs to GnRH/LH system, endogenous activation of Y2 receptors takes place as servomechanism to prevent excessive secretion of gonadotropins. Accordingly, in these conditions, blockade of Y2 receptors resulted in further increases in gonadotropin secretion. Of note, inhibitory roles of Y2 receptor activation have previously been reported (3, 8, 16, 22, 34, 45) in a wide variety of biological processes such as food intake, gastrointestinal motility, cardiovascular regulation, or neuronal excitability.

Y2 receptor subtype is the predominant Y receptor in the brain and is also present at the pituitary level (14, 40). In the present work we have analyzed the possible contribution of hypothalamic and pituitary Y2 receptors in the suppression of gonadotropin secretion that is induced by central administration of PYY13-36. To this end, the effects of this peptide on

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**Fig. 7. Effects of PYY13-36 (10^{-10}, 10^{-8}, and 10^{-6} M) on basal (top) and gonadotropin-releasing hormone (GnRH)-stimulated (bottom) levels of LH and FSH (ng/ml) secreted by hemipituitaries obtained from adult males. Values are expressed as means ± SE (for 10–12 samples/group). **P ≤ 0.01 vs. hemipituitaries incubated in absence of PYY3-36 (ANOVA followed by Student-Newman-Keuls test).**
GnRH and gonadotropin release were explored using incubations of hypothalamic and pituitary tissue, respectively. Our results demonstrate an inhibitory effect of PYY\textsubscript{13-36} on GnRH release, which is coincident with data reported previously by our group (14, 40) on the ability of PYY\textsubscript{3-36} to decrease hypothalamic GnRH secretion in prepubertal and adult male rats, thus suggesting the involvement of Y\textsubscript{2} receptor activation in mediating such action. The inhibition of GnRH release might be directly exerted on GnRH neurons or mediated through blockade of different neurotransmitters such as glutamate, NPY, or noradrenaline, which are well-known stimulatory inputs for GnRH neurons (23, 25, 29, 35, 36, 46) and whose release is inhibited after activation of Y\textsubscript{2} receptors (9, 11, 27, 30). Admittedly, the mechanism(s) involved in the reduction of GnRH release by PYY\textsubscript{13-36} needs to be characterized further. Yet, the functional relevance of this phenomenon in the control of the gonadotrophic axis by Y\textsubscript{2} receptors is reinforced by the absence of inhibitory effects of PYY\textsubscript{13-36} on basal LH release directly at the pituitary level. In contrast, GnRH-stimulated gonadotropin responses were potentiated in the presence of PYY\textsubscript{13-36}, suggesting a complex, dual mode of action of Y\textsubscript{2} receptors in the control of gonadotropin secretion at different levels of the hypothalamic-pituitary unit.

In conclusion, present experiments have demonstrated that activation of Y\textsubscript{2} receptors inhibits gonadotropin secretion in vivo, an action that is apparently not conducted at the pituitary but is rather dependent on changes in GnRH release at the hypothalamus. In addition, our data have also disclosed the divergence in some of the gonadotrophic effects of PYY\textsubscript{3-36} (physiological agonist of Y\textsubscript{2} and Y\textsubscript{5} receptors) and PYY\textsubscript{13-36} (pharmacological agonist of Y\textsubscript{2} receptors), providing further proof for the complexity of Y receptor-mediated actions in the control of reproductive axis. Finally, our results strongly suggest that constitutive activation of Y\textsubscript{2} receptors is likely to take place after removal of testicular inhibitory inputs on the GnRH/gonadotropin system, a finding whose physiological relevance as servomechanism for the restrain of gonadotropin (hyper) secretion warrants further investigation.

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