Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat

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Departments of 1Physiology and Biophysics and 2Obstetrics and Gynecology, University of Washington, Seattle, Washington; 3Department of Cell Biology, Physiology, and Immunology, University of Córdoba, 4CIBER Fisiopatología de la Obesidad y Nutrición, and 5Instituto Maimonides de Investigaciones Biomédicas de Córdoba, Córdoba, Spain

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Navarro VM, Castellano JM, McConkey SM, Pineda R, Ruiz-Pino F, Pinilla L, Clifton DK, Tena-Sempere M, Steiner RA. Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat. Am J Physiol Endocrinol Metab 300: E202–E210, 2011. First published November 2, 2010; doi:10.1152/ajpendo.00517.2010.— Neurokinin B (NKB) and its cognate receptor neurokinin 3 (NK3R) play a critical role in reproduction. NKB and NK3R are coexpressed with dynorphin (Dyn) and kisspeptin (Kiss1) genes in neurons of the arcuate nucleus (Arc). However, the mechanisms of action of NKB as a cotransmitter with kisspeptin and dynorphin remain poorly understood. We explored the role of NKB in the control of LH secretion in the female rat as follows. 1) We examined the effect of an NKB agonist (senktide, 600 pmol, administered into the lateral cerebral ventricle) on luteinizing hormone (LH) secretion. In the presence of physiological levels of estradiol (E2), senktide induced a profound increase in serum levels of LH and a 10-fold increase in the number of Kiss1 neurons expressing c-fos in the Arc (P < 0.01 for both). 2) We mapped the distribution of NKB and NK3R mRNAs in the central forebrain and found that both are widely expressed, with intense expression in several hypothalamic nuclei that control reproduction, including the Arc. 3) We studied the effect of E2 on the expression of NKB and NK3R mRNAs in the Arc and found that E2 inhibits the expression of both genes (P < 0.01) and that the expression of NKB and NK3R reaches its nadir on the afternoon of proestrus (when circulating levels of E2 are high). These observations suggest that NKB/NK3R signaling plays an important role in the E2-dependent negative-feedback control of gonadotropin-releasing hormone (GnRH) and LH in mammals.

In the Arc of sheep and rodents, NKB is coexpressed with kisspeptin and dynorphin, which are encoded by the Kiss1 and preprodynorphin (Dyn) genes, respectively (4, 15, 24). Kisspeptin is a potent GnRH secretagogue (25), and GnRH neurons express the kisspeptin receptor (Kiss1r, aka GPR54) (17), which plays a critical role in the neuroendocrine regulation of GnRH and LH secretion (8, 25, 31). Kisspeptin neurons in the Arc express estrogen receptor-α and are thought to be direct targets for the negative-feedback action of E2 on GnRH secretion (32), Kiss1r/NK3R cells in the Arc are surrounded by a dense network of NKB-containing fibers (4), and they express NK3R, at least in the mouse (24), perhaps reflecting autonomic processes that control the rhythmic activity of this cellular network. Thus, from different species, fragments of a model have begun to emerge that would directly link a NKB/NK3R signaling pathway in Kiss1 neurons to the regulation of GnRH secretion; however, critical elements of the model are missing.

The purpose of this study was to investigate the proposition that NKB induces the release of GnRH by activating Kiss1 neurons, which serve as a conduit for the E2-dependent negative-feedback control of GnRH/LH secretion. 1) We tested the hypothesis that NKB regulates GnRH secretion by assessing the effect of the NKB agonist senktide on LH release in the female rat. 2) To determine whether kisspeptin/NKB neurons are possible targets for the action of senktide on GnRH/LH secretion, we assessed whether senktide could activate c-fos expression in Kiss1 neurons. 3) To elucidate the anatomic circuitry that links NKB/NK3R signaling to E2-dependent regulation of GnRH/LH secretion, we mapped the distribution of NKB and NK3R mRNAs in the brain and evaluated whether their expression is regulated by E2.

MATERIALS AND METHODS

Animals and Drugs

Female Wistar rats were bred in the vivarium at the University of Córdoba. The animals were maintained under constant conditions of light (14 h of light, lights on at 0700) and temperature (22°C) and were weaned at 21 days postpartum: they were housed in groups of five rats per cage with free access to standard rat chow and tap water. For hormone tests involving intracerebroventricular cannulation, the rats were caged individually from the day before cannula implantation until termination of experiments. Correct positioning of the cannulas was checked by visual inspection (to exclude animals showing obvi-
ous displacement or detachment) and confirmed at necropsy. Experimental procedures were approved by the University of Córdoba Ethical Committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals. Rat/mouse kisspeptin-10 (Kp-10) was obtained from Phoenix Pharmaceuticals (Belmont, CA). The NK3R agonist senktide was purchased from Sigma-Aldrich. The dose of senktide was selected on the basis of previous references as maximally effective in inducing gonadotropin responses in the rat (4).

**OVX and Steroid Replacement**

OVX was performed on adult female rats. Briefly, bilateral lumbar incisions were made while the animals were under fluorouracil anesthesia. Vasculature to the ovary was sutured, the ovary was removed, and wound clips were used to close the incision. Immediately after OVX, capsules filled with oil (sham) or with E2 + oil were implanted subcutaneously via a small midscapular incision at the base of the neck; wound clips were used to close the incision. For E2 implantation, Silastic tubing (20 mm long, 0.062 cm ID, 0.125 cm OD; Dow Corning) was used. Crystalline E2 (Sigma) at a low (physiological) dose of 100 µg/mL was dissolved in olive oil, as previously described (35). After capsules were filled with E2 in oil, the end of the capsule was sealed with silicone cement and allowed to cure overnight. On the day before surgery, implants were washed twice for 10 min in changes of 100% ethanol and then placed in sterile physiological saline overnight.

**Tissue Preparation**

Blood was centrifuged to isolate the serum, which was stored at −20°C until hormone measurements. Uteri were removed and weighed to provide an additional marker of plasma E2 levels (and their biological effect). Brains were removed for in situ hybridization (ISH), frozen on dry ice, and then stored at −80°C until sectioned. Five sets of 20-µm sections in the coronal plane were cut on a cryostat (from the diagonal band of Broca to the mammillary bodies), thaw-mounted onto SuperFrost Plus slides (VWR Scientific), and stored at −80°C. A single set of slides was used for ISH (adjacent sections 100 µm apart).

**RIA for LH and E2**

Serum LH levels were measured in 25- to 50-µL samples using a double-antibody method and RIA kits (supplied by Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 was labeled with 125I with the use of Iodo-gen tubes (Pierce, Rockford, IL) according to the manufacturer’s instructions. Hormone concentrations were expressed, with the reference preparation LH-RP-3 used as a standard. Intra- and interassay coefficients of variation were <8 and 10%, respectively. The sensitivity of the LH assay was 5 pg/tube. E2 levels were measured in 100-µL aliquots of serum samples with the use of a commercial, ultrasensitive E2 RIA kit (Immunotech-Beckman Coulter, Prague, Czech Republic) according to the manufacturer’s instructions. The sensitivity of the E2 assay was 0.6 pg/tube.

**Detection of Kiss1, NKB, NK3R, and c-fos mRNAs**

The probes used for detection of Kiss1, NKB, NK3R, and c-fos mRNA are described elsewhere (11, 24). Sense probes for every transcript were used as controls for the specificity of the ISH procedures, which are outlined below.

**Single-Labeled ISH of NKB and NK3R mRNAs**

NKB and NK3R mRNA sense and antisense probes were transcribed with T7 or T3 polymerase (Fermentas), as described previously (24). Briefly, radiolabeled probes were synthesized in vitro by inclusion of the following ingredients in a volume of 20 µL: 250 Ci of [35S]UTP (Perkin Elmer Life and Analytical Sciences), 1 µg of PCR product, 0.5 mM each ATP, CTP, and GTP, and 40 U of polymerase. Residual DNA was digested with 4 U of DNase (Ambion), and the DNase reaction was terminated by addition of 2 µL of 0.5 M EDTA, pH 8.0. The riboprobes were separated from unincorporated nucleotides with NucAway spin columns (Ambion). Slides with mouse hypothalamic sections from the different experimental groups were processed as reported previously (7, 16).

**Double-Labeled ISH**

Antisense rat Kiss1 and c-fos probes were transcribed from linearized pAMP1 plasmid containing the mouse Kiss1 or the rat c-fos insert with T7 polymerase (Fermentas) (11, 16). Radiolabeled riboprobe for c-fos was synthesized as described above for the NKB and NK3R riboprobes. Digoxigenin-labeled Kiss1 antisense riboprobe was synthesized with T7 RNA polymerase and digoxigenin labeling mix (Roche) according to the manufacturer’s instructions. Slides were processed for double-labeled ISH, as described previously (17). Slides were stored at 4°C and developed 8 days later.

**Quantification and Analysis of NKB, NK3R, Kiss1, and c-fos mRNAs**

The brain sections were analyzed unilaterally. Slides from all the animals were assigned a random three-letter code, arranged alphabetically, and read under dark-field illumination with custom-designed software designed to count the total number of cells and the number of silver grains (corresponding to radiolabeled Kiss1, NKB, NK3R, or c-fos mRNA) over each cell (5). Kiss1 mRNA-containing cells were visualized under fluorescent illumination, and custom-designed software was used to count the number of silver grains over each Kiss1 cell. The number of cells reported for each experiment represents the number of cells within the coronal sections containing the hypothalamic nucleus studied for each set, not the total number of cells in this specific nucleus. The starting and ending points of quantification were determined according to the atlas of Paxinos and Franklin (26a). Signal-to-background ratios for individual cells were calculated; an individual cell with a signal-to-background ratio of ≥3 was considered to be double-labeled. For each animal, the number of double-labeled cells was calculated as a percentage of the total number of particular mRNA-positive cells and then averaged across animals to produce a mean ± SE.

**Statistical Analysis**

Values are means ± SE for each group. In addition, when appropriate, integrated LH secretory responses are expressed as the area under the curve (AUC), calculated following the trapezoidal rule (27), over a 120-min period after administration of senktide. Two-way ANOVA, followed by Bonferroni’s post hoc comparison and t-tests, was used to assess variation among experimental groups. Significance level was set at P ≤ 0.05. All analyses were performed with Statview 5.0.1 for Macintosh (SAS Institute).

**Experimental Designs**

Experiments 1a and 1b: LH response to NKB agonist in intact and OVX animals treated with E2. Although it has been demonstrated in the rat and mouse that the NKB agonist senktide inhibits LH secretion in untreated OVX animals and has no discernable effect on LH in OVX animals treated with exceedingly high doses of E2 (24), the effect of senktide has not been assessed in intact or OVX animals (rodents) treated with physiological levels of E2. In experiments 1a and 1b, we assessed the ability of senktide to modulate LH secretion in vivo under two experimental conditions: animals (killed on diestrus 1 and proestrus) were left intact, and a capsule containing a low physiological dose of E2 was immediately implanted subcutaneously.
NKB agonist stimulates LH in female rats

RESULTS

Action of the NKB Agonist Senktide on LH Secretion in Intact Female Rats

In normal intact animals, we observed that senktide induced a robust increase in serum levels of LH on diestrus 1 and proestrus. At diestrus, the values were as follows: 1.1 ± 0.2 and 3.6 ± 0.4 ng/ml for vehicle and senktide, respectively, at 20 min (P < 0.01), 0.6 ± 0.2 and 1.4 ± 0.4 ng/ml for vehicle and senktide, respectively, at 60 min (P < 0.01), and 0.4 ± 0.1 and 0.9 ± 0.2 ng/ml for vehicle and senktide, respectively, at 120 min (P < 0.01; Fig. 1A). At proestrus, the values were as follows: 1.8 ± 0.2 and 3.6 ± 0.4 ng/ml for vehicle and senktide, respectively, at 20 min (P < 0.01), 1.4 ± 0.3 and
Effect of NKB Agonist in OVX Animals With and Without E₂ Replacement

Adult OVX rats injected with 600 pmol of senktide showed a significant decrease in LH release 120 min after treatment: 3.7 ± 0.4 and 2.1 ± 0.2 ng/ml for vehicle and senktide, respectively (P < 0.01; Fig. 2A). The overall release of LH, as represented by the AUC, was significantly different between groups: 464 ± 39 and 388 ± 33 AUC for vehicle and senktide, respectively (P < 0.01; Fig. 2A). In OVX animals treated with a low dose of E₂, senktide evoked a significant increase in LH release at all time points: 1.9 ± 0.4 and 6.6 ± 0.8 ng/ml for vehicle and senktide, respectively, at 20 min (P < 0.01), 2.9 ± 0.5 and 5.3 ± 1.2 ng/ml for vehicle and senktide, respectively, at 60 min (P < 0.01), and 1.0 ± 0.2 and 3.1 ± 1.2 ng/ml for vehicle and senktide, respectively, at 120 min (P < 0.01; Fig. 2B). The overall release of LH, as represented by the AUC, was significantly different between groups: 252 ± 49 and 584 ± 99 AUC for vehicle and senktide, respectively (P < 0.01; Fig. 2B). Circulating E₂ levels in OVX + E₂ animals matched those of proestrus, whereas OVX + sham animals showed E₂ levels equivalent to the diestrus phase. Similarly, uterine weight showed a significant increase in OVX + E₂ animals compared with the OVX + sham group (Table 1).

Coexpression of c-fos and Kiss1 mRNA in the Arc After Senktide Treatment

The aim of this experiment was to determine whether Kiss1 neuronal expression is activated by NKB. The percentage of Kiss1 neurons expressing c-fos in OVX + E₂ rats after senktide injection was assessed by double-labeled ISH. In the Arc, after the senktide injection, ~40% of the Kiss1 neurons were found to coexpress c-fos, reflecting a 10-fold increase in the coexpression of c-fos and Kiss1 compared with animals treated with vehicle alone: 39.4 ± 8.1% vs. 4.5 ± 1.7% (P < 0.01; Fig. 3). No expression of c-fos was detected in Kiss1 neurons in the anteroventral paraventricular nucleus following any of the treatments (data not shown). As anticipated, senktide stimulated LH secretion, as previously shown: 0.9 ± 0.3 and 3.0 ± 0.3 ng/ml for vehicle and senktide, respectively (P < 0.01).

Distribution of NKB mRNA in the Hypothalamus and Its Regulation by E₂

Figure 4 (top) shows areas of the brain where NKB mRNA was clearly detectable in OVX + sham and OVX + E₂ animals. NKB mRNA was expressed in the cerebral cortex, the bed nucleus of the stria terminalis, the anterocortical amygdaloid nucleus, the supraoptic nucleus (SON), the basolateral nucleus of the amygdala, the medial habenular nucleus (MH),

Table 1. LH, E₂ levels, and uterine weights in experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Proestrus</th>
<th>Diestrus</th>
<th>OVX</th>
<th>OVX + E₂</th>
</tr>
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<tbody>
<tr>
<td>LH, ng/ml</td>
<td>0.9 ± 0.1 (11)</td>
<td>0.9 ± 0.2 (10)</td>
<td>5.2 ± 0.8* (9)</td>
<td>2.0 ± 0.4 (10)</td>
</tr>
<tr>
<td>E₂, pg/ml</td>
<td>12 ± 2* (14)</td>
<td>&lt;6* (11)</td>
<td>&lt;6* (9)</td>
<td>12 ± 3* (6)</td>
</tr>
<tr>
<td>Uterine wt, mg/100 g body wt</td>
<td>46.3 ± 1.7 (10)</td>
<td>153.6 ± 4.2* (10)</td>
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</table>

Values are means ± SE of number of rats in parentheses. LH, luteinizing hormone; E₂, estradiol; OVX, ovariectomized. *P < 0.01 (1-way ANOVA). Values represented with different letters (a, b) are statistically significant (P < 0.05).
the lateral hypothalamus (LHA), the zona incerta, and the Arc. Within the hypothalamus, expression was found to be concentrated in two regions: the Arc and the LHA. As a function of cycle stage, we found significantly reduced levels of NKB mRNA in the Arc during the afternoon of proestrus (when E2 levels are highest) relative to the morning of diestrus 1 (P < 0.05) and the morning of proestrus (P < 0.05; Fig. 5A), whereas no differences were detected in levels of NKB mRNA in the LHA as a function of cycle stage. To test whether E2 might be responsible for the differences in NKB expression we had observed in different cycle stages, we compared the expression of NKB in OVX animals with subcutaneously implanted empty (sham) capsules or E2-filled capsules (OVX vs. OVX/E2) and found that levels of NKB mRNA in the Arc were significantly reduced by E2 treatment (P < 0.05; Fig. 5B). Despite the equivalent levels of plasma E2 in animals in proestrus and OVX + E2, the expression of NKB was significantly reduced only in the OVX + E2 group compared with diestrous and OVX animals, suggesting the presence of additional, steroid-independent factors that stimulate NKB expression.

**Distribution of NK3R mRNA in the Rat Hypothalamus and Its Regulation by E2**

Cells expressing NK3R mRNA were observed in discrete regions of the brain, including the cerebral cortex, the suprachiasmatic nucleus, ventromedial hypothalamic nucleus (VMH), paraventricular hypothalamic nucleus, medial habenular nucleus, LHA, SON, and Arc (Fig. 4, bottom). No difference in the general distribution of the transcript was found between the range of E2 treatments in OVX animals (OVX + sham vs.
OVX + E₂). In intact animals, the expression of NK3R in the Arc was at its maximum on the morning of diestrus 1 (when E₂ levels are low), as was the case for NKB. Moreover, levels of NK3R were markedly reduced on proestrus but, in this case, in the morning and afternoon (compared with diestrus 1; Fig. 6). Similarly, the expression of NK3R in the Arc was significantly reduced by E₂ (Fig. 6). The expression of NK3R in most other hypothalamic regions (e.g., LHA, SON, and VMH) was unaffected by E₂, with one notable exception: the paraventricular hypothalamic nucleus, where there appeared to be a modest induction of NK3R by E₂ (P < 0.05; data not shown).

**DISCUSSION**

Previous studies have demonstrated that NKB agonists inhibit LH secretion in OVX rats but have no effect on LH secretion in OVX mice treated with supraphysiological levels of E₂ (24, 30). However, when administered to intact ewes during the follicular phase, NKB stimulates LH secretion (3). Thus, under extremes in circulating levels of E₂ (i.e., very high or low), NKB/senktide inhibits LH secretion, at least in rats and mice. Neurotransmitters having the opposite effect on LH secretion in OVX and intact animals (physiological levels of E₂) have long been recognized [e.g., neuropeptide Y (37)]. We puzzled whether this paradox might be reconciled by differential effects of E₂ on NK3R signaling. We have demonstrated in the rat that, in the presence of physiological levels of E₂ (intact or OVX with physiological E₂ restoration), the NKB agonist senktide stimulates LH secretion. We have also confirmed that, in the absence of sex steroids in the rat (sham-treated OVX animals), senktide exerts an inhibitory effect on LH release, as previously reported (30).

It would appear that the facilitatory effect of NKB requires a relatively narrow physiological range of circulating E₂, which somehow alters the NKB/NK3R signaling pathway. At
first glance, the fact that E\textsubscript{2} levels in the diestrous females were as low as those in the OVX group seems to argue against a role for E\textsubscript{2} in this process. However, LH levels are very low during diestrous, indicating that they are still under the inhibitory influence of higher E\textsubscript{2} levels that occurred earlier in the cycle. In fact, LH levels in female rats remain under the inhibitory influence of E\textsubscript{2} for \textasciitilde 1 wk after removal of their ovaries (14); therefore, it is plausible that the same E\textsubscript{2}-dependent mechanism that restrains GnRH/LH secretion during diestrous also sustains the ability of NKB (sentkide) to induce GnRH/LH release.

Within the hypothalamus, E\textsubscript{2} has profound effects on the expression of NKB and NK\textsubscript{3}R. In mice, the expression of NK\textsubscript{3}R mRNA in the Arc appears to be particularly sensitive to the inhibitory effects of E\textsubscript{2}, as documented also here in the rat (Fig. 6), which correlates with the relative insensitivity of the LH response to sentkide in animals treated with high doses of E\textsubscript{2} (24). However, additional regulation at the level of translation or the rate of elimination of the protein cannot be ruled out. This may also account for the lack of LH stimulation by NKB or the rate of elimination of the protein cannot be ruled out. We note that, in the absence of E\textsubscript{2} (OVX animals), the NK\textsubscript{3}R mRNA and should thus be responsive to exogenous ligand inhibiting GnRH/LH secretion; however, this hypothesis remains to be tested.

We and others have argued that Kiss1 neurons in the Arc serve as the nodal point for the negative-feedback regulation of GnRH secretion by sex steroids (25). However, it has been recently recognized that Kiss1 neurons in this region have several cotransmitters. Indeed, NKB is coexpressed with kisspeptin and dynorphin in the Arc in many species, including the rat, sheep, and mouse (4, 15, 24). We have suggested that NKB acts autocytically on these kisspeptin/NKB/dynorphin cells to feed back and stimulate kisspeptin release (24, 36). How could this explanation account for the paradoxical inhibitory effect of NKB on LH release observed in the absence of E\textsubscript{2}, i.e., OVX animals, which have high levels of NK\textsubscript{3}R mRNA and should thus be responsive to exogenous NKB? We note that, in the absence of E\textsubscript{2} (OVX animals), the expression of NKB mRNA is extraordinarily high (24). We postulate that, under these circumstances, elevated levels of endogenous ligand flooding the receptor may destabilize the Kiss1(NKB/NK\textsubscript{3}R)-GnRH pulse generator, so that additional exogenous ligand inhibits GnRH/LH secretion; however, this hypothesis remains to be tested.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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