The $GABA_B$ Antagonist CGP 52432 Attenuates the Stimulatory Effect of the $GABA_B$ Agonist SKF 97541 on Luteinizing Hormone Secretion in the Male Sheep

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The objectives of this study were to determine if the γ-aminobutyric acid $(GABA)_B$ agonist, 3-aminopropyl (methyl) phosphinic acid (SKF97541), would increase luteinizing hormone (LH) secretion when infused by microdialysis into the medial basal hypothalamus (MBH) of the castrated ram, and to determine if the action of SKF97541 would be attenuated by co-infusion of the $GABA_B$ antagonist CGP52432. Initial experiments established that infusion of SKF alone, at concentrations as low as 5 μM, increased mean LH, LH pulse amplitude, and in some cases, pulse interval. In the last experiment, animals were treated with artificial cerebrospinal fluid (CSF) alone, SKF alone (30 μM), 3-[[(3, 4-dichlorophenol) methyl] amino] propyl] diethoxymethyl) phosphinic acid (CGP) alone (500 μM), or SKF plus CGP. SKF increased both mean LH and LH pulse amplitude as compared with CSF. CGP alone had no significant effect on LH, but it attenuated the effect of SKF on mean LH. These observations indicate that the stimulatory effects of $GABA_B$ agonists on LH pulse patterns are mediated through $GABA_B$ receptors and provide further evidence that $GABA_B$ receptors located in the MBH can regulate pulsatile GnRH-LH release.

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Secretion of GnRH is regulated by external environmental factors as well as endogenous steroid hormones. Several neuronal peptides and amines serve as mediators of these regulatory factors, but the specific neural pathways and mechanisms involved are only partially defined (1, 2).

γ-Aminobutyric acid (GABA) is one of those mediators. A role for GABA is indicated by the presence of synapses between GABA and GnRH neurons in the preoptic area, and by numerous studies showing that administration of GABA or GABA analogues alter luteinizing hormone (LH) secretion (e.g., Refs. 3–7).

GABA is considered as primarily an inhibitory neurotransmitter that acts via three major receptor types: the $GABA_A$, $GABA_B$, and $GABA_C$ (8, 9). Activation of the $GABA_B$ receptor causes membrane hyperpolarization by changes in K^+ or Ca^{++} flux and reduction of neuronal firing. Consequently, a prevailing concept is that GABA suppresses GnRH secretion. This concept is supported by observations that GABA concentrations in the preoptic area fall coincident with onset of the LH surge in ewes (10), and that activity of glutamic acid decarboxylase (GAD) in the preoptic area (POA), the rate-limiting enzyme in GABA synthesis, falls after orchidectomy, and is elevated by testosterone (11, 12).

The specific site or sites at which GABA acts to regulate GnRH are not defined, but the fact that GABA and GABA receptors are widely distributed in the hypothalamus (13–15) makes it possible for existence of multiple control sites and mechanisms. Although most studies have focused on the POA, several observations also suggest the medial basal hypothalamus (MBH) as a site important for GABA regulation of GnRH (16–21). Recent elegant studies by Bilger *et al.* (22) revealed that tetracycline-dependent release of GABA from grafted astrocytes in the median eminence of the rat disrupts estrous cycles, possibly due to a stimulatory action of GABA. The specific site of this effect is not known, but other studies have suggested the presence of GABA receptors on GnRH neurons (23, 24) and that GABA agonists can alter GnRH release from neuronal cell lines (25).

Ferreira *et al.* (21) reported that infusion of the $GABA_B$ agonist baclofen into the MBH of castrated male sheep increased LH pulse amplitude without affecting pulse frequency. Additionally, infusion of baclofen into the MBH rapidly induced the appearance of robust GnRH and LH

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pulses in intact rams and elevated mean LH in testosterone-treated castrated rams (26). Although the responses to baclofen were consistent across animal models, the effects were unexpected and their physiological relevance is not clear. Although baclofen appears to affect only $GABA_B$ receptors (9), there is concern that the effects on LH were due to non-specific actions on other transmitter systems. To help address this issue, it is important to determine if another $GABA_B$ agonist will elevate LH secretion and if that effect can be attenuated, or blocked, by a $GABA_B$ antagonist. Accordingly, we tested the effects of the potent $GABA_B$ agonist 3-aminopropyl (methyl) phosphinic acid (SKF97541) and the $GABA_B$ antagonist 3-[[(3, 4-dichlorophenol) methyl] amino] propyl] diethoxymethyl) phosphinic acid (CGP52432) separately and together on basal LH secretion in castrated rams.

Materials and Methods

Adult rams, predominantly of the Suffolk breed, that had been castrated for at least 3 months were maintained outdoors at the Veterinary Research Farm (Urbana, IL; latitude 40°N) until a few days before undergoing surgery for bilateral placement of guide cannulae into the brain. Thereafter, they were housed indoors in a building with windows. The natural lighting was supplemented by artificial lighting appropriate to the season. They were fed a pelleted ration formulated by the Animal Science Department at the University of Illinois (Champaign-Urbana) and were given free access to water. The experimental protocol was approved by the Institutional Committee on Laboratory Animal Care and was conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Surgery. Surgery for bilateral placement of the guide cannulae was carried out under aseptic conditions using procedures previously described (21). Anesthesia was induced with sodium thiopental and was maintained with 3% to 4% halothane. The animal's head was secured firmly in a stereotaxic instrument (Kopf Instruments, Tujunga, CA). After an incision, a circular piece of skull (2.5 cm in diameter) was removed, and the sagittal sinus was doubly ligated. The sinus was then retracted, and 0.15 ml of a radiopaque dye (Conray 400; Mallinkrodt Inc., St. Louis, MO) was injected into the third ventricle. Lateral radiographs that outline the ventricle were used to aid in the placement of guide cannulae. The final placements were made using an x, y, z manipulator and additional radiographs. Twenty-gauge stainless steel guide cannulae 62 mm long with stylets extending an additional 1 mm were then placed bilaterally into the MBH. The tips of the stylets were placed 2.8 to 3.0 mm above the floor of the ventricle, 1.3 mm anterior to the most anterior portion of the posterior wall of the infundibular recess, and 2.25 mm lateral to midline. Given that the microdialysis probes extended 3 mm beyond the guide tubes, the dorsoventral target of the probe tip was 0.8 to 1.0 mm above the floor of the ventricle. The cannulae and a protective cap were anchored to the skull with dental acrylic and screws, and the incision was then closed.

Dialysis Probe and Dialysis Buffer. The microdialysis probe had a nitrocellulose hollow fiber dialysis membrane with a molecular mass cut-off of 6 kDa (Spectra/Por; Spectrum, Gardena, CA). The probe was of the concentric design adapted for use in sheep with modifications previously described (21). It was constructed in our laboratory from 24-gauge stainless steel tubing through which a fused silica tubing passed (Polymicro Technologies, Phoenix, AZ) and exited from the microline inlet. The silica tubing extended 1.75 mm from the stainless guide around which the dialysis membrane (length of 2.5 mm) was fixed. The distal end of the dialysis membrane was sealed with epoxy (Devcon Corp., Riviera Beach, FL). The final length of dialysis membrane in direct contact with brain tissue was 2 mm. It should be noted that the maximal dorsoventral dimension of the ventromedial nucleus is approximately 3.5 mm in sheep (27). Drugs were dissolved in an artificial cerebrospinal fluid (CSF), which consisted of 127.6 m*M* NaCl, 2.5 m*M* KCL, 0.69 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 2.3 m*M* NaH_2PO_4, and 9.7 m*M* Na_2HPO_4 (pH 7.4).

Experimental Design. *Experiment 1.* The objective of this experiment was to determine if the $GABA_B$ agonist SKF97541 (Tocris Cookson Ltd., Ballwin, MO) would increase circulating LH in castrated rams and to select an effective dose for future studies. Two trials were conducted. In the first, three concentrations were tested: 0, 5, and 20 μ*M*. This trial was conducted during November and December. Natural lighting was supplemented by artificial lighting for 10 hr each day (lights on at 0700 hr and off at 1700 hr). In the second trial, conducted during January and February, three concentrations were tested: 20, 100, and 500 μ*M*. Natural lighting was supplemented by artificial lighting for 16 hr each day (lights on at 0400 hr and lights off at 2000 hr).

In both trials, dose and perfusion order were assigned according to pairs of balanced 3 × 3 Latin squares such that each of the six animals was to receive each of the three doses in a balanced order. Each animal had bilateral guide cannulae implanted into the MBH. After a 14-day recovery period, groups of three animals each were placed into adjoining perfusion pens. A catheter was inserted into the jugular vein and was attached to silicone tubing to permit remote sampling. After an 18- 24-hr acclimation period, the stylets were removed from the guide tubes and were replaced with microdialysis probes filled with aCSF. The probes were then connected to infusion syringes with polyethylene tubing. CSF was infused through the probes at a rate of 2 μl/min for 3 hr. After 3 hr, the syringes and tubing were switched to ones containing the drug, and infusion was continued for 4 hr. Blood samples were taken from the jugular vein at 9-min intervals throughout the infusions. The infusions were then terminated, the probes were replaced with stylets, and the animals were returned to their regular pens. They were injected with Liquamycin (LA 200; Pfizer,

New York, NY) on both the day before perfusion and at the end of the perfusion. After a 7-day recovery period, the perfusion sessions were repeated, and by the end of the series, each animal was to be given each dose of the drug. After the last infusion, the animals were euthanized and the hypothalami were collected for histological preparation and evaluation of the probe tip location.

Experiment 2. The objectives were to determine if the $GABA_B$ antagonist CGP52432 (Tocris Cookson) affected LH release and if it blocked the effect of SKF97541. This experiment was conducted during March and April. Natural lighting was supplemented by artificial lighting for 16 hr each day (lights on at 0400 hr and lights off at 2000 hr). The four treatments were CSF, SKF (30 μM), CGP (500 μM), and CGP plus SKF. Treatments were assigned according to two balanced 4 × 4 Latin squares such that each of eight sheep received each of the four treatments in balanced order. The chosen dose of SKF was based on the results of the previous experiment, and the dose of CGP was based on published data regarding affinity (28) and in order to exceed the dose of SKF at least 10-fold on a molar basis. The protocol was similar to that of Experiment 1.

Hormone Assay. Plasma samples were assayed in duplicate for LH using a previously described radioimmunoassay validated for use in our laboratory (29). The sensitivity was 2 ng/ml NIH LH S-20 at 90% binding. The intraassay coefficient of variation was 3.7% and the interassay coefficients of variation were 8.4%, 4.2%, and 6.3% for low, medium, and high internal standards, respectively. Values for LH interpulse interval (IPI) and LH pulse amplitude were determined using the Pulsar algorithm (30).

Histology. At the end of the experiment, the animals were euthanized. The brains were removed after perfusion via carotid artery with saline, followed by 10% formalin fixative, after which the hypothalami were isolated and immersed in fixative. The sections collected were histologically processed and stained with Luxol fast blue to localize probe placement. Evaluation of probe placement was made with the aid of diagrams from Lehman *et al.* (31).

Analysis of Data. Data were analyzed in two ways. For Trial 2, Experiment 1, LH pulse parameters during the first 3 hr (control) were compared with those during the last 3 hr (drug) of infusion using two-tailed Student's *t* test for paired observations. For all other data, the differences ("delta") in each parameter between the first 3 hr and the last 3 hr of infusion were calculated. Subsequently, the mean differences induced by each treatment were compared using analysis of variance (ANOVA) for repeated measures followed by Newman-Keuls tests (32). All analyses were conducted with the aid of a computer program (GB-STAT; Dynamic Microsystems, Silver Spring, MD).

Results

Histology. A schematic representation of the area of probe placement and extent of variation in 13 animals is shown in Figure 1. These schematic drawings show location of bilateral probes placed in the MBH. Locations of probes in six animals excluded from analysis owing either to probe damage or lateral misplacement are not shown.

Effect of SKF. In the first trial, both doses of SKF increased pulse amplitude as compared with CSF ($P < 0.05$), but neither dose increased mean LH as compared with CSF (Fig. 2). The 20-μM dose of SKF increased ($P < 0.01$) IPI when compared with CSF (Figs. 2 and 3).

In the second trial (data not shown), the first animal infused with the 500-μM dose of SKF became noticeably agitated and restless during perfusion, thus perfusion was terminated and this dose was not used again. Both mean LH ($P < 0.02$) and LH pulse amplitude ($P < 0.01$) were higher during infusion of 20 μM SKF than during infusion of CSF. The 100-μM dose had similar effects; however, due to smaller sample size ($n = 4$; two data sets were lost due to probe damage) and high variance, the effects did not reach statistical significance ($P = 0.20$). There was no detectable effect of either dose on IPI.

Effects of SKF-CGP Combination. Observations made in the first experiment that the effects of either 5 or 20 μM SKF on Δ mean LH did not differ from CSF suggested that a threshold was being approached. For this reason, a 30-μM dose was used in this experiment.

Across treatment, comparison revealed main effects of treatment on mean LH ($P < 0.001$). SKF increased ($P < 0.01$) Δ mean LH compared with each of the other treatments (Fig. 4). However, there were no differences among

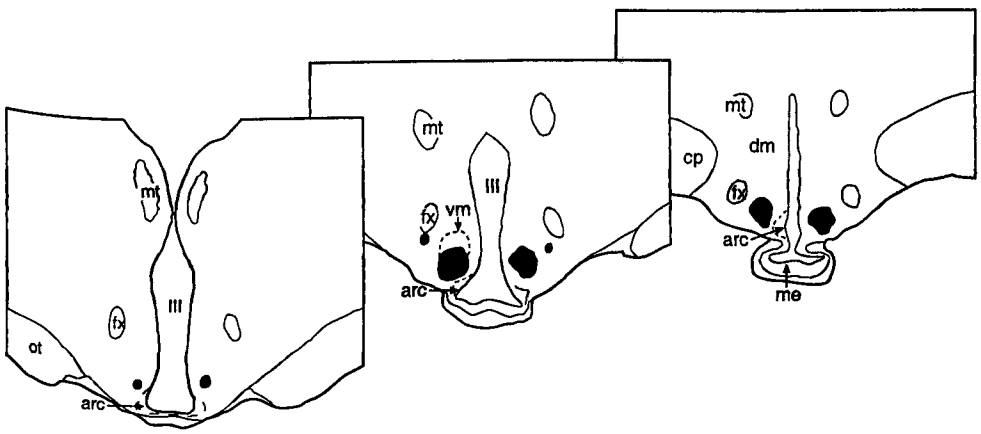

Figure 1. Diagram showing composite location for all experiments of probe placement in castrated rams in which the target was the MBH. The shaded areas represent the composite locations of the tips of the 2-mm dialysis membrane. ARC, acuate nucleus; cp, cerebral peduncle; dm, dorsomedial nucleus; III, third ventricle; fx, fornix; me, median eminence; mt, mammillothalamic tract; ot, optic tract; vm, ventromedial nucleus.

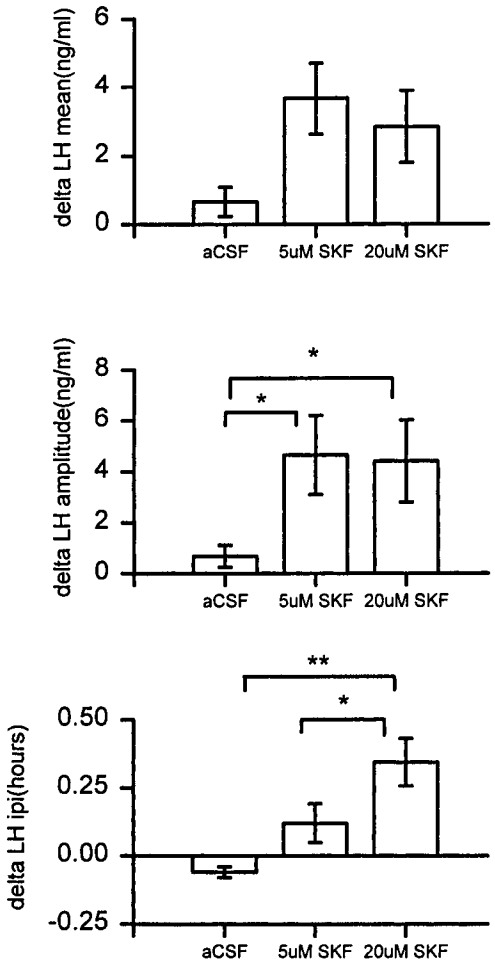

Figure 2. Changes (delta) in LH pulse parameters in castrated rams subjected to bilateral microdialysis infusions of either aCSF only, aCSF-5 μM SKF97541, or aCSF-20 μM SKF97541 into the MBH. Delta was calculated as values obtained during first 3 hr of aCSF infusion versus last 3 hr of drug infusion. $^{*}P < 0.05$, $^{**}P < 0.01$ ($n = 5$).

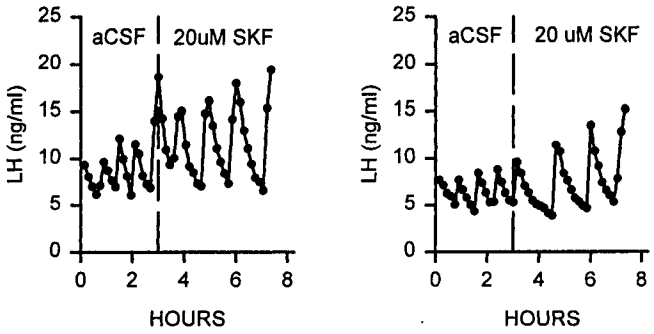

Figure 3. Profiles of circulating LH in two castrated rams subjected to bilateral microdialysis infusion of aCSF for 3 hr followed by 20 μM SKF97541 for 4 hr into MBH. This experiment was performed in December. Note: the combined increase in LH pulse amplitude coupled with reduced pulse frequency (increased IPI).

responses to the other three treatments, i.e., CGP alone was without significant effect, yet blocked the effect ($P < 0.01$) of SKF on mean LH. There also was a main effect of treatment ($P < 0.01$) on Δ LH pulse amplitude. SKF increased ($P < 0.05$) Δ pulse amplitude compared with CSF. Although

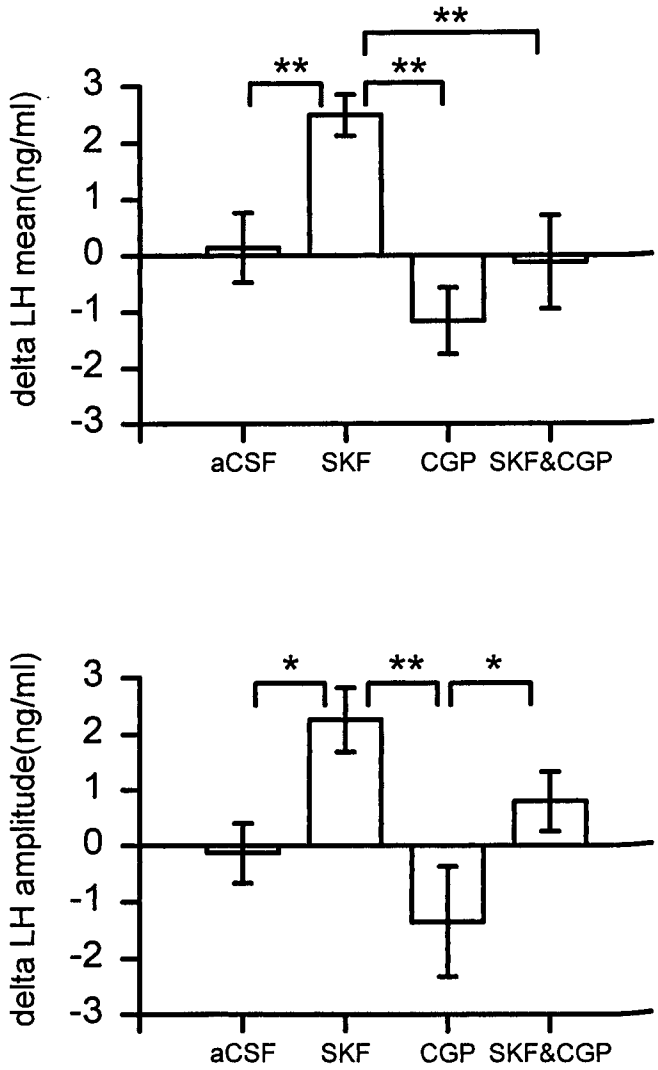

Figure 4. Change (delta) of LH pulse parameters in castrated rams subjected to bilateral microdialysis infusions of either aCSF only, aCSF-SKF97541 (30 μM), aCSF-CGP (500 μM), or aCSF-SKF + CGP into the MBH. Delta is calculated as values obtained during the first 3 hr of CSF infusion versus last 3 hr of drug infusion. $^{*}P < 0.05$ ($n = 8$).

CGP alone appeared to suppress LH pulse amplitude in some animals, overall, the effect of CGP on pulse amplitude did not differ significantly from that of CSF, and CGP-SKF did not differ from either CSF or SKF alone. There was no effect of treatment on IPI.

Discussion

These results show that the $GABA_B$ agonist SKF97541, like baclofen (21), elevates mean LH and pulse amplitude and, under some conditions, may increase IPI. They also show that the effects of SKF97541 on mean LH were attenuated or blocked by the antagonist CGP52432, which itself did not significantly affect LH pulse patterns. Collectively, the results provide additional evidence that specific activation of $GABA_B$ receptors in the MBH of the male sheep increases LH release and are consistent with the hy-

pothesis that $GABA_B$ receptors in the MBH regulate LH release in the male sheep.

Within the range of doses used, there was no obvious dose-response relationship between SKF and LH release. Although a 20-fold dose range was tested, it is likely that concentrations of less than 5 μM would have been partially effective. Given that delivery efficiency of the probes for similar drugs was approximately 20% (21), and that concentration decreased with distance from infusion site, it is likely that nanomolar concentrations of SKF were reaching the active site. Thus, the partial effectiveness of even the lowest dose attests to the high potency of this drug (33).

The ability of SKF to increase the LH IPI in some animals in Experiment 1, Trial 1 is notable for two reasons. First, the effect appeared clear in those animals (Fig. 3), and thus likely was not just a statistical aberration. Second, this was the only experiment of several using baclofen (21) or SKF in which an effect on IPI in castrated animals was noted. This experimental trial was conducted in the winter at the height of the breeding season in animals kept under a 10:14-hr light:dark photoperiod, whereas the other experiments were conducted during the non-breeding season and on animals exposed to a 16:8-hr light:dark photoperiod. Although there are insufficient data to resolve this issue, it is noteworthy that Scott *et al.* (34) presented evidence that the effect of baclofen injections into the preoptic area of ewes differed between the breeding and anestrous seasons. Thus, our results may indicate that the specific role of the $GABA_B$ system in controlling LH pulse parameters in the ram varies with season or photoperiod.

Some of the possible mechanisms by which baclofen and SKF increase LH release have been discussed previously (21, 26) and thus, will not be fully readdressed here. However, the recent report that estradiol decreased the hyperpolarizing response of GABAergic neurons to baclofen (35) is noteworthy. The authors' interpretation was that estrogen may exert rapid negative feedback on GnRH secretion by attenuating the ability of $GABA_B$ auto-receptors to suppress endogenous GABA release. That observation also suggests a key role for GABA and $GABA_B$ receptors in mediating steroid negative feedback. We view the results and interpretation of that study as consistent with our studies in indicating that activation of $GABA_B$ auto-receptors may reduce endogenous GABA release, and thus elevate GnRH release, whereas attenuation of $GABA_B$ auto-receptor function may result in suppression of GnRH release. However, one difficulty with that interpretation is that within our system, CGP 52432 alone, at a relatively high dose (33), did not significantly or uniformly suppress LH (GnRH) in castrated animals, although it appeared to do so in some individuals. As noted previously (36), one interpretation of this response is that in the castrated ram, there is relatively low secretion of GABA at the relevant sites and hence little activation of the relevant $GABA_B$ auto-receptors. Thus, their blockade could have only a minor effect on GABA release. This could explain the slight response to simple $GABA_B$ blockade versus the relatively robust response observed in response to $GABA_B$ activation.

In summary, these data extend previous observations that locally infused baclofen elevates GnRH and LH release in the male sheep by showing that the same effects are exerted by another $GABA_B$ agonist, and importantly, that the effects are blocked by a specific antagonist. These studies provide evidence that the effects of baclofen and SKF 97541 are exerted through $GABA_B$ receptors and hence provide additional support for the concept that this system may play a functional role in regulating GnRH-LH secretion.

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