# Evidence for a Celiac Ganglion-Ovarian Kisspeptin Neural Network in the Rat: Intraovarian Anti-Kisspeptin Delays Vaginal Opening and Alters Estrous Cyclicity

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Kisspeptin and its receptor GPR54 have been described as key hypothalamic components in the regulation of GnRH secretion. Kisspeptin is also present in several regions of the central nervous system and the peripheral organs and has recently been identified in the superior ganglion. Herein, we tested the possibility that ovarian kisspeptin is regulated by the sympathetic nervous system and participates locally in the regulation of ovarian function. Both ovarian and celiac ganglion kisspeptin mRNA levels increase during development, whereas kisspeptin peptide levels and plasma levels decrease during development. In the celiac ganglion, kisspeptin colocalized with tyrosine hydroxylase, indicating potential kisspeptin synthesis and transport within the sympathetic neurons. A continuous (64 h) cold stress induced marked changes within the kisspeptin neural system along the celiac ganglion-ovary axis. *In vitro* incubation with the β-adrenergic agonist isoproterenol increased ovarian kisspeptin mRNA and peptide levels, and this increase was inhibited by treatment with the β-antagonist propranolol. Sectioning the superior ovarian nerve altered the feedback information within the kisspeptin celiac ganglion-ovary axis. *In vivo* administration of a kisspeptin antagonist to the left ovarian bursa of 22- to 50-d-old unilaterally ovariectomized rats delayed the vaginal opening, decreased the percentage of estrous cyclicity, and decreased plasma, ovarian, and celiac ganglion kisspeptin concentrations but did not modify the LH plasma levels. These results indicate that the intraovarian kisspeptin system may be regulated by sympathetic nerve activity and that the peptide, either from a neural or ovarian origin, is required for proper coordinated ovarian function. **(*Endocrinology* 153: 4966–4977, 2012)**

Currently, it is widely accepted that kisspeptin, the product of the *kiss1* gene, and its cognate receptor GPR54 play a key role in the reproductive physiology of multiple species, including humans (1–6). However, the function of kisspeptin beyond the hypothalamic-pituitary axis is poorly understood. Kisspeptin is expressed in certain neural areas such as the medulla oblongata and the spinal cord (7) and has also recently been identified in the superior cervical ganglion (8). These findings raise the possibility that kisspeptin could be associated with the well-described hypothalamic-celiac ganglion-ovary sympathetic circuit, the neurons of which originate at the paraventricular nucleus of the hypothalamus (9, 10). In addition, kisspeptin mRNA is expressed in the ovary of different species (11–13), and its expression changes throughout the estrous cycle, with higher levels observed in late proestrus (12). These and other findings (13, 14) indicate that kisspeptin may participate in ovulation-associated processes. The localization of kisspeptin mRNA in the ovary (theca layer and granulosa cells) is very similar

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Abbreviations: SON, Superior ovarian nerve; SONX, surgical sectioning of the SON.

to the localization of β-adrenergic receptors. This may indicate that kisspeptin expression could respond to β-adrenergic stimulation, suggesting a possible interaction between norepinephrine and kisspeptin in ovarian physiology.

Importantly, these adrenergic fibers regulate the secretion of sex steroids and follicular development (15, 16). We demonstrated that adrenergic innervation is present in the rat ovary at the time of birth but is only fully activated at the time of puberty (17), suggesting that the sympathetic system could participate as an intraovarian regulator in the processes that lead to puberty. Currently, it is accepted that the ovarian sympathetic nervous system can generate changes in follicular development with an increase in cyst formation (18–20), but the exact mechanism involved is unknown. A role for this system was revealed because an acute cold stress (21) increased the noradrenaline content of the celiac ganglion and ovary. Considering that intraovarian kisspeptin changes during the estrous cycle as it has also been demonstrated to occur with norepinephrine, both of them could have functional and/or morphological relations that could be relevant to ovary function.

Therefore, in this paper, we focused on the neural relation between celiac ganglion and the ovary to investigate whether kisspeptin 1) changes during puberty and reaches the ovary via a neural pathway through the celiac ganglion; 2) participates with ovarian sympathetic activation in response to a cold stress; and 3) participates in the onset of puberty and in the regulation of estrous cyclicity.

## Materials and Methods

### Animals

The ovaries, celiac ganglion, hypothalamus, and plasma were isolated from infantile (15 d of age), peripubertal (30 d of age), and adult (60 d of age) Sprague Dawley rats derived from a stock maintained at the University of Chile. A total of 118 rats was used. The animals were obtained from the facilities at the University of Chile and maintained in individual cages at 23 C under a 12-h light, 12-h dark cycle (lights on at 0700 h), and food and water was available *ad libitum*. The rats were killed at the end of each procedure in the morning by decapitation. The hypothalamus (medium basal hypothalamus-arcuate nucleus), celiac ganglion (ventral surface of the aorta between the celiac and superior mesenteric arteries), and the ovaries were rapidly removed, weighed, and frozen at −80 C until their use for RNA or protein analysis. A second group of samples was fixed in Bouin´s fixative solution, embedded in paraffin, and serially sectioned at 6 μm for immunohistochemistry assays. The last group of samples was used for *in vitro* incubation of the ovaries.

All animal procedures were approved by the Institutional Ethic Committee of the Faculty of Chemistry and Pharmaceutical Sciences, Universidad de Chile, in accordance with national guidelines (CONICYT Guide for the Care and Use of Laboratory Animals).

### Experimental designs

#### *Experiment 1: Developmental expression of kisspeptin mRNA and peptide*

This was determined in ovaries and celiac ganglion of infantile (15 d old), prepuberal rats (30 d old), and adult (60 d old) rats. One ovary was used for kisspeptin peptide determination and the other was used for total RNA isolation. Because celiac ganglion could not be surgically separated in equal parts (either morphological or functional), to determine the mRNA and peptide, we used 16 rats per group. In adult rats, daily vaginal lavages were performed, and only rats with two consecutive 4-d regular estrous cycles were used. Rats were killed at 1000 h when they were in diestrus.

#### *Experiment 2: Presence of kisspeptin in the ovary and its colocalization with tyrosine hydroxylase in the celiac ganglion*

Cycling adult rats in diestrous phase were chosen. Estrous cyclicity was follow as in experiment 1. Rats were killed at 1000 h, and the ovaries and celiac ganglion were fixed and prepared for the determinations. To confirm the kisspeptin presence in the granulosa and theca cells, the granulosa cells were collected as described previously (22, 23).

#### *Experiment 3: Effect of stress on kisspeptin mRNA and peptide expression*

We used three different approximations: 1) a continuous 64-h acute exposure to cold stress protocol (4 C), a procedure that activates ovarian sympathetic nerves (21, 24); 2) to mimic a noradrenergic stimulation in the ovary, we incubated *in vitro* the ovary of adult cycling rat in the presence of isoproterenol, or isoproterenol plus propranolol; and 3) to eliminate the neuronal communication between the celiac ganglion and the ovary, we disrupted neuronal communication by surgical section of the superior ovarian nerve (22, 25).

#### *Experiment 4: Role of kisspeptin during puberty*

To determine the relative role of kisspeptin acting at the ovary level, either during puberty or during estrous cycling activity. We used *in vivo* intraovarian administration of kisspeptin antagonist (p234) by means of a miniosmotic pump during 28 d to prepubertal rats.

#### *Acute cold stress procedure*

Rats undergoing cold stress were placed in individual cages in a room maintained at 4 C for 64 h with regular 12-h light, 12-h /dark cycles. Control and experimental animals were killed by decapitation 1 h after the end of the stress session.

### Transection of the superior ovarian nerve

The superior ovarian nerve (SON) was selected for transection because it predominantly innervates the endocrine components of the ovary, in contrast to the plexus nerve, which mainly innervates the ovarian vasculature (26). The surgical procedures were performed as previously described (22). Briefly, surgical

sectioning of the SON (SONX) rats were anesthetized with a ketamine (60 mg/kg)/xylazine (10 mg/kg) mixture, the ovaries were exposed through a dorsal incision, and the SON was sectioned with a microcautery tool as previously described (27). Experiments were performed 15 d after the surgical procedure, which corresponded to our observation of the lowest ovarian sympathetic activity (28).

## Separation of the granulosa cells and the residual ovary

The granulosa cells were collected as described previously (22, 23). Briefly, ovaries were punctured with a needle, and the cell suspension was carefully collected into Krebs bicarbonate buffer. The cells were transferred to a 1.5-ml plastic tube, pelleted by centrifugation at 250 × g, and washed three times with Krebs bicarbonate buffer. Both the suspension of granulosa cells and the rest of the ovary (residual ovary) were used for the extraction of total RNA and for kisspeptin and 18S mRNA expression, as described below. To assess the purity of the preparation, we measured mRNA expression (real-time PCR) for the FSH receptor in the granulosa cell fraction and in the residual ovary (the FSH receptor is only expressed in granulosa cells) as previously described (23). More than 99% of the FSH mRNA was present in the granulosa cell fraction.

## Intraovarian administration of a kisspeptin antagonist (p234) in prepubertal rats

Rats were implanted using an Alzet miniosmotic pump (model 2004, Alza Corp., Palo Alto, CA) loaded with 100 nmol/200 μl of kisspeptin antagonist (p234, Phoenix Pharmaceutical, Inc., Mountain View, CA) at a delivery rate of 0.25 μl/h/28 d. Control animals were infused with saline solution. The procedure was done as we have previously described (29), in brief the osmotic pump was connected to SILASTIC (Dow Corning Corp., Midland, MI) brand tubing (inner diameter, 0.025 inch; outer diameter, 0.047 inch) loaded with the antagonist solution. The cannula was inserted underneath the bursa of the left ovary and kept in place with a drop of cyanoacrylate and sutures attached to the ipsilateral uterine horn. To eliminate the possible contribution of the contralateral ovary, all control and antagonist-treated animals were hemiovariectomized at the moment of the minipump implant. At the end of the first 28-d period (when the pumps stopped delivering the antagonist), the pumps were left in the animal for a total of 50 d to control the estrous cycling activity. Estrous cyclicity was monitored by daily vaginal lavages. At the end of the experiments, the rats were killed by decapitation; the intrabursal location of the cannula's tip was verified by visual inspection.

## Real-time PCR

Total RNA was extracted as described by Chomczynski and Sacchi (30) from the hypothalamus, celiac ganglion, and whole ovary, and the granulosa and theca-interstitial cells were obtained as described above. Total RNA (5 μg) was subjected to RT. After that a standard real-time PCR mix was prepared with a protocol of 40 cycles using a IQ5 thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA). Primer-specific amplification and quantification cycles were run at 95 C for 20 sec, 62.5 C for 20 sec (60.5 C for GPR54 and 60 C for 18S), 72 C for 20 sec, and a final extension of 72 C for 10 min. The fluorescence intensity of the double-strand specific SYBR Green I was read at the end of each elongation step. Then, the amounts of specific initial template mRNA were calculated by determining the time point at which the linear increase of sample PCR product started relative to the corresponding points of a standard curve obtained by serial dilution of known copy numbers of the corresponding control tissue. The *kiss1* gene was designed according to data from GenBank. The forward oligodeoxynucleotide primer used to detect *kiss1* mRNA was 5′-CCG GAC CCC AGG AAC TCG T-3′ (NM_181692.1); the reverse primer sequence was 5′-CGT AGC GCA GGC CAA AGG AG-3′. The forward oligodeoxynucleotide primer used to detect *GPR54* mRNA was 5′-TGT GCA AAT TCG TCA ACT ACA TCC-3′ (NM_023992.1); the reverse primer was 5′-AGC ACC GGG GCG GAA ACA GCT GC-3′. To normalize the quantification of *kiss1* and *GPR54* mRNA, the amount of ribosomal *18S* mRNA was measured in each protocol using a commercially available RT primer pair (Ambion, Inc., Austin, TX).

## Immunohistochemistry

The sections (6 μm) were deparaffinized in xylene and rehydrated in graded ethanol series. Subsequently, the sections were submitted to antigen retrieval in a preheated water bath containing a sodium citrate buffer dish (1.8 mM citric acid; 8.2 mM sodium citrate, pH 6.0) at 90 C for 30 min. The endogenous peroxidase activity was quenched after washing with PBS and by incubation in a solution of 3% $H_2O_2$ in methanol. The sections were blocked with normal serum and incubated overnight at 4 C with two primary, kisspeptin-13 rabbit antiserum [Bachem AG, Bubendorf, Switzerland, 1:800, previously tested (12) and Phoenix Pharmaceuticals, Inc., Mountain View, CA; 1:600] followed by a second antibody (antirabbit biotinylated γ-globulin, 1:200; Vector Laboratories, Burlingame, CA), and avidin-biotin complex peroxidase (Vector Laboratories). The binding was visualized using 3′3-diaminobenzidine HCl (Vector Laboratories). As a control for specificity, some sections that had been preabsorbed overnight at 4 C with kisspeptin-10 peptide, 1 μg/ml, (Phoenix Pharmaceuticals, Inc.) were incubated with antikisspeptin.

## Double immunohistochemistry for kisspeptin and TH

Deparaffination, hydration, and antigen retrieval were performed as detailed above. The sections were then incubated overnight at 4 C with the primary kisspeptin-13 rabbit antiserum (Bachem AG) diluted 1:800 in 3% normal goat serum in PBS. After rinsing in PBS, the sections were incubated with a secondary antibody against rabbit [ab6717 (Abcam, Cambridge, MA) conjugated with fluorescein isothiocyanate] diluted 1:200 in 3% normal goat normal serum in PBS overnight at 4 C. After several washes in PBS, the ovary sections were incubated overnight at 4 C in mouse anti-TH (MA118038; Pierce Chemical Co., Rockford, IL) diluted 1:5000 in 3% normal goat serum in PBS, washed three times in PBS, and incubated overnight with a secondary antibody against mouse [antibody 6787 (Abcam), conjugated with Texas Red] diluted 1:200 in 3% normal goat serum in PBS. Finally, the sections were washed in PBS and mounted with PROLONG antifade (P36930; Invitrogen, Carlsbad, CA). Fluorescence was observed using a Zeiss Axioscope 20 fluorescence microscope (Carl Zeiss, Thornwood, NY), after which the images were obtained and then analyzed with NIH ImageJ software

to determine the positive double staining for tyrosine hydroxylase and kisspeptin cells. Controls for immunostaining specificity consisted of 1) the omission of the primary antibody and the application of the secondary antibody alone and 2) the use of inappropriate excitation wavelengths.

### Hormone assays

Kisspeptin determination was established by EIA (Phoenix Pharmaceuticals, Inc., Mountain View, CA). The ovary and celiac ganglion kisspeptin measurements required an extraction procedure that was performed using the suggested method by the manufacturers (31). Briefly, the tissues were homogenized in lysis buffer (10 mM Tris, pH 7.4) and centrifuged for 13,000 rpm for 15 min at 4 C. The supernatant was loaded in a Phoenix Peptide sep-column (C18, RK-Sepcol-2, Phoenix Pharmaceuticals, Inc.) previously equilibrated [1 ml of buffer A (RK-BA-1, Phoenix Pharmaceuticals, Inc.), once and 3 ml of buffer B three times]. The peptides were eluted with 3 ml of buffer B (RK-BB-1; Phoenix Pharmaceuticals, Inc.) and then evaporated to dryness. The pellet was stored at −80 C until the assay day and was dissolved in 250 $\mu$l of 1× assay buffer provided by the EIA kit. For kisspeptin determination, the serum was loaded in the sep-column as described above. We tested the cross-reactivity between the antagonist and kisspeptin. We did not find cross-reactivity at any concentration used either alone or as a mixture with kisspeptin. The minimal detectable value for kisspeptin was 0.08 ng/ml. Intra- and intervariance was 8.6% and 5.5%, respectively.

Progesterone, testosterone, and estradiol were measured by enzyme immunoassay following the manufacturer's instructions (Alpco Diagnostics, Windham, NH). Intraassay and interassay variations were less than 11%, 7%, and 10% for progesterone, testosterone, and estradiol, respectively. The minimal detectable value for progesterone was 2.5 pg, for testosterone it was 1.25 pg, and for estradiol it was 0.5 pg. LH levels were determined by enzymoimmunometric assay following the manufacturer´s instructions (LH Detect; Repropharm, Nouzilly, France); intraassay and interassay variations were less than 10%; the minimal detectable value of LH was 0.2 ng/ml.

### Statistics

The differences between age groups were assessed by ANOVA followed by the Student-Newman-Keuls multiple range test for unequal replications. Differences between the two groups were analyzed using a Student's $t$ test. $P < 0.05$ was considered to be statistically significant.

## Results

### Kisspeptin levels change in the celiac ganglion and ovary during development

Kisspeptin mRNA in the celiac ganglion and the ovary was expressed at low levels in prepubertal rats and increased to higher levels in the 60-d-old rat ($P < 0.001$) (Fig. 1, A and C). Kisspeptin concentrations in the celiac ganglion and ovary followed an inverse correlation; con-

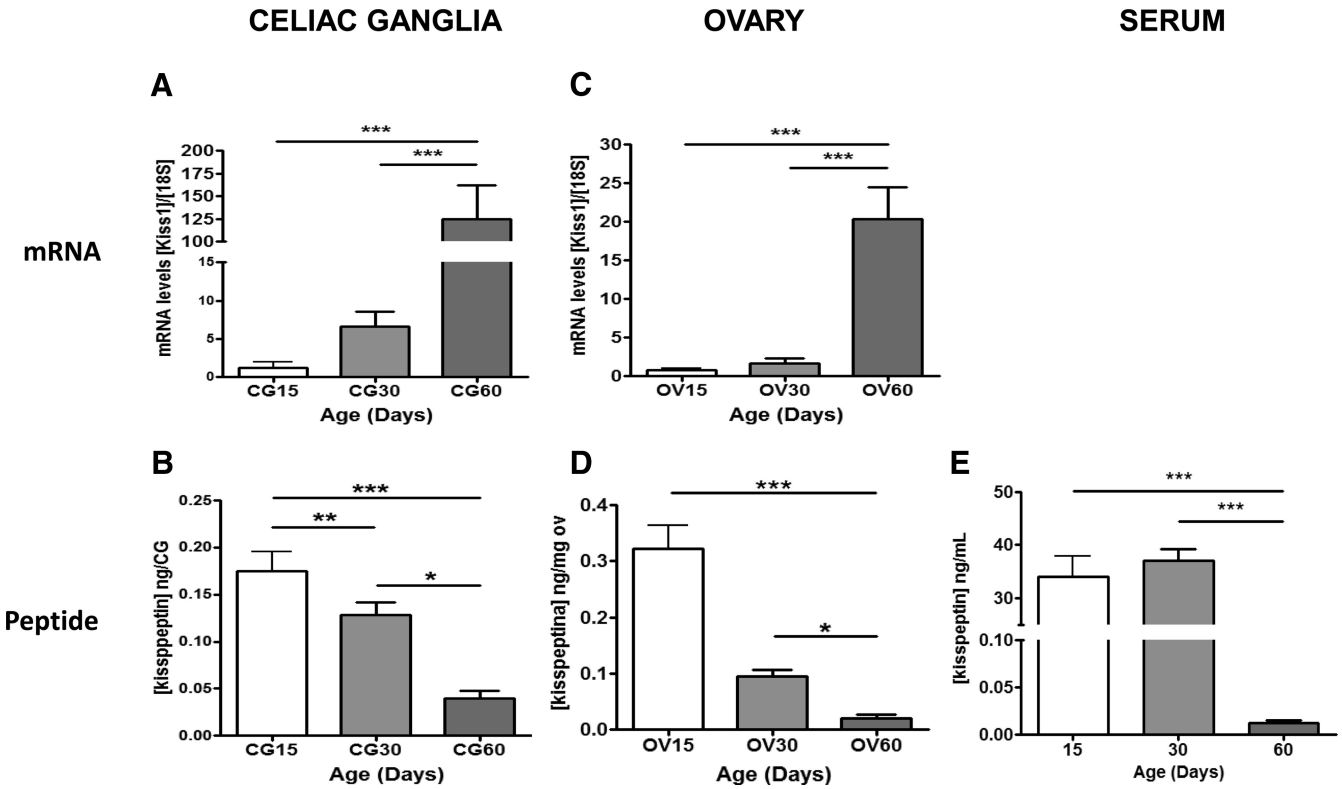


**FIG. 1.** Developmental changes in the mRNA expression and peptide concentration in the celiac ganglia (A and B), the ovary (C and D), and plasma (E). Each *bar* represents the celiac ganglia (CG), the ovary (OV), or plasma of 15, 30, and 60 d of age. Kisspeptin mRNA was normalized using the ribosomal 18S mRNA. Data correspond to the mean ± SEM of eight rats. Each graph was analyzed using an ANOVA test followed by Student-Newman-Keuls posttest. *Asterisks* represent statistically significantly differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

centrations were highest at 15 d of age and decreased to low levels in the 60-d-old rats. The serum levels of kisspeptin were maintained at high levels in the prepubertal stages but decreased in the adult animals (Fig. 1, B, D, and E).

Figure 2 presents the immunolocalization of kisspeptin in the ovary and in the celiac ganglion. Because of its ability to recognize all isoforms of the kisspeptin family, we used the same antibody against kisspeptin-10 that was previously used by Castellano *et al.* (12) in the ovaries of Wistar rats. Strong cytoplasmic immunoreactivity was observed in the neuronal somas of the celiac ganglion (Fig. 2B). In the ovary, a positive stain for kisspeptin was present in several ovarian structures, including the corpus luteum, interstitial gland, and growing and preovulatory follicles. In the follicles, the inner theca and the granulosa cells were positively stained (Fig. 2A). As negative controls, the celiac ganglion and ovary tissues were incubated only with the secondary antibody (Fig. 2, C and D). To test the specificity of the primary antibody, we performed an additional control using preabsorption of the primary antibody with the kisspeptin-10 peptide (Fig. 2E).

To demonstrate the intrafollicular localization, we prepared a granulosa-rich fraction, which is composed primarily of granulosa cells and the residual ovary, which principally contains theca cells, as described previously (32, 33). Kisspeptin mRNA expression was significantly higher in the granulosa cell fraction compared with theca cells ($P < 0.05$, Fig. 2F), thereby identifying the granulosa cells as a major site of synthesis. However, *GPR54* mRNA was equally distributed between the granulosa cells and residual ovary (Fig. 2G).

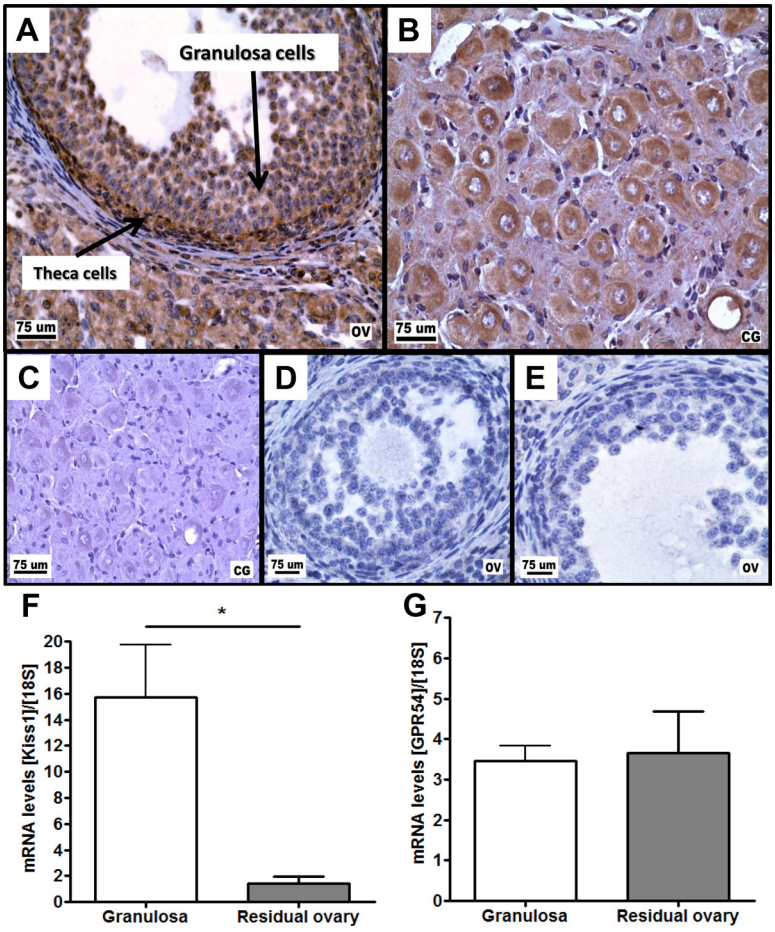


**FIG. 2.** Kisspeptin immunoreactivity in ovary sections from adult rats (A) and neuronal somas of the celiac ganglion (CG) (B) of the same rats. Strong cytoplasmic immunostaning was present in the granulosa and theca cells of antral follicles (A) and in somas in the celiac ganglion (B). Negative control without primary antibody for celiac ganglion is presented in panel C and for the ovary in panel D. Ovarian preadsorption of the primary antibody with kisspeptin-10 peptide is presented in panel E, kisspeptin (panel F) and GPR54 (panel G) mRNA in a granulosa cell-enriched fraction and residual ovary containing theca cells. The data were normalized using the ribosomal 18S mRNA. *Bars,* 75 $\mu$m. Each *bar* represents the mean ± SEM of five rats. *Asterisks* represent statistically significantly differences as analyzed by Student's *t* test for paired data (*, $P < 0.05$).

## Colocalization of kisspeptin and tyrosine hydroxylase in the celiac ganglion

To test the possibility of colocalization of kisspeptin with tyrosine hydroxylase, we performed a double immunohistochemistry and double immunofluorescence stain for kisspeptin and tyrosine hydroxylase. Both immunohistochemistry (Fig. 3, A and B) and immunofluorescence (Fig. 3, C–E) demonstrated many neurons in which kisspeptin and tyrosine hydroxylase neurons localize together.

## Cold stress activates the kisspeptin system in the celiac ganglion-ovary axis

Previously, our laboratory (19, 20) and other investigators (18) have shown that cold stress activates the ovarian sympathetic nerves and leads to marked changes in ovarian function and morphology. We used the same stress procedure to determine whether we can activate the kisspeptin celiac ganglion-ovary axis. A decrease in the kisspeptin mRNA expression level (Fig. 4A) was found in hypothalamus, and no changes were observed in the peptide concentration (Fig. 4B). In contrast, in the celiac ganglion, both the mRNA and peptide levels increased significantly (Fig. 4, C and D). Furthermore, an inverse relation was found between the mRNA and peptide levels in the ovary: as the mRNA level decreased

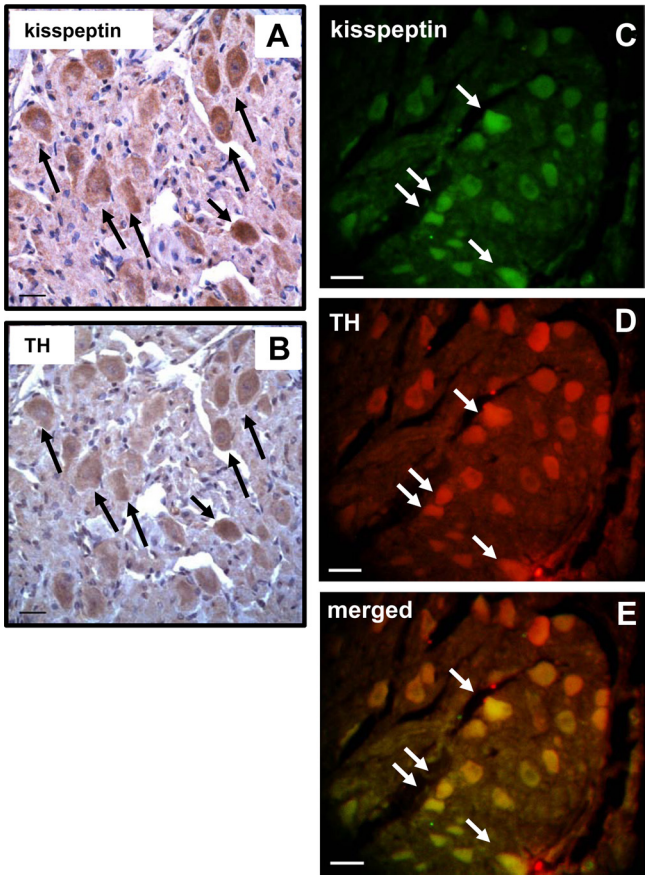


**FIG. 3.** Colocalization of kisspeptin with tyrosine hydroxylase (TH) cells in celiac ganglion. Kisspeptin (A) and TH (B) immunohistochemistry in the celiac ganglion. Immunofluoresnce detection showing the colocalization (*white arrows*) of kisspeptin (C) and TH (D) immunoreactivities in cells of the celiac ganglion (*merged image* is shown in E). *Bars*, 75 $\mu$m.

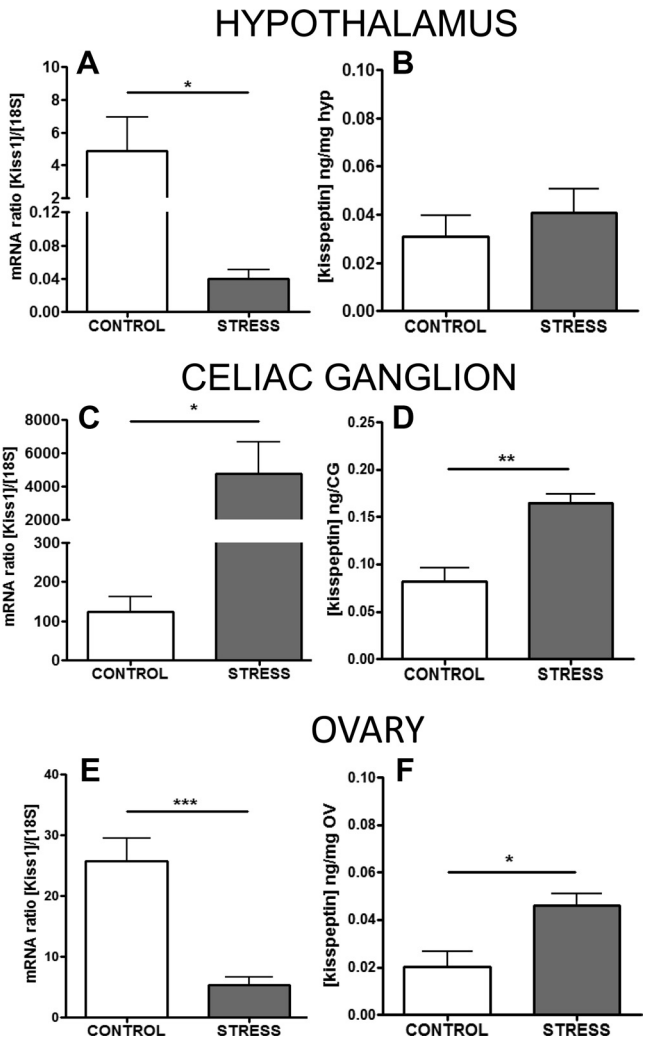


**FIG. 4.** Effect of 64-h cold stress over the kisspeptin mRNA and peptide concentration in the hypothalamus (A and B), celiac ganglion (CG) (C and D), and the ovary (OV) (E and F). The mRNA data were normalized using the ribosomal 18S mRNA. Each *bar* represents the mean ± SEM of five rats. Each *graph* was analyzed using an ANOVA test followed by Student-Newman-Keuls posttest. *Asterisks* represent a statistically significantly differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

(Fig. 4E), the peptide concentration increased significantly (Fig. 4F), whereas serum peptide levels did not change under stress (0.01 ± 0.003 ng/ml *vs.* 0.014 ± 0.006 ng/ml control *vs.* stress; n = 5).

To understand whether the previous results were a consequence of an increase in sympathetic activity, we performed an *in vitro* incubation of the ovary with a $\beta$-adrenergic agonist. Figure 5A indicates that an 8-h ovarian incubation with 20 $\mu$M isoproterenol, a $\beta_2$-adrenergic agonist, increased the kisspeptin mRNA expression level ($P < 0.05$), an effect that was blocked with propranolol, a nonspecific $\beta$-adrenergic antagonist, at a concentration of 200 $\mu$M. Similar results were found after an 8-h incubation to measure the amount of the peptide (Fig. 5B).

## Ovarian superior nerve sectioning modifies the kisspeptin celiac ganglion-ovary axis

Because an increase in sympathetic activity alters ovarian function (22, 28), we investigated the effect of SON denervation on this sympathetic activity and ovarian kisspeptin mRNA and peptide levels. Figure 6 demonstrates that the denervation produced a marked decrease in the ovarian and celiac ganglion kisspeptin mRNA levels (Fig. 6, A and C). Protein levels significantly rose after denervation (Fig. 6, B and D). No changes in LH were detected, and, in addition, no changes were observed in progesterone or estradiol plasma levels. Testosterone levels significantly decreased (115.7 ± 36.4 pg/ml for control *vs.* 11.9 ± 5.9 pg/ml for SONX; $P < 0.01$), and intriguingly, kisspeptin serum levels significantly increased (0.78 ± 0.74 ng/ml for control *vs.* 5.26 ± 0.39 ng/ml for SONX).

## Effect of *in vivo* intraovarian administration of a kisspeptin antagonist (p234)

Based on previous evidence (12) that kisspeptin can participate in processes related to follicular development

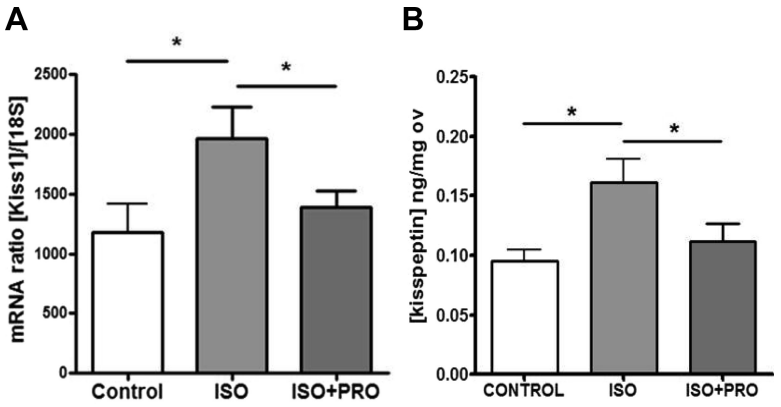


**FIG. 5.** A, Kisspeptin mRNA expression after 8-h incubation of the ovaries (OV) from control and stressed rats with 20 $\mu$M isoproterenol (ISO) or with 20 $\mu$M isoproterenol + 200 $\mu$M-propranolol (PRO). The mRNA data were normalized using the ribosomal 18S mRNA. B, Values for the peptide determination. Each *graph* was analyzed using an ANOVA test followed by Student-Newman-Keuls posttest. Each *bar* represents the mean ± SEM of five rats. *Asterisks* represent statistically significantly differences (*, $P < 0.05$).

and ovulation, we investigated the role of ovarian kisspeptin during puberty and ovulation. To this end, we used a miniosmotic pump to deliver a kisspeptin antagonist into the left ovary for 28 d, starting on d 22 of age, after removing the right ovary. Control ovariectomized rats infused with saline did not exhibit an alteration in the age of vaginal opening compared with the age of vaginal opening in the untreated rats of our colony. However, rats that received a local chronic antagonist had a delay in vaginal opening by 2 d ($P < 0.05$) (Fig. 7, A and B). The day when first estrus followed by a diestrus occurred (indicating puberty) was delayed (36.1 ± 1.1 d for control and 47.7 ± 0.4 d for antagonist-treated rats; $P < 0.01$; mean ± SEM of n = 9 rats). These rats exhibited marked irregularities in estrous cycling activity (Fig. 7, C and D) that were observed when the data are presented as percentages of the number of estrous cycles observed in control rats compared with the theoretical number of estrous cycles during the observation period (considering a theoretical 4-d estrous cycle in which 1 d is spent in proestrus, 1 d is spent in estrus, and 2 d are spent in diestrus: diestrus 1 and -2). We found a decrease in the number of days that rats stayed in proestrus (3.8 ± 0.4 d for control *vs.* 1.3 ± 0.2 d for kisspeptin antagonist; $P < 0.001$) with no changes in the number of days in estrus (4.6 ± 0.4 d for controls *vs.* 4.8 ± 0.7 d for kisspeptin antagonist) and diestrus (8.2 ± 0.6 d for control *vs.* 9.2 ± 0.5 d for kisspeptin antagonist). To control a possible central effect of the locally administrated antagonist, we measured the plasma concentrations of LH, testosterone, estradiol, and progesterone at the end of the experiment. The results indicate that serum levels of LH (0.6 ± 0.1 ng/ml *vs.* kisspeptin antagonist 0.54 ± 0.1 ng/ml) and testosterone (29.9 ± 10.2 pg/ml *vs.* kisspeptin antagonist 26.3 ± 6.8 pg/ml) did not differ from those of the controls, whereas serum levels of estradiol (30.1 ± 3.9 pg/ml *vs.* kisspeptin antagonist 11.4 ± 1.8 pg/ml; $P < 0.05$) and progesterone (34.5 ± 10.2 pg/ml *vs.* kisspeptin antagonist 13.6 ± 2.0 pg ml; $P < 0.05$) decreased more than 50%. In addition there was a clear decrease in the kisspeptin concentra-

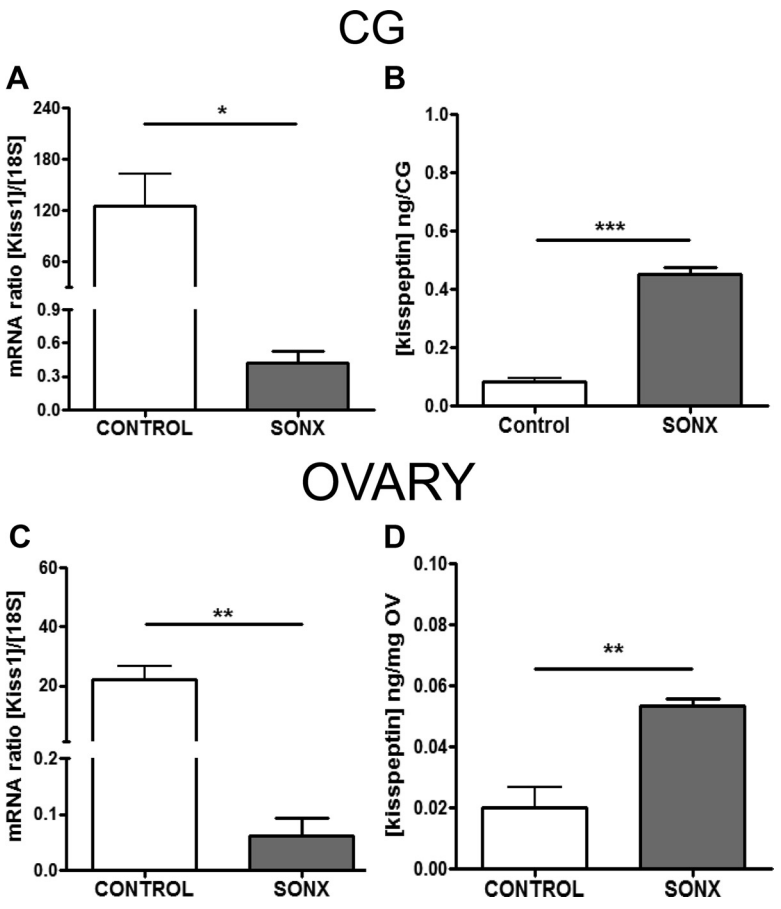


**FIG. 6.** Effect of SONX on ovarian kisspeptin mRNA and peptide concentration in the celiac ganglion (CG) (A and B) and the ovary (OV) (C and D). The mRNA data were normalized using the ribosomal 18S mRNA. Each *bar* represents the mean ± SEM of five rats. Each *graph* was analyzed using an ANOVA test followed by Student-Newman-Keuls posttest. *Asterisks* represent statistically significantly differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

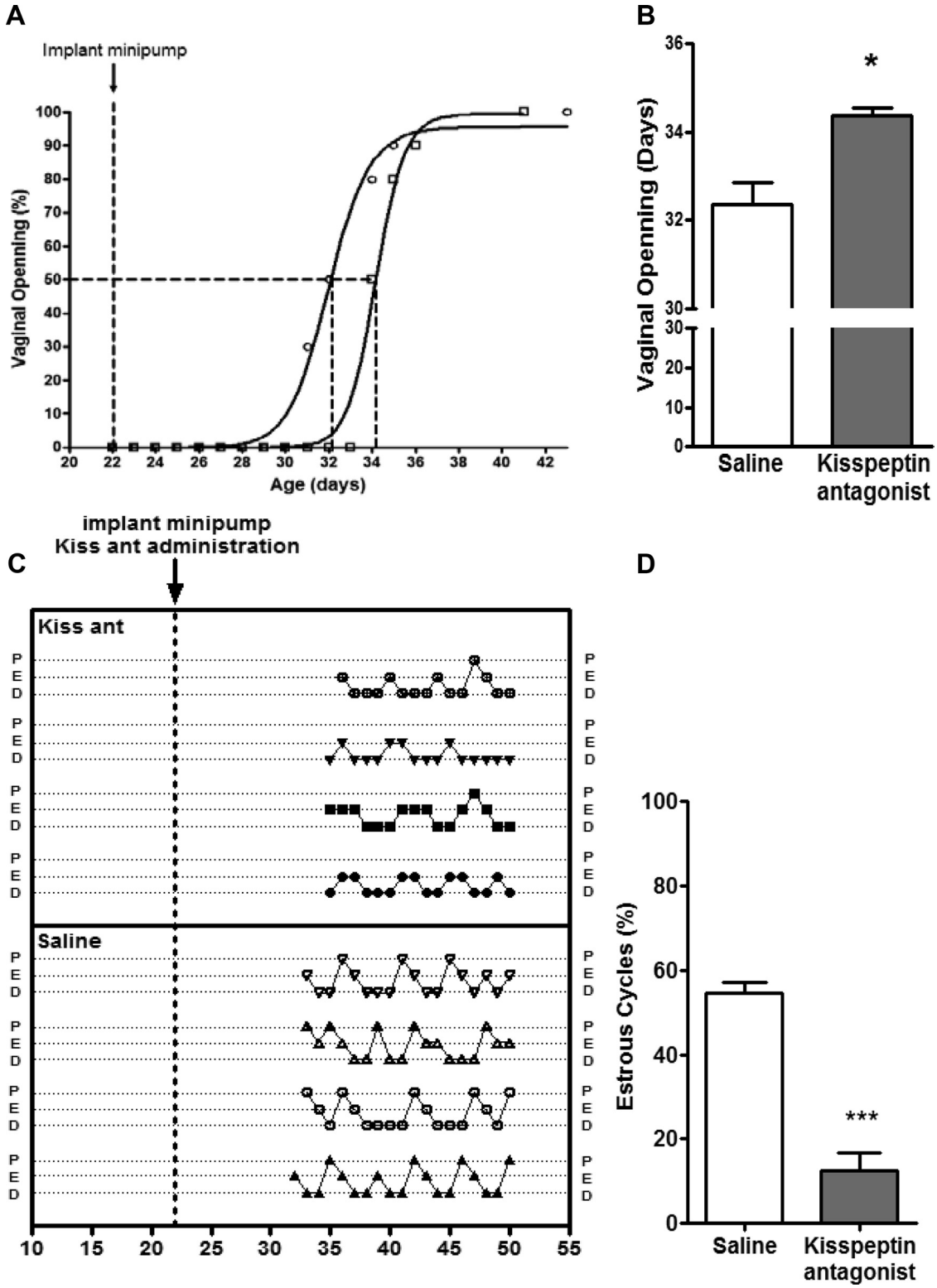


**FIG. 7.** Effect of *in vivo* intraovarian administration of the kisspeptin antagonist (Kiss ant) (p234) on the age of vaginal opening and in the estrous cycling activity. A, Percentages of rats showing vaginal opening as a function of age in the control (saline) (*squares*) and kisspeptin antagonist-exposed (p234) rats (*circles*). B, Mean day of vaginal opening in control (n = 10) and kisspeptin antagonist (n = 10) animals. C, Representative estrous cycle patterns between d 22 and 50 of age of control (n = 10) and kisspeptin antagonist-exposed animals (n = 10). D, Number of normal estrous cycles expressed as a percentage of the theoretical number of cycles (considering that each estrous cycle lasts 4 d) from vaginal opening up to 50 d of age for control and kisspeptin antagonist-exposed rats. D, Diestrus; E, estrus; P, proestrus. One theoretical estrous cycle corresponds to a P followed by an E and 2 d in D. *Asterisks* represent a statistically significantly differences as analyzed by Student's *t* test for paired data (*, $P < 0.05$; ***, $P < 0.001$).

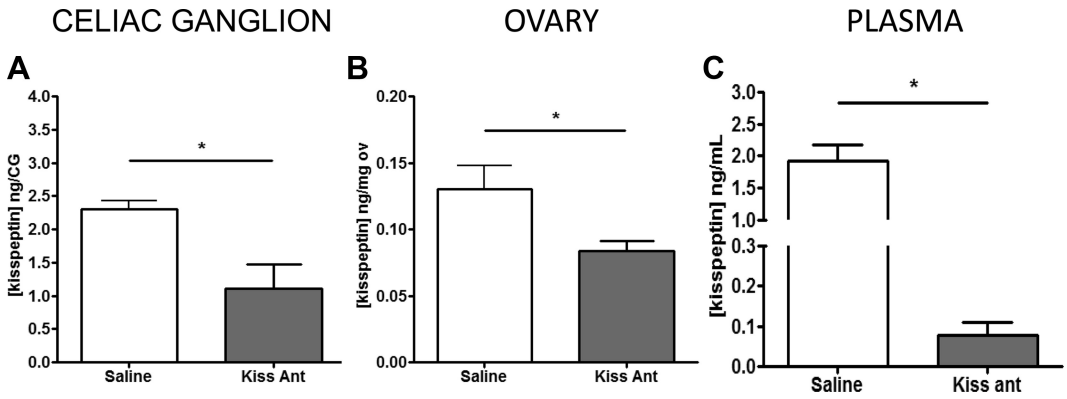


**FIG. 8.** Effect of the kisspeptin antagonist (Kiss ant) administration (p234) on the kisspeptin concentration in the celiac ganglion (CG) (A), the ovary (OV) (B), and plasma (C). Each *bar* represents the mean ± SEM of five rats. Each graph was analyzed using an ANOVA test followed by Student-Newman-Keuls posttest. *Asterisks* represent statistically significantly differences (*, $P < 0.05$).

tion in the celiac ganglion (Fig. 8A), ovary (Fig. 8B) and particularly in the plasma (Fig. 8C), which reached very low levels ($P < 0.01$).

## Discussion

In this work, we describe a novel kisspeptinergic circuit formed by the celiac ganglion and the ovary that it is likely under hypothalamic control. In the celiac ganglion, the system is only expressed at the kisspeptin mRNA and protein level and not at the receptor. The lack of receptor expression (at least as determined by the sensitivity of our method) suggests that the celiac ganglion is only a site of synthesis of the peptide, which is then transported to other organs, including the ovary through the SON, which acts as a neurotransmitter in this organ.

### Developmental expression of kisspeptin mRNA and peptide

The increasing levels of ovarian kisspeptin mRNA from the infantile stage to puberty confirmed previous data on detection of kisspeptin mRNA (12). In addition, the presence of a similar relationship in the celiac ganglion suggested a possible functional role for the kisspeptin system in the control of ovarian function, particularly in light that the celiac ganglion is the origin of the SON, which includes most of the nerve fibers that innervate the secretory cells of the ovary. The inverse relationship between the levels of kisspeptin mRNA and kisspeptin protein in both organs as the rat matures could represent posttranslational modifications of kisspeptin expression as has been previously demonstrated to occur in the hypothalamus of lactating rats (34). Although we cannot exclude contamination from other tissues or plasma, it is unlikely to occur due to the cleaning and washing of the tissue before it is processed. If this is the case and we accept that there is a direct relationship between the levels of mRNA and peptide concentrations in different tissues, more mRNA represents increased peptide production, then the increase in mRNA found in the prepubertal ovary and in the celiac ganglion could represent the biosynthetic response to a high secretory activity of the peptide associated with the appearance of ovulation. In fact, the increased peptide levels found in the serum as the rat approaches puberty (whereas in the ovary there are low levels of the peptide), could be the result of increased releasing activity from the terminal organ (*i.e.* the ovary or other organs that change its activity during the peripubertal period) and from the nerve terminals of the neuronal bodies of the celiac ganglion. If this were the case, we can suggest that the increased sympathetic nerve activity previously described in the rat ovary during the peripubertal period (17) is closely associated with the kisspeptinergic system in the celiac ganglion or may even belong to the same neuronal compartment. The fact that, during adulthood, all ganglion, ovary, and plasma levels are lower than during the peripubertal stage in contrast to humans, in which there is no decrease after puberty, could represent differences in the morphological characteristics of the ovaries: whereas the human ovary is monoovulatory, rats are multiovulatory, and more than 90% of the total area of the ovary after puberty is formed by corpus luteum, a compartment that has lower immunoreactivity to kisspeptin.

### Presence of the peptide in the ovary and celiac ganglion in adult rats and its colocalization with tyrosine hydroxylase in the celiac ganglion

Our results strongly suggest that both kisspeptin and tyrosine hydroxylase are present in the same neurons of the celiac ganglion. Although a similar finding was reported for the colocalization of kisspeptin with tyrosine hydroxylase neurons in the periventricular area of the

third ventricle in the mouse (35), it has been difficult to demonstrate the same distribution in the rat (36), strongly suggesting a species difference. However, the fact that the postganglionic nerves in the celiac ganglion innervate multiples organs of the gastrointestinal tract (37) makes it possible that neuronal kisspeptin could be transported to other tissue targets and may also contribute to the plasma levels of kisspeptin. This theory is supported by the work of Ohtaki *et al.* (38), who described the preferential increase in kisspeptin mRNA expression in the cecum and the colon, two regions innervated by the celiac ganglion.

The ovaries of the Sprague Dawley rats exhibited specific immunoreactivity to kisspeptin in various ovarian compartments, including the luteal cells of the corpus luteum (low reactivity), interstitial glands, and follicles at different stages of development. Additionally, we also observed strong immunoreactivity in both the theca and granulosa cells of growing and antral follicles. Castellano *et al.* (12) reported preferential expression of kisspeptin in theca cells of antral follicles and low expression in granulosa cells. To confirm this difference, we repeated this analysis in one ovary of Wistar rats. In these rats, we found a similar distribution of immunoreactivity as the one described by Castellano *et al.*, strongly suggesting a difference in the expression of kisspeptin between Wistar and Sprague Dawley rats (data not shown). Analogously, differences in the presence of intraovarian sympathetic neurons between both strains of rats have been reported previously (39).

### Effect of stress on kisspeptin mRNA and peptide expression through the celiac ganglion-ovary axis

Considering the localization of kisspeptin in the postganglionic sympathetic neurons, we used a cold-stress paradigm to activate these sympathetic neurons. Previous work in our laboratory has shown that cold stress activates the hypothalamus (10, 21, 24) and the sympathetic nerve system innervating the ovary without altering the adrenal axis (40). This effect can be blocked by the *in vivo* administration of MK-801 (an *N*-methyl-D-aspartate receptor blocker) in the magnocellular region of the paraventricular nucleus of the hypothalamus (24), suggesting a central origin for this stimulation. The increase in the concentration of the peptide in the celiac ganglion and ovary strongly suggests that the activation of the sympathetic network from the paraventricular nucleus to the ovary (9) alters the kisspeptin system. Because we measured the concentration of the peptide in total hypothalamus, it did not permit us to conclude that a possible hypothalamic nucleus could be involved in the stress activation. The activation in the celiac ganglion-ovary nerve connection, however, was further supported by changes not only in the peptide concentrations but also in the kisspeptin mRNA levels at these sites. The increase in kisspeptin mRNA and peptide induced by $\beta$-adrenergic stimulation *in vitro* strongly suggests 1) that a direct cause-effect between mRNA and peptide occurs *in vitro* but not *in vivo*, and 2) that at least a fraction of ovarian kisspeptin may be associated with a sympathetic nerve-dependent activation of the intraovarian expression of kisspeptin. This possibility is strongly supported by our data that SONX decreases the firing rate of sympathetic neurons in the ovary and decreases kisspeptin mRNA, raising the interesting possibility of neural control of ovarian kisspeptin expression. The increase in ovarian kisspeptin concentration in contrast to the decreased levels of mRNA suggests a decreased efflux of kisspeptin from the ovary by retrograde flux and, as a consequence, an increased efflux of ovarian kisspeptin to extracellular sites. This possibility is supported by the increase in the serum levels of the peptide after SONX.

### Role of ovarian kisspeptin during puberty or estrous cycling activity

The role of kisspeptin in the reproductive system and puberty is well established, but participation of a kisspeptinergic system in follicular development remains unexplored. The delay in vaginal opening (an index of puberty in the rat) after the direct ovarian administration of a kisspeptin antagonist from prepuberty to adulthood clearly demonstrates that the ovary depends on kisspeptin to initiate a normal reproductive cycle. This last assertion was experimentally supported by the marked irregularities in the estrous cycling activity of the rats treated with intraovarian kisspeptin antagonist. Although we cannot discard a central effect of the antagonist, our data suggest that the antagonist did not reach the hypothalamus in a sufficient concentration to alter the LH surge. Previous data have demonstrated that systemic administration of 5 nmol of the same kisspeptin antagonist (41) blocked the response to kisspeptin-10, without affecting basal LH secretion. The fact that 1) centrally applied antagonists inhibit the preovulatory surge of the gonadotropins (41), 2) the kisspeptin antagonist modified the onset of puberty (our study), and 3) we found these effects with a constant ovarian infusion of 0.13 nmol/h (0.25 $\mu$l/h), a concentration that administered intracerebroventricularly does not inhibit the LH surge in castrated male rats (42), strongly suggests that the effects described above are rather the results of a local action of the antagonist. As expected, because the ovulation process was modified, the progesterone and estradiol serum levels were low. The close association between the large decrease in the plasma levels of kisspeptin and estradiol and the changes in estrous cycling

activity confirm the importance of kisspeptin to ovarian follicular cycling activity. In addition, the decreased levels of ovarian kisspeptin as a result of a poor follicular development (low estradiol levels) could result in an increased efflux of kisspeptin from the celiac ganglion to the ovary, a possibility that remains to be proved.

In conclusion, the results of the present work have shown the following: 1) the presence of kisspeptin mRNA and protein in the celiac ganglion and the ovary contributes to a novel circuit that regulates ovarian function and is manifest as physiological changes along pubertal development to adulthood; 2) sympathetic activation by a continuous 64-h cold stress through the hypothalamic neurons activates by cold principally involves, the celiac ganglion-ovary axis of the kisspeptin network; 3) the adrenergic innervation of the ovary regulates ovarian kisspeptin expression; and 4) direct ovarian administration of a kisspeptin antagonist *in vivo* delays vaginal opening and alters the estrous cycle, further supporting the importance of this peptide in ovarian function. These data, in addition to those recently published involving central kisspeptin in ovarian pathologies such as ovarian cysts (43), also suggest a peripheral nerve-kisspeptin relation that could participate in the development of the pathology.

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