Disparate Changes in Kisspeptin and Neurokinin B Expression in the Arcuate Nucleus After Sex Steroid Manipulation Reveal Differential Regulation of the Two KNDy Peptides in Rats

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Kisspeptin, neurokinin B (NKB) and dynorphin A are coexpressed in a population of neurons in the arcuate nucleus (ARC), termed KNDy neurons, which were recently recognized as important elements for the generation of GnRH pulses. However, the topographic distribution of these peptides and their regulated expression by sex steroids are still not well understood. In this study, detailed examination of NKB and kisspeptin immunoreactivity in the rat ARC was carried out, including comparison between sexes, with and without sex steroid replacement. Neurons expressing kisspeptin and NKB were more prominent in the caudal ARC of females, whereas neurons expressing NKB, but not kisspeptin, were the most abundant in the male. Sex steroid manipulation revealed differential regulation of kisspeptin and NKB; although kisspeptin immunoreactive (ir) cells increased in response to gonadectomy, NKB remained unchanged. Furthermore, the number of NKB-ir cells increased upon sex steroid replacement compared with gonadectomy, whereas kisspeptin did not, suggesting that sex steroids differently regulate these peptides. In addition, only in females did the density of kisspeptin- and NKB-ir fibers in the ARC increase upon sex steroid replacement in relation to sham and ovariectomy, respectively, suggesting sex-specific regulation of release. In conclusion, our observations reveal sex differences in the number of kisspeptin- and NKB-ir cells, which are more prominent in the caudal ARC. The divergent regulation of kisspeptin and NKB peptide contents in the ARC as a function of sex and steroid milieu enlarge our understanding on how these neuropeptides are posttranscriptionally regulated in KNDy neurons.

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The initial KNDy neuron concept implies that KNDy neurons are a homogenous population of neurons, but actually the expression of the three KNDy peptides only overlaps partially. In the intact ewe for instance, 80% of kisspeptin-immunoreactive (ir) cells coexpress NKB, 73% of NKB-ir neurons coexpress kisspeptin, 94% kisspeptin-ir neurons coexpress dynorphin A, and 86% of NKB-ir neurons coexpress dynorphin A (9, 10). In mice, approximately 90% of Kiss1 mRNA-expressing cells also express Tac2 and Dyn mRNA, whereas the percentage of Tac2 mRNA-expressing cells coexpressing Kiss1 mRNA was not assessed (2). In rats, 99% of NKB-ir neurons coexpress dynorphin A, whereas only 65% of dynorphin-ir neurons coexpress NKB (7). KNDy neurons have also been reported in both ovine, murine, and human males (1, 11, 12); however in males, the overlap in expression is even less prominent, with more than double NKB immunoreactivity compared with kisspeptin in the human (12–14) and more than double Tac2 mRNA-expressing cells compared with Kiss1 mRNA-expressing cells in mice (11), suggesting a population of NKB cells not expressing kisspeptin.

The regulation of KNDy neurons by sex steroids has been investigated mainly at the mRNA level, and it is well documented that expression levels in the ARC increase upon gonadectomy and decrease upon steroid replacement for both Kiss1 (15–17) and Tac2 mRNA in rodents (15, 18, 19). However, it is noteworthy that Kiss1 mRNA is more sensitive to sex steroids than Tac2 mRNA because Kiss1 mRNA is reduced in mice at low levels of estradiol replacement, whereas Tac2 mRNA is decreased only at a higher estradiol replacement level (20).

Sex differences in the expression levels of KNDy peptides have been more scarcely investigated, but immunocytochemical studies suggest that kisspeptin and NKB contents are higher in the female than in the male ARC of rats and humans (12, 21, 22). However, although one study reported more Kiss1 mRNA expressing neurons in the female rat at some postdevelopmental stages (23), a majority of studies has found no sex difference in the expression of Kiss1 and Tac2 mRNAs in mice and rats (15, 21, 24), which suggest posttranscriptional regulatory mechanisms so that sex differences become evident at the peptide level. This raises some interesting aspects as to whether the two peptides are regulated by sex steroids in the same manner. In fact, sex steroids have been shown to influence gonadotropin responses to the two peptides differentially. Thus, NKB has been shown to be either inhibitory or stimulatory on LH release, dependent on the sex steroid milieu in both mice, rats, monkeys, and sheep (2, 11, 25–30), whereas kisspeptin always stimulates LH release, although the magnitude of such responses is blunted in absence of estrogen effects (31).

All in all, the available evidence strongly suggests that the KNDy neurons are controlled by complex regulatory mechanisms, involving possibly differential translation, transport and release. In the light of this complex regulation, we investigate in detail the anatomical distribution and regulation of kisspeptin and NKB by sex steroid manipulation to localize sensitive subpopulations of neurons and to characterize the posttranscriptional dynamics of these two peptides.

Materials and Methods

Animals and drugs

Male and female Wistar rats were bred and raised in the animal facility of the University of Córdoba, at which they were kept under constant conditions of light-dark cycles (14 h of light from 7:00 AM) and temperature (22°C), with free access to standard laboratory chow and tap water. The 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. Adult male and female rats (aged 5–6 mo; females ~230 g and males ~280 g) were randomly assigned into three groups per sex (females, n = 6–8 per group; males 8–10 per group): sham female, ovariectomized (OVX), OVX and estradiol replaced (OVX + E); sham male, orchidectomized (ORX), and ORX and T replaced (ORX + T). The estrous cycle of the female sham group was followed by vaginal cytology to confirm regular estrous cyclicity. Three weeks after surgery, the animals were perfused and the brains were processed as described below. Sham females were perfused in diestrus 1.

Gonadectomy and hormone replacement were performed under ketamine/xylazine anesthesia; female rats underwent bilateral ovariec-tomy via abdominal incisions, and male rats were castrated via a single abdominal incision. Immediately after gonadectomy, capsules filled with 17β-estradiol (abbreviated estradiol or E) or T were implanted sc via a small incision at the base of the neck. SILASTIC brand tubing (Dow Corning Corp; 1.98 mm inner diameter, 3.18 mm outer diameter) was used for capsule preparation. For E replacement, crystalline estradiol (E8875; Sigma Chemical) was dissolved in olive oil (100 μg/mL) and filled in capsules of 20 mm effective length. For T replacement, crystalline T (86500; Sigma Chemical) was filled in capsules of 10 mm effective length. Capsules were filled as described previously (11, 32). Based on previous studies (32, 33) and our detection of slightly higher levels of circulating LH in sex steroid-replaced groups relative to sham (data not shown), we consider that the doses chosen enabled the replacement approximately in the physiological range. Sham-operated animals served as controls, and olive oil-filled and empty capsules were used in females and males, respectively, not receiving hormone replacement.

Detailed mapping of the number of kisspeptin- and NKB-ir neurons across the ARC was evaluated in male and female rats subjected to sex steroid manipulation in adulthood to assess whether kisspeptin and NKB are differentially expressed be-
tween males and females and to what extent these are differenti-
ately regulated by sex steroids. Furthermore, kisspeptin and
NKB colocalization was assessed by dual labeling, as described
below.

**Tissue preparation**

Animal were deeply anesthetized with pentobarbital (50 mg/ 
kg) and perfused transcardially with 0.9% saline for 5 minutes,
followed by 4% paraformaldehyde-phosphate buffer for 10 
minutes (0.1 M; pH 7.4). The brains were rapidly and carefully
isolated from the skull and postfixed in the same fixative over-
night and thereafter kept in 0.05 M PBS at 4°C until immuno-
cytochemical processing.

**Immunocytochemistry**

The fixed brains were infiltrated with 30% sucrose-PBS so-
lution before sectioning into four parallel series of 40 μm free-
floating coronal sections through the entire rostrocaudal exten-
sion of the ARC; the sections of each series were thus separated
by 160 μm. For the mapping of immunoreactive neurons, one
series of sections was used to evaluate kisspeptin immunoreac-
tivity and the adjacent series was evaluated for NKB immuno-
reactivity using 3,3′-diaminobenzidine (DAB) as chromagen, as
previously described (30, 34). For evaluation of kisspeptin im-
munoreactivity, a purified polyclonal primary antiserum (JLV-1,
diluted 1:200, Table 1) was used. This antiserum is raised against
rat kisspeptin-52 in rabbit and has been shown not to cross-react
with a number of arginine-phenylalanine (RF)-amides (21, 35),
and the overlap of JLV-1 immunoreactivity and
*Kiss1* mRNA
localization, evaluated by in situ hybridization, has been deter-
mined in rats (34). For detection of NKB immunoreactivity, a
purified polyclonal antiserum raised against NKB in rabbit (IS-
39, diluted 1:5000, Table 1), which has also been previously
characterized, was used (36, 37). Further validation of IS-39
includes overlapping immunoreactivity and mRNA, as deter-
mined by in situ hybridization, and lack of binding to six related
peptides in a displacement assay (38).

For each experiment, all sections reacted for the same antigen
were reacted simultaneously. For the DAB staining procedure, a
series of sections was washed in PBS, and endogen peroxidases
were blocked in 1% H₂O₂ in PBS for 10 minutes, followed by
blockade of nonspecific binding in PBS containing 0.3% Triton
X-100, 5% swine serum, and 1% BSA for 20 minutes. The sections
were then incubated in primary antiserum diluted in PBS contain-
ing 0.3% Triton X-100 and 1% BSA and gently shaken
overnight at 4°C. After washing in PBS containing 0.1% Triton
X-100 (T-PBS), sections were incubated 1 hour in biotinylated
donkey antirabbit (1:1000; Jackson Labs) in PBS containing
0.3% Triton X-100 and 1% BSA. After another T-PBS wash, the
sections were incubated in 0.4% avidin-biotin-peroxidase com-
plex (Vector Elite Kit; Vector Laboratories) diluted in T-PBS and
thereafter developed in 0.05% DAB (Sigma-Aldrich) with
0.05% H₂O₂ in Tris-HCl buffer [0.05 M (pH 7.6), 5°C] for 10
minutes. The sections were mounted in a rostral-to-caudal order
and coverslipped in Pertex (HistoLab).

For each section, the number of NKB- or kisspeptin-ir cells in
both hemispheres was quantified under bright-field illumination
(Axioskop 2plus; Zeiss) with special attention to anatomical cues
to map the correct rostrocaudal position of each section. The
identities of the rats were blinded through the immunocytochem-
ical procedure and during quantification.

For quantification of immunoreactive fiber density, photom-
icrographs of the section 2.44 mm posterior to bregma were
used. This section contains only a few immunoreactive cells, and
OD therefore reflects the amount of immunoreactive fibers in the
rostral ARC, rather than cell bodies. The section was selected
based on anatomical cues, with the shape of the ARC and the
median eminence and the retraction of the optical nerves as the
major anchor points. Photomicrographs were taken at ×10 mag-
nification under identical light and microscope settings using the

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**Table 1. Antibody Information**

<table>
<thead>
<tr>
<th>Peptide Target</th>
<th>Antigen Sequence</th>
<th>Name of Antibody</th>
<th>Source</th>
<th>Species Raised in Monoclonal or Polyclonal</th>
<th>Dilution Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKB</td>
<td>N-terminal extension of the NKB precursor Rodent kisspeptin-52</td>
<td>IS-39</td>
<td>Professor P. Ciofi, INSERM Unité 862, Bordeaux, France</td>
<td>Rabbit, polyclonal</td>
<td>1:5000 and 1:4000</td>
</tr>
<tr>
<td>Kisspeptin</td>
<td>Rodent kisspeptin-52</td>
<td>JLV-1</td>
<td>Professor J. Mikkelsen, Neurobiology Research Unit, University Hospital Rigshospitalet, Copenhagen, Denmark</td>
<td>Rabbit, polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Kisspeptin</td>
<td>N-terminal rodent kisspeptin-52</td>
<td>AC067</td>
<td>Dr A. Caraty, Physiologie Reproduction et Comportements-Institut National de la Recherche Agronomique, Nouzilly, France</td>
<td>Sheep, polyclonal</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
Zeiss microscope Axioskop 2plus (Carl Zeiss Imaging Solutions) and a PixelLINK PL-A622C camera (PixelLINK), and the photomicrographs were analyzed using the software ImageJ (National Institutes of Health, Bethesda, Maryland), measuring OD unilaterally in a triangle covering the ARC. The OD outside the ARC, close to the ventromedial hypothalamic nucleus, which contained no immunoreactive fibers, served as background measure and was subtracted OD of the ARC. The identities of all photomicrographs were blinded during analysis, and all photomicrographs were analyzed simultaneously.

**Fluorescent double labeling**

To evaluate coexpression of kisspeptin and NKB, the section 3.88 mm posterior to bregma, which displays the largest sex difference in expression, was stained for both kisspeptin and NKB using double immunofluorescence. The kisspeptin antiserum AC067 against the N-terminal part of rat kisspeptin-52 is raised in sheep and is therefore appropriate for double labeling with the NKB antibody IS-39, which is raised in rabbit (Table 1). AC067 has been characterized previously and specificity has been validated in mouse knockout tissue (39). However, because it was reported that this antibody is suitable for staining of kisspeptin in the ARC of rats, only if the rats are gonadectomized (39), which was also found in our studies of antibody testing, we double labeled only the OVX and ORX groups. For the double-labeling procedure, the sections were washed thoroughly in PBS, and nonspecific binding was then blocked in blocking buffer (5% normal swine serum and 0.3% Triton X-100 in PBS) for 30 minutes. The sections were thereafter transferred directly to the primary antiserum AC067 diluted 1:1000 in blocking buffer. After incubation overnight at 4°C, sections were washed in PBS + 0.3% Triton X-100 and then incubated 3 hours in fluorochrome-coupled secondary antibody (Alexa Fluor 488 antirabbit IgG; Invitrogen) diluted 1:333 in blocking buffer.

From this point, the wells were covered at all times to protect the fluorochrome. The sections were washed thoroughly in PBS + 0.3% Triton X-100, and the protocol was repeated using the polyclonal NKB antiserum IS-39 (diluted 1:4000) and the secondary antibody Alexa Fluor 568 antirabbit IgG (Invitrogen). After washing in PBS, the sections were mounted and coverslipped with Fluoromount-G (Southern Biotech). Photomicrographs were taken at ×20 magnification using a Zeiss Imager Z.1 microscope with the software AxioVision 4.7.3 (Carl Zeiss Imaging Solutions), and an AxioCam MRm camera (Carl Zeiss Imaging Solutions). Although we found that the number of immunoreactive cells was comparable for DAB and fluorescence when detecting NKB, this was not the case for kisspeptin; for kisspeptin, we found fewer cells using AC067 and fluorescence compared with JLV-1 and DAB, even in the gonadectomized condition, and quantification of the number of cells expressing kisspeptin would therefore be underestimated (and the cells expressing NKB and not kisspeptin thus overestimated). Double labeling using AC067 is therefore used only as qualitative support for our detailed findings using DAB labeling.

**Statistical analysis**

Differences between the three groups were analyzed using a one-way ANOVA followed by Tukey’s multiple comparisons test. Due to the caudal peak in NKB- and kisspeptin-ir cells in the females, the ARC was divided into a rostral part (3.56 mm posterior to bregma and rostral hereof) and a caudal part (caudal of 3.56 mm posterior to bregma), which were also analyzed separately using a one-way ANOVA followed by Tukey’s multiple comparisons test to reveal whether sex differences and sex steroid sensitivity differed in the rostral and caudal populations. Further subdivisions of the rostrocaudal area did not provide any additional information, and this division was therefore considered appropriate and sufficient. Differences between the sham male and sham female groups were analyzed using a Student’s t test. For all analyses P < .05 was considered statistical significant. Data are presented as mean ± SEM. Mapping of cell numbers in the ARC are presented both as the total cell count for the entire ARC and as a rostrocaudal mapping of the total cell count of each section.

**Results**

**Sex differences in kisspeptin- and NKB-ir cells in the ARC**

We investigated the detailed distribution of kisspeptin- and NKB-ir cells throughout the ARC in male and diestrous female (sham) rats to identify sex differences. Overall, the female ARC contained both more kisspeptin-ir (Figure 1A, P < .0001) and NKB-ir cells (Figure 1B, P < .05) than the ARC of males. In females, a steady rostrocaudal increase in the number of kisspeptin- and NKB-ir cells was found, and from 3.56 mm posterior to bregma, a marked increase in cell numbers occurred for both peptides, peaking 3.88 mm posterior to bregma (Figure 1A and B). In males, the number of kisspeptin-ir cells was very low throughout the extent of the ARC, and no peak was observed in the sham males (Figure 1A). Accordingly, the females had more kisspeptin-ir cells in both the rostral (P < .0001) and the caudal ARC (P < .0001). For NKB, the number of immunoreactive cells in males was similar to female levels in the rostral ARC (P < .44). In the caudal ARC of the male, there was a peak in NKB-ir cells, which is in contrast to male kisspeptin expression. However, the caudal peak was less prominent than in the female, as was the total number of NKB-ir cells (Figure 1B, P < .01).

Kisspeptin and NKB double labeling of the caudal ARC in OVX and ORX rats supported the finding of more kisspeptin- and NKB-ir neurons in the female compared with the male and suggested that kisspeptin and NKB are coexpressed to a large extent in OVX females, whereas in ORX males, neurons expressing NKB, but not kisspeptin, were pronounced (Figure 2). Concomitantly, these expression patterns suggest that there are more neurons expressing both NKB and kisspeptin in the caudal ARC of females and unveil the existence of a subpopulation of cells, which is more pronounced in the male than in the female ARC.
expressing only NKB and not kisspeptin; we define these as NKB-only neurons.

**Kisspeptin- and NKB-ir cells are differentially regulated by adult sex steroid manipulation**

To investigate the sex steroid feedback on kisspeptin- and NKB-ir neurons, we subjected one group of male and female rats to gonadectomy and another group to gonadectomy with physiological levels of sex steroid replacement for a total of 3 weeks. The number of kisspeptin-ir cells in the ARC increased after gonadectomy in both females (Figure 3A, \( P < .05 \)) and males (Figure 3C, \( P < .001 \)), and sex steroid replacement tended to reduce the number of kisspeptin-ir cells in OVX females, although such effect did not reach statistical significance. In the male, the kisspeptin levels in the ORX+T group was reduced compared with ORX (Figure 3C, \( P < .001 \)) but did not reach the sham male levels (\( P < .05 \)).

Examination of the number of kisspeptin-ir neurons along the rostrocaudal axis (females, Figure 3B; males, Figure 3D) revealed that gonadectomy increased the number of kisspeptin-ir cells across the entire ARC, whereas steroid replacement lowered the number of kisspeptin-ir cells across the entire ARC in both males and females. Separate analyses of the rostral and caudal ARC also revealed no regional differences in sex steroid sensitivity in both the males and females (data not shown).

Regulation of NKB-ir cells by adult sex steroid manipulation was strikingly different from the regulation of kisspeptin. First, the number of NKB-ir cells was not significantly affected by gonadectomy in either the female (Figure 3E) or the male (Figure 3G). Second, sex steroid replacement resulted in an increase in the number of NKB-ir cells relative to the gonadectomized group in both sexes (females, \( P < .01 \); males, \( P < .001 \)), and in addition, the OVX+E group had more NKB-ir cells relative to sham (Figure 2G, \( P < .05 \)). The detailed expression profiles (females, Figure 3F; males, Figure 3H) showed that, as for kisspeptin, NKB expression was equally affected by
changes in sex steroid levels across the extension of the ARC. The equal sensitivity of the rostral and caudal ARC to sex steroid manipulation was confirmed when analyzing the rostral and caudal ARC separately (data not shown).

Sex steroids increase kisspeptin- and NKB-ir fiber density in the female ARC

To assess whether changes in the number of immunoreactive cells were also reflected in the density of fibers in the ARC, OD was measured in a rostral ARC section (2.44 mm posterior to bregma). This section was chosen because it is the most rostral section containing immunoreactive fibers in the distinct pattern characterizing the rostral ARC but only a few immunoreactive cells, and OD is therefore an indirect measure of fiber density. The area rostral to this section represents the very rostral ARC and contains few fibers, all lining the third ventricle, and the triangular shape of the ARC is not evident in these sections, making the section 2.44 mm posterior to bregma the first representative ARC section. A significant sex difference in fiber density was observed in sham animals for kisspeptin \((P < .0001)\) but not for NKB \((P < .075)\). However, both kisspeptin and NKB were sensitive to sex steroid manipulation in the female, in which the OVX+E group had higher fiber density compared with sham or OVX, respectively (Figure 4, A and C, \(P < .05\)). In contrast, male fiber density was not affected by sex steroid manipulation for either kisspeptin or NKB (Figure 4, B and D). Although the degree of colabeled fibers was not assessed, we found higher OD values for kisspeptin relative to NKB, indicating more kisspeptin-ir fibers in the rostral ARC (Figure 4).

Discussion

The KNDy neuron model has been the subject of intense debate recently, and it has been recognized that sex and species differences may be more pronounced than initially assumed \((1, 12, 14, 21)\). Whereas dynorphin has long been recognized to be involved in several functions unrelated to reproduction, such as anxiety and stress \((40)\), NKB and in particular kisspeptin predominantly participate in reproductive control. We therefore focused our analyses in the distribution and regulation of kisspeptin and NKB, as the two putative stimulatory elements of KNDy neurons, to document differences in their patterns of expression that may reveal differential regulation and functions (including non-KNDy actions). Nonetheless, implementation of analogous analyses to dynorphin, although technically demanding (eg, triple kisspeptin, NKB, and dynorphin labeling), might be informative as well and would merit specific investigation.

Our topographical mapping of kisspeptin- and NKB-ir neurons in the ARC of intact male and female rats shows that although there is a sex difference only in the number of kisspeptin-ir cells and not of NKB-ir cells in the rostral ARC, there is a pronounced sex difference in the caudal ARC, with the female expressing both more kisspeptin- and NKB-ir cells. Based on our finding of extensive overlap in kisspeptin and NKB expression in the OVX female, as revealed by double labeling, these neurons are suggested to be KNDy neurons.

Such a caudal population of kisspeptin- and NKB-expressing neurons has also been described in ewes \((1, 41, 42)\) and in OVX monkeys \((43)\). Because the caudal KNDy neurons show the largest sex difference in neuron numbers, it is tempting to hypothesize that they play a specific role in the control of gonadotropin secretion in the female. Yet, to our knowledge, and in contrast to sheep \((41, 42, 44)\), no functional evidence for a role of caudal ARC kisspeptin neurons in the generation of the preovulatory surge has been presented in rodents.

Our finding of more NKB- and kisspeptin-ir cells in adult females compared with males is supported by previous findings in sheep, rats, and humans \((1, 12, 21, 22, \ldots)\).
Furthermore, our finding of sex differences in kisspeptin fiber density and not in NKB fiber density in the ARC is in line with reports in rats and humans (12, 37). Considering the higher numbers of NKB-ir cells in the female over the male, this could suggest that a fraction of female NKB-ir neurons project to areas outside the ARC.

Our data are the first to reveal increased NKB-ir neurons in OVX/E relative to OVX rats; a finding that is not in line with studies at the mRNA level, in which hormone replacement resulted in a decrease in Tac2 mRNA expression in male and female mice and in male rats (11, 15, 18, 19). Because no mRNA studies have been performed in this study, a direct comparison of mRNA and peptide levels is not possible. However, the apparent contrast in mRNA vs peptide levels could suggest that sex steroids, in addition to inhibiting transcription, also inhibit NKB release. The divergent mRNA and protein is unlikely to stem from differences in the methodology of in situ hybridization and immunohistochemistry because the findings are in different directions and therefore cannot be a question of sensitivity. It remains a possibility that NKB release from NKB-ir fibers projecting to areas outside the ARC, eg, as described for the involvement of NKB in regulation of body temperature (46), is regulated differently.

Contrary to NKB, kisspeptin-ir cells decreased upon sex steroid replacement to a level intermediate of sham and gonadectomized levels, which is in line with Kiss1 mRNA in mice (17), thus suggesting a primary transcriptional regulation of kisspeptin expression so that a reduction of Kiss1 mRNA would directly translate into reduced kisspeptin. To our knowledge, we are the first to quantify kisspeptin- and NKB-ir fibers in the ARC upon sex steroid replacement, and the increased fiber density of kisspeptin and NKB upon sex steroid replacement in relation to sham and OVX, respectively, specifically in the female suggests that the release of kisspeptin and NKB is repressed in the OVX+E state. Such suppression in secretion of both neuropeptides would result in increased fiber density. Other possible explanations could be estradiol-mediated changes in peptide cleavage resulting in changed antibody affinity, or estradiol-mediated increase in peptide stability. Although the mechanisms causing disparate findings in mRNA and peptide levels in this condition are not understood, divergent mRNA and peptide levels have also been described for kisspeptin in the AVPV, but not the

Figure 3. Kisspeptin- and NKB-ir neurons in the ARC after adult sex steroid manipulation. Total number of immunoreactive cells in the ARC (left panel) as well as the topographical mapping across the extension of the ARC (right panel) are presented for kisspeptin in females (A and B) and males (C and D) and for NKB in females (E and F) and males (G and H). Gonadectomy increased kisspeptin-ir cells, and sex steroid replacement lowered kisspeptin expression across the extension of the ARC in both males and females. In contrast, NKB expression was unchanged upon gonadectomy and increased in both sexes after sex steroid replacement across the entire ARC. The sham groups are the same as in Figure 1. One-way ANOVA followed by Tukey’s post hoc test was performed (A, C, E, and G). *, P < .05; **, P < .01; ***, P < .001.
Interestingly, the increase in kisspeptin and NKB fiber density in the OVX+E group was not found in the ORX+T group, suggesting a female-specific mechanism of fiber density regulation. This regulation may be important for rapid changes in release and could serve a function in regulation of the estrous cycle.

In line with findings in humans, in which the decrease in the negative sex steroid feedback associated to menopause had a greater effect on kisspeptin compared with the NKB levels (12, 13), we document here that kisspeptin is more sensitive than NKB to gonadectomy. Differential regulation of *Tac2* and *Kiss1* mRNA has also been reported in mice, in which pubertal lack of sex steroids markedly increased *Kiss1* expression, whereas *Tac2* expression was not affected (20). Likewise, in adult OVX mice, lower sex steroid replacement was required to suppress *Kiss1* expression as compared with *Tac2* expression (20). Species differences in the sensitivity of NKB to sex steroid feedback likely exist; thus, whereas we here find no difference in NKB-ir cells upon gonadectomy, previous data in humans documented an increase in NKB-ir cells upon decreased negative sex steroid feedback (48), and in sheep, both pre- and postpubertal gonadectomy increased the number of NKB-ir cells in the ARC (49).

Of interest, our analyses documented more NKB-ir cells compared with kisspeptin-ir cells in the male rat, observations that are in line with findings in male mice (11) and humans (12, 14). In the same line, our data support the existence of a population of NKB-ir cells that do not co-express kisspeptin, which is much more prominent in the ARC, of lactating rats (47). Interestingly, the increase in kisspeptin and NKB fiber density in the OVX+E group was not found in the ORX+T group, suggesting a female-specific mechanism of fiber density regulation. This regulation may be important for rapid changes in release and could serve a function in regulation of the estrous cycle.

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Of interest, our analyses documented more NKB-ir cells compared with kisspeptin-ir cells in the male rat, observations that are in line with findings in male mice (11) and humans (12, 14). In the same line, our data support the existence of a population of NKB-ir cells that do not co-express kisspeptin, which is much more prominent in the ARC.
male rat. It is possible that the major function of the NKB-only neurons is to integrate in the KNDy neuronal network to ensure optimal coordination through NKB-NKB receptor signaling. Indeed, the lack of coexpression of kisspeptin and NKB in the very same cells does not invalidate the concept that NKB signaling may modulate (in a paracrine manner) the release of kisspeptin from adjacent kisspeptin or KNDy neurons. Nevertheless, the projection of NKB-ir fibers to several areas other than the ARC itself also allows speculation of multiple additional functions for NKB eg., in homeostasis, behavior, and neuroendocrine regulation (50). Of note, the existence of NKB-only neurons in the ARC of males was documented by colocalization experiments, even in ORX rats, in which, due to the withdrawal of negative feedback, there is an increase in the number of kisspeptin-ir cells, without changes in the total number of the NKB cells.

Our study also documents a small fraction of cells in the ARC expressing kisspeptin but not NKB. Our data in rats, and a previous study in mice (48), demonstrate that kisspeptin-only neurons in the ARC exist in low numbers. The function of such a population awaits to be elucidated, but kisspeptin neurons project to several limbic structures (51) so that a role for this population other than its participation in the control of KNDy neuron function cannot be ruled out. Notably, the abundance (and eventual function) of this population is likely to vary across species. Thus, in agonadal male monkeys, the kisspeptin-only population seems to be rather significant (only 50% of kisspeptin-ir cells coexpress NKB) whereas an NKB-only population apparently does not exist (52). In contrast, in the goat there is an almost complete overlap of kisspeptin and NKB-ir cells (6).

Although, as stated above, the lack of coexpression of kisspeptin and NKB does not preclude the potential interplay of these neuropeptides in shaping GnRH pulses, as proposed by the KNDy neuron concept, our anatomical analyses highlight the neurochemical diversity of kisspeptin and NKB neuronal populations in the ARC. These findings are in line with recent reports in young men (14), in whom NKB-only and kisspeptin-only cells were identified. Such a condition might invalidate global assumptions derived from the consideration of the KNDy neuronal population as a homogenous set of neurons, expressing all the KNDy neuropeptides and receptors. For instance, based on this assumption, some conclusions on the roles of KNDy neurons have been inferred, when in fact only one KNDy peptide/mRNA/receptor was investigated (41, 50, 53, 54). This might lead to an overestimation of the real function of KNDy neurons. For instance, it is possible that it is the NKB-only population and not the KNDy neuron population that participates in the control of body temperature during menopause (53).

In conclusion, we provide here conclusive evidence for the differential regulation of kisspeptin and NKB by sex steroid manipulation, with kisspeptin-ir cells increasing in response to gonadectomy but NKB-ir cells raising in sex steroid replaced animals vs gonadectomized rats of both sexes; hence, in contrast to kisspeptin-ir cells, changes of NKB-ir cells do not parallel existing data on mRNA regulation. Furthermore, sex steroid replacement increases kisspeptin and NKB fiber density exclusively in females. Finally, we suggest the existence of a population of NKB-only neurons that is more prominent in the male and a population of neurons coexpressing kisspeptin and NKB, which is more pronounced in females. Collectively these findings reveal the complex (if not divergent) regulation of NKB and kisspeptin neuropeptides in the ARC, which may not fit perfectly well, and would help to refine, our current consensus view on the KNDy neuron model.

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