Behavioral/Systems/Cognitive

Regulation of Gonadotropin-Releasing Hormone Secretion by Kisspeptin/Dynorphin/Neurokinin B Neurons in the Arcuate Nucleus of the Mouse

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Kisspeptin is encoded by the *Kiss1* gene, and kisspeptin signaling plays a critical role in reproduction. In rodents, kisspeptin neurons in the arcuate nucleus (Arc) provide tonic drive to gonadotropin-releasing hormone (GnRH) neurons, which in turn supports basal luteinizing hormone (LH) secretion. Our objectives were to determine whether *preprodynorphin* (*Dyn*) and *neurokinin B* (*NKB*) are coexpressed in *Kiss1* neurons in the mouse and to evaluate its physiological significance. Using *in situ* hybridization, we found that *Kiss1* neurons in the Arc of female mice not only express the *Dyn* and *NKB* genes but also the *NKB receptor* gene (*NK3*) and the *Dyn receptor* [the κ opioid receptor (*KOR*)] gene. We also found that expression of the *Dyn*, *NKB*, *KOR*, and *NK3* in the Arc are inhibited by estradiol, as has been established for *Kiss1*, and confirmed that Dyn and NKB inhibit LH secretion. Moreover, using *Dyn* and *KOR* knock-out mice, we found that long-term disruption of *Dyn/KOR* signaling compromises the rise of LH after ovariectomy. We propose a model whereby NKB and dynorphin act autosynaptically on kisspeptin neurons in the Arc to synchronize and shape the pulsatile secretion of kisspeptin and drive the release of GnRH from fibers in the median eminence.

Introduction

Kisspeptin signaling plays a critical role in the neuroendocrine regulation of reproduction (Popa et al., 2008). Kisspeptin activates gonadotropin-releasing hormone (GnRH) neurons (Gottsch et al., 2004; Han et al., 2005), which are the final common pathway by which the brain regulates the gonadotropin secretion, and pulsatile secretion of kisspeptin and GnRH are temporally linked (Keen et al., 2008). The *Kiss1* gene encodes kisspeptin, which is expressed by neurons in the arcuate nucleus (Arc) of the hypothalamus (Smith et al., 2005a,b), the nodal point for controlling the negative feedback regulation of GnRH secretion by estradiol (E_2) and for generating pulsatile GnRH secretion (Freeman, 2005). *Kiss1* neurons in the Arc express estrogen receptor-α (ERα) (Smith et al., 2005b), which mediates negative feedback and the inhibition of *Kiss1* expression in the Arc (Smith et al., 2005b; Glidewell-Kenney et al., 2007). Thus, *Kiss1* neurons in the Arc may be conduits for receiving E_2 signaling from the gonad and relaying that information to GnRH neurons (Smith et al., 2005b). However, *Kiss1* neurons in the Arc also express several cotransmitters, which may have important, independent functions.

In the sheep, *Kiss1* neurons in the Arc coexpress dynorphin A (Dyn) and neurokinin B (NKB) (Goodman et al., 2007), and both Dyn and NKB have been implicated in the regulation of luteinizing hormone (LH) secretion. Dyn, which acts via the κ opioid receptor (KOR) (Chavkin et al., 1982), inhibits LH release (Schulz et al., 1981; Kinoshita et al., 1982), as does NKB (Sandoval-Guzmán and Rance, 2004). Moreover, disabling mutations of either *NKB* (known as *TAC3* in humans) or the *NKB type 3 receptor* gene (*NK3*) (known as *TAC3R* in humans) are associated with reproductive failure (Topaloglu et al., 2009). Thus, three neuropeptides are produced by a single population of cells in the Arc (in the sheep), and all three have been independently linked to GnRH secretion. However, the nature of the interaction among these cotransmitters and their physiological significance remains a mystery. We postulated that kisspeptin, Dyn, and NKB work together to generate episodic kisspeptin secretion, which drives the pulsatile secretion of GnRH, based on several lines of reasoning. First, in the rat, Dyn/NKB-containing fibers contact Dyn/NKB-containing soma in the Arc (Burke et al., 2006), suggesting that recurrent collaterals shape and synchronize periodic firing of *Kiss1/Dyn/NKB* neurons. Second, Dyn/NKB-containing fibers project from the Arc and terminate near GnRH fibers or terminals in the median eminence (ME) (Burke et al., 2006). Finally, GnRH neurons express both the kisspeptin receptor (*Kiss1r*) and NK3 (Irwig et al., 2004; Krajewski et al., 2005). The studies described here were designed to test this

Received April 1, 2009; revised June 6, 2009; accepted July 30, 2009.

This research was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development/National Institutes of Health (NICHD/NIH) through Cooperative Agreement U54 HD12629 (to the University of Washington Center for Research in Reproduction and Contraception), NICHD/NIH Grant R01 HD27142, National Institute on Drug Abuse/NIH Grants R01 DA016898 and KO5 DA020570, the Fulbright/MEC Program of the United States Department of State and Ministerio Español de Ciencia e Innovación, the Marie Curie Outgoing International Fellowship supported by the 7th Frame Programme of the European Union, and the Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN) of Japan. We are grateful for the technical assistance provided by Sarah McConkey, Sarah Ahmad, and Dan Messinger at the University of Washington (Seattle, WA) and Brigitte Mann at Northwestern University (Evanston, IL). We thank Drs. Martin Kelly and Oline Ronnekleiv at the Oregon Health and Science University (Portland, OR) for their constructive comments on a previous draft of this manuscript.

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DOI:10.1523/JNEUROSCI.1569-09.2009

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model. First, we evaluated whether *Dyn* and *NKB* are coexpressed in *Kiss1* neurons in the Arc of the mouse. Second, we determined whether *Kiss1* neurons express *KOR* and *NK3*. Third, we tested whether *Dyn* and *NKB* are targets for regulation by E_2, and fourth, we studied the effects of KOR and NK3 agonists and the impact of genetically targeted deletions of *Dyn* and *KOR* on GnRH/LH secretion.

Materials and Methods

Animals

Animals were housed at the University of Washington, and all procedures and surgeries were approved by the Animal Care Committee of the University of Washington School of Medicine in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Adult female C57BL/6 mice (8 weeks old) were purchased from The Jackson Laboratory. C57BL/6 mice with a *prodynorphin* (*Dyn*) gene deletion were generated as described previously (McLaughlin et al., 2003), and homozygous (−/−) and paired wild-type (+/+) littermate controls were generated by heterozygote crosses. Homozygous *KOR* gene deletion (−/−) mice (C57BL/6 background) were prepared by homologous recombination as described previously (Clarke et al., 2002) and provided for this study. Female *ERα* null mice (C57BL/6 background) were produced by breeding heterozygote pairs, each carrying a single copy of the disrupted *ERα* gene, as described previously (Lubahn et al., 1993; Smith et al., 2005b). Animals were housed in groups of three to five, maintained on a 14/10 light cycle with lights on at 4:00 A.M. and had access to standard rodent chow and water *ad libitum*.

Ovariectomy and steroid replacement

Ovariectomy (OVX) was performed on adult female mice through bilateral lumbar incisions, while maintaining the animals under isoflurane inhalation anesthesia (Abbott Laboratory) delivered by a vaporizer (Veterinary Anesthesia Systems). Vasculature to the ovary was sutured, and wound clips were used to close the incision. Immediately after OVX, oil-filled capsules (sham) or E_2 plus oil-filled capsules were implanted subcutaneously via a small midscapular incision at the base of the neck; wound clips were used to close the incision. For E_2 implants, SILASTIC tubing (inner diameter, 1.47 mm; outer diameter, 1.95 mm; Dow Corning) was cut to 9 mm; one end was sealed with silicone cement and allowed to cure overnight. Crystalline E_2 (Sigma) at a dose of 1 mg/ml was dissolved in safflower oil based on previous studies (Miller et al., 1995; Smith et al., 2005b; Dungan et al., 2007). After capsules were filled with E_2 in oil, the end of the capsule was sealed with silicone cement and allowed to cure overnight. The day before surgery, implants were washed two times for 10 min in changes of 100% ethanol and then placed in sterile physiological saline overnight.

Tissue preparation

Blood was centrifuged for 5 min (3000 rpm), and the serum was stored at −20°C until hormone measurements. 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 was used for *in situ* hybridization (adjacent sections 100 μm apart).

Radioimmunoassays

Serum levels of LH were measured at Northwestern University (Evanston, IL). Reagents for the LH assay were obtained from NIH. For LH, the antiserum used was anti-rLH–S-11, and the standard was rLH–RP3. The assay sensitivity was 0.2 ng/ml, and the intra-assay coefficient of variation was 4%.

Detection of Kiss1 *mRNA*

The Kiss1 probe used for detection of *Kiss1* mRNA was described previously (Gottsch et al., 2004). The *Kiss1*-specific sequence of the probe spans bases 76-486 of the mouse cDNA sequence (GenBank accession number AF472576). The procedure for ISH is outlined below.

Detection of Dyn *mRNA*

The Dyn probe used for detection of *Dyn* mRNA was described previously (Gottsch et al., 2009). The Dyn-specific sequence of the probe spans bases 132-540 of the mouse cDNA sequence (GenBank accession number NM_018863). The procedure for *in situ* hybridization is outlined below.

Detection of KOR, NKB, *and* NK3 *mRNAs*

Total RNA was extracted from mouse brain using an RNAqueous kit (Ambion). RNA was reverse transcribed into cDNA with a RetroScript kit (Ambion) primed with oligodeoxythymidine for subsequent PCR. Primers were designed based on the published sequence of the *KOR* mouse gene (GenBank accession number NM_011011) with forward primers starting at 127 bp and reverse primers starting at 964 bp, *NKB* mouse gene (GenBank accession number NM_009312) with forward primers starting at 177 bp and reverse primers starting at 440 bp, and *NK3* mouse gene (GenBank accession number NM_021382) with forward primers starting at 286 bp and reverse primers starting at 691 bp. Primers were custom synthesized (OPERON). PCR reactions contained the following in a volume of 25 μl:2 μl of reverse transcriptase reaction product, 0.2 μM of each primer, 12.5 μl of RediTaq polymerase (Sigma-Aldrich), and 8.5 μl of water. Reactions were performed in a PTC-100 thermal cycler (MJ Research) using the following protocol: cDNA was denatured for 5 min at 94°C, and then 35 cycles were performed at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min, with a final 5 min extension at 72°C. After electrophoresis on a 2% agarose (w/v) gel, a single DNA fragment was obtained of approximately the expected size and gel purified with a QiaQuick gel extraction kit (Qiagen). The PCR product was confirmed to be the mouse *KOR*, *NKB*, or *NK3* probe by sequencing. Clamp polymerase sequences for T7 or T3 polymerase were added for the final primer product sequence and transcribed for ISH.

Single-label in situ *hybridization of* Dyn *mRNA*

Dyn mRNA sense and antisense probes were transcribed with T7 or T3 polymerase (Fermentas) as described previously by Gottsch et al. (2009). Briefly, radiolabeled probes were synthesized *in vitro* by inclusion of the following ingredients in a volume of 20 μl:250 μCi [^{33}P]UTP (PerkinElmer Life and Analytical Sciences), 1 μg of linearized DNA (or 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 (Cunningham et al., 2002; Gottsch et al., 2004).

Double-label in situ *hybridization*

Antisense mouse *Kiss1* probe was transcribed from linearized pAMP1 plasmid containing the mouse *Kiss1* insert with T7 polymerase (Fermentas) (Gottsch et al., 2004). The cDNA template for the *Dyn*, *KOR*, *NKB*, and *NK3* riboprobes were generated by PCR with primers that were designed to contain promoters for T7 RNA polymerase in the antisense direction and T3 RNA polymerase in the sense direction. Radiolabeled riboprobes for *KOR*, *NKB*, and *NK3* were synthesized as described above for the *Dyn* riboprobe. Digoxigenin (DIG)-labeled *Kiss1* antisense riboprobe was synthesized with T7 RNA polymerase and DIG labeling mix (Roche) according to the instructions of the manufacturer. Slides were processed for double-labeled *in situ* hybridization as described previously (Irwig et al., 2004). Slides were stored at 4°C and developed 8–12 d later.

Quantification and analysis of Kiss1, Dyn, KOR, NKB, *and* NK3 *mRNAs*

The brain sections were analyzed bilaterally unless otherwise specified. Slides from all of the animals were assigned a random three-letter code, alphabetized, 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 Dyn, *KOR*, *NKB*, or *NK3* mRNA) over each cell (Chowen et al., 1990). *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 depending on which radiolabeled probe was used. The number of cells reported for each experiment represents the number of cells within the coronal sections containing the Arc nucleus for each set, not the total number of cells in the Arc. The starting and ending point of quantification was determined according to Paxinos and Franklin (2001). Signal-to-background ratios (SBRs) for individual cells were calculated; an individual cell was considered to be double labeled if it had an SBR of three or more. For each animal, the number of double-labeled cells was calculated as a percentage of the total number of *Kiss1* mRNA-positive cells and then averaged across animals to produce a mean ± SEM.

Statistical analysis

All data are expressed as the mean ± SEM for each group. One-way ANOVA was used to assess variation among experimental groups in each experiment. Significance level was set at $p < 0.05$. All analyses were performed with Statview 5.0.1 for Macintosh (SAS Institute).

Experimental design

Experiment 1: coexpression of Kiss1/Dyn *mRNAs in the forebrain.* The purpose of this experiment was to determine whether *Kiss1* neurons in the mouse female forebrain [Arc and anteroventral periventricular nucleus (AVPV)] coexpress *Dyn* mRNA. Wild-type (WT) mice were divided into two groups ($n = 5$ per group): OVX + sham and OVX + E_2 replacement. At approximately 9:00 A.M. on 7 d after castration, mice were anesthetized with isoflurane, blood was collected for LH radioimmunoassays by retro-orbital bleeding to confirm gonadectomy, and mice were killed by decapitation. Brains were collected as described above.

Experiment 2: regulation of Dyn *mRNA by E_2.* The purpose of this experiment was to analyze the regulation of *Dyn* mRNA by E_2 and determine whether E_2 can regulate this expression in *ERα* knock-out (KO) female mice. Seven female *ERα* KO mice and seven WT littermates were ovariectomized, and four from each group received E_2 replacement. Tissue collection and preparation for *Dyn* mRNA *in situ* hybridization was performed as described in experiment 1.

Experiment 3: coexpression of Kiss1/KOR *mRNAs in the Arc.* The purpose of this experiment was to determine whether *Kiss1* and *KOR* are coexpressed in cells in the Arc and to assess the possible regulation of *KOR* mRNA by E_2. We performed double-labeled *in situ* hybridization on a set of coronal sections from brains of OVX + sham and OVX + E_2 adult female mice ($n = 8$ per group), generated as described above. *Kiss1* mRNA was visualized via DIG-labeled riboprobes.

Experiment 4: coexpression of Kiss1/NKB/NK3 *mRNAs in the AVPV and the Arc.* The purpose of this experiment was to determine whether *Kiss1*, *NKB*, and *NK3* are coexpressed in cells in the Arc (as well as the AVPV for *Kiss1/NKB*) and to assess their possible regulation by E_2. We performed double-labeled *in situ* hybridization on a set of coronal sections from brains used in the previous experiment (OVX + sham and OVX + E_2 mice; $n = 8$ per group). *Kiss1* mRNA was visualized by the use of DIG-labeled riboprobes.

Experiment 5: postcastration LH response in adult female Dyn *KO and* KOR *KO mice.* The purpose of this experiment was to determine the effect of long-term deficiency of either *Dyn* or *KOR* in controlling the GnRH/LH response to reduced circulating levels of E_2. Ten adult female *Dyn* KO mice, 8 adult female *KOR* KO mice, and 10 adult WT littermates (between 8 and 12 weeks of age) were ovariectomized, and blood was collected after 7 d as described in experiment 1 (for LH determinations). This experiment showed a reduced rise in serum levels of LH after OVX in *Dyn* KO and *KOR* KOs compared with their respective WT controls. To investigate whether this phenomenon might be attributable to a decrease in kisspeptin drive to GnRH neurons, WT and *Dyn* KO animals (five adult female mice for each group) were subjected to the same protocol described above. At 1 week after OVX, brain levels of *Kiss1* mRNA were measured and compared between genotypes by quantitative ISH (after confirming that the postcastration rise in LH was compromised in the *Dyn* KOs).

Experiment 6: response of LH serum levels to U50488. The purpose of this experiment was to assess the effect of the KOR agonist U50488 [(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-1-pyrrolidinyl)-cyclo-hexyl] benzeneacetamide] (Tocris Biosciences) on serum LH levels in adult (8 weeks old) female WT mice, in the presence and absence of E_2. Animals were ovariectomized as described above ($n = 10$ per group) and allowed to recover for 7 d. Each group received an intraperitoneal injection of vehicle (0.9% NaCl) or U50488 (5 $mg \cdot kg^{-1} \cdot ml^{-1}$). After 30 min, animals were bled and serum was collected as described in experiment 1. In addition, adult WT OVX mice were implanted with E_2-containing capsules and treated intraperitoneally with vehicle or U50488 ($n = 10$ per group) following the same protocol.

Experiment 7: LH response to NK3 agonist. The purpose of this experiment was to assess the effect of the NK3 agonist senktide (Sigma-Aldrich) on LH secretion in adult (8 weeks old) female WT mice, in the presence and absence of E_2. Animals were ovariectomized and implanted with empty capsules (OVX + sham) or E_2 capsules (OVX + E_2) as described above ($n = 7$–10 per group). Each group was equally divided and received an intracerebroventricular injection (Gottsch et al., 2004) of vehicle (0.9% NaCl) or senktide (600 pmol/3 μl) as described previously (Sandoval-Guzmán and Rance, 2004). After 30 min, the animals were bled and serum was collected as described in experiment 1.

Results

Coexpression of *Kiss1/Dyn* genes and the regulation of *Dyn* gene by E_2

The percentage of *Kiss1* neurons expressing *Dyn* in OVX mice with and without E_2 treatment was assessed by double-labeled ISH. In the Arc, nearly all (92%) of the *Kiss1* neurons coexpressed *Dyn* (Fig. 1*A*). *Dyn* was also found in some *Kiss1*-containing cells in the AVPV, but the extent of coexpression was only 33%. Levels of *Dyn* mRNA in *Kiss1* cells of the AVPV—as indicated by the number of silver grains per cell—was low, amounting to <10% of that found in *Kiss1* cells of the Arc (OVX + sham AVPV, <2 gpc; OVX + E_2 AVPV, <2 gpc; OVX + sham Arc, 15 ± 2 gpc; OVX + E_2 Arc, 17 ± 2 gpc). E_2 treatment did not affect the percentage of *Dyn* coexpressing *Kiss1* cells in either the Arc or AVPV.

Single-labeled ISH was used to evaluate the ability of E_2 to regulate *Dyn* expression in adult WT and *ERα* KO mice. As shown in Figure 1*B*, the number of *Dyn* mRNA-containing cells in the Arc was high in OVX WT mice (574 ± 27), and E_2 treatment reduced the number of cells by ~80% (112 ± 21; $p < 0.01$). E_2 also reduced the per cell content of *Dyn* mRNA in the Arc (OVX + sham, 125 ± 4 gpc vs OVX + E_2, 44 ± 4 gpc; $p < 0.01$). *Dyn* expression in the Arc of *ERα* KO mice was unaffected by E_2 treatment (Fig. 1*B*), both in terms of cell numbers (*ERα* KO OVX + sham, 365 ± 62 cells vs *ERα* KO OVX + E_2, 330 ± 122 cells; $p = 0.17$) and gpc (*ERα* KO OVX + sham, 181 ± 13 gpc vs *ERα* KO OVX + E_2, 133 ± 22 gpc; $p = 0.66$).

Coexpression of *Kiss1/KOR* genes and regulation of *KOR* by E_2

The coexpression of *KOR* mRNA in *Kiss1* neurons of OVX mice with and without E_2 was also determined by double-labeled ISH. *KOR* mRNA was found in ~20% of the *Kiss1* neurons in the Arc, regardless of E_2 treatment. Although this would seem to be a relatively small fraction of the total number of *Kiss1* cells, it is significant and may reflect the relatively weak limits of detectability of the *KOR* riboprobe. Using the single-labeled approach, we found that E_2 inhibited the expression of *KOR* in cells inside the Arc, presumably including some that do not contain *Kiss1* (OVX + sham, 57 ± 11 cells vs OVX + E_2, 19 ± 4 cells; $p < 0.02$) (Fig.

2). There were several hypothalamic areas that showed a greater expression of *KOR* than the Arc, including the ventromedial nucleus, dorsomedial nucleus, and the caudal part of the periventricular nucleus (data not shown).

Coexpression of *Kiss1*, *NKB*, and *NK3* genes and regulation by E_2

In the Arc, double-labeled ISH revealed that ~90% of the *Kiss1* neurons of OVX mice also expressed *NKB* mRNA (regardless of E_2 treatment) (Fig. 3*A*). Some cells in the Arc that contained *NKB* did not colabel with *Kiss1* (data not shown). Only ~10% of the *Kiss1* neurons in the AVPV coexpressed *NKB*, and the level of *NKB* expression in those cells was much less than in the Arc (OVX + sham AVPV, <1 gpc; OVX + E_2 AVPV, <1 gpc; OVX + sham Arc, 28 ± 5 gpc; OVX + E_2 Arc, 30 ± 35 gpc). It has been reported previously that *NKB* in the Arc is regulated by E_2 (Danzer et al., 1999; Goubillon et al., 2000; Pillon et al., 2003; Dellovade and Merchenthaler, 2004). In the present work, we studied the effect of E_2 specifically in *Kiss1/NKB* colabeled neurons. We found that E_2 replacement decreased the population of these neurons in the Arc of OVX animals by 53% when compared with the OVX + sham group (OVX + sham, 308 ± 57 vs 145 ± 18 cells; $p = 0.027$) (Fig. 3*C*).

We also found that virtually all (96%) of the *Kiss1* neurons in the Arc of OVX mice coexpressed high levels of *NK3* mRNA in the absence of E_2 (Fig. 3*B*). Within the Arc, *NK3* appeared to be expressed exclusively in *Kiss1* neurons (data not shown). In the presence of E_2, there were too few detectable *Kiss1*-expressing cells to determine the degree of *NK3* mRNA coexpression. In any case, there were no visible clusters of silver grains in the Arc of E_2-treated animals, suggesting that *NK3* mRNA was profoundly suppressed by E_2, as was the case for *Kiss1* mRNA in this region.

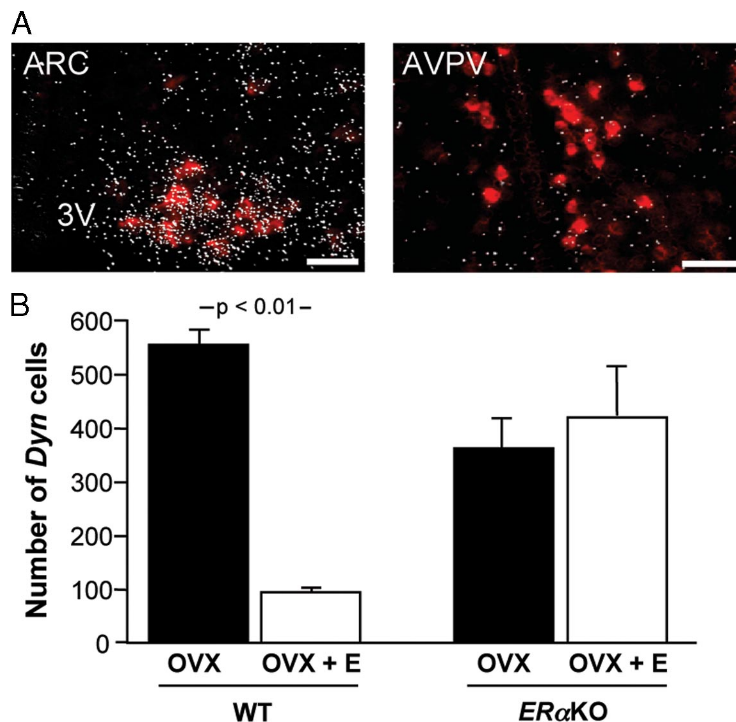

Figure 1. ***A***, Representative photomicrographs showing coexpression of *Kiss1* mRNA with *Dyn* in the Arc and AVPV of the female mouse. *Kiss1* mRNA-expressing cells are fluorescent with Vector Red substrate, and clusters of silver grains reflect the presence of *Dyn* mRNA. Scale bars, 50 μm. ***B***, Effect of E_2 replacement on the number of *Dyn*-expressing cells in coronal sections of the Arc in OVX *ERα* KO and WT controls. Data are presented as the mean ± SEM.

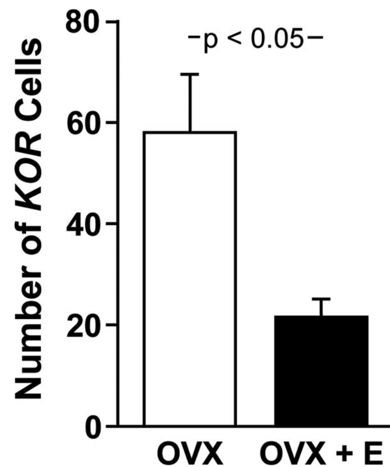

Figure 2. Effect of E_2 replacement on the number of *KOR*-expressing cells in coronal sections of the Arc in OVX WT mice. Data are presented as the mean ± SEM.

Effect of a long-term deficit of *Dyn/KOR* signaling on the LH response to OVX

Serum LH levels in adult WT, *Dyn* KO, and *KOR* KO female mice were elevated 7 d after OVX compared with those in diestrus animals. However, the post-OVX LH levels observed in both *Dyn* KO and *KOR* KO mice were only 37 and 51% of the LH level in the WT group (WT, 1.95 ± 0.3 ng/ml; *Dyn* KO, 0.73 ± 0.09 ng/ml; $p < 0.01$; *KOR* KO, 1.0 ± 0.3 ng/ml; $p < 0.05$) (Fig. 4). In OVX mice treated with E_2, LH levels were not significantly different between WT and *Dyn* KO (WT, 0.202 ± 0.001 ng/ml vs *Dyn* KO, 0.27 ± 0.04 ng/ml; $p = 0.29$).

To determine whether reduced expression of *Kiss1* in *Dyn* KOs might account for their reduced LH response to OVX, we compared levels of *Kiss1* mRNA between WT and *Dyn* KOs, following the same protocol used to assess serum LH levels. We confirmed that, 7 d after OVX, *Dyn* KOs had reduced LH levels compared with WTs (WT, 1.93 ± 0.34 ng/ml vs *Dyn* KO, 0.92 ± 0.26 ng/ml; $p < 0.05$). Furthermore, we found no significant difference in *Kiss1* mRNA between genotypes (WT, 59.2 ± 18.17 cells vs *Dyn* KO, 65.0 ± 15.93 cells; $p = 0.81$).

Action of KOR and NK3 agonists on LH secretion

Although it has been shown in rats that KOR and NK3 agonists inhibit LH release, this has not been demonstrated in the mouse. In OVX (7 d) female mice, we found that administration of either the KOR agonist U50488 or the NK3 agonist senktide reduced serum LH levels after 30 min (vehicle + OVX, 1.92 ± 0.27 ng/ml vs U50488 + OVX, 0.88 ± 0.29 ng/ml; $p < 0.01$; vehicle + OVX, 1.60 ± 0.53 ng/ml vs senktide + OVX, 0.81 ± 0.17 ng/ml; $p < 0.01$) (Fig. 5). When this study was repeated in E_2-treated OVX mice, serum levels of LH were exceedingly low (at or near the

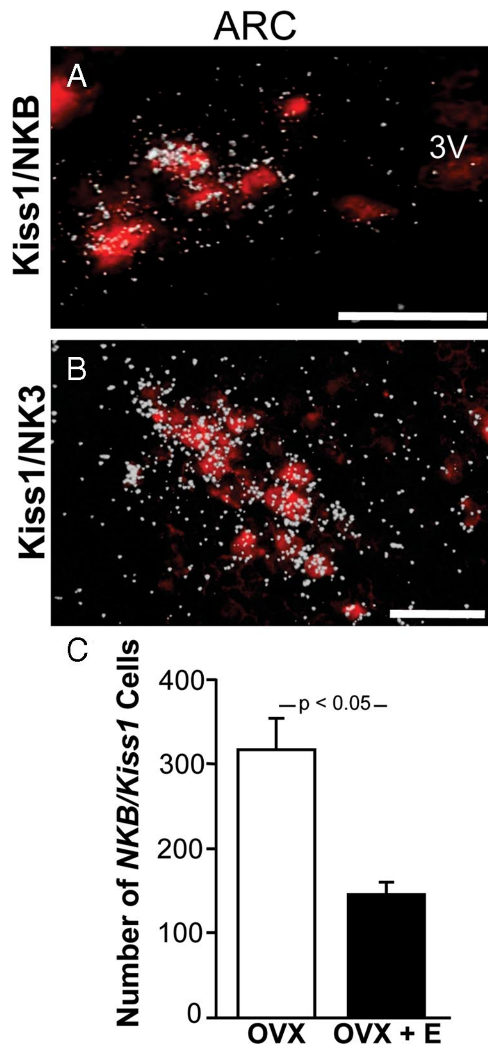

Figure 3. Representative photomicrographs showing coexpression of *Kiss1* mRNA with *NKB* (***A***) and *NK3* (***B***) in the Arc of the female mouse. *Kiss1* mRNA-expressing cells are fluorescent with Vector Red substrate, and clusters of silver grains reflect the presence of *NKB* mRNA (***A***) or *NK3* mRNA (***B***). Scale bars, 50 μm. Effect of E_2 replacement on the number of *Kiss1/NKB* cells in coronal sections of the Arc in OVX WT mice. Data are presented as the mean ± SEM (***C***).

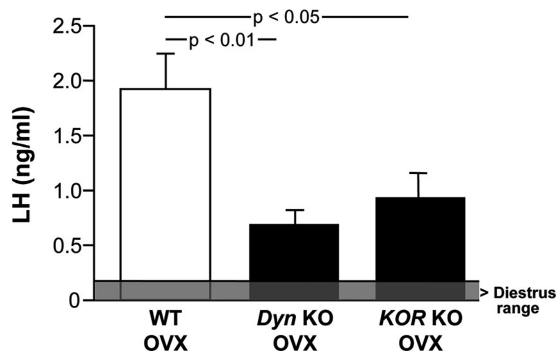

Figure 4. Serum LH levels in adult female WT, *Dyn* KO, and *KOR* KO mice 7 d after OVX compared with diestrus LH levels. Data are presented as the mean ± SEM.

minimum detectable limits of the assay) and were indistinguishable among groups, regardless of treatment (vehicle + OVX + E_2, 0.28 ± 0.09 ng/ml vs U50488 + OVX + E_2, 0.24 ± 0.03 ng/ml; senktide + OVX + E_2, 0.20 ± 0.0 ng/ml) (Fig. 5).

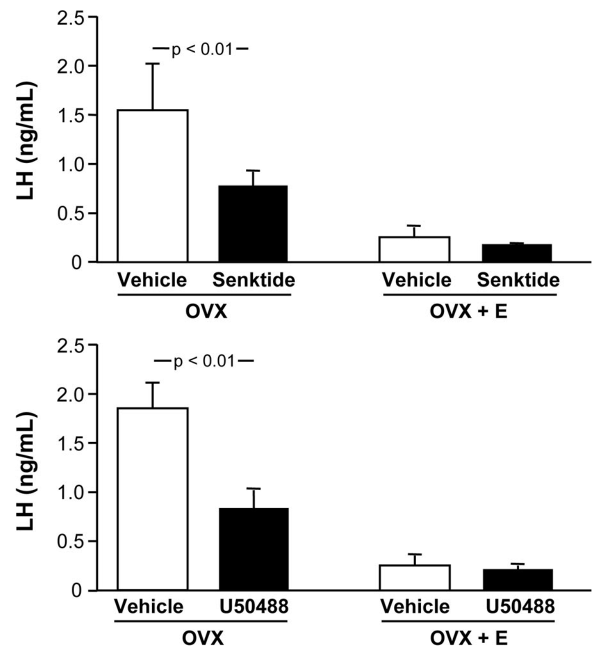

Figure 5. Serum LH levels in female WT mice (OVX + sham and OVX + E_2) 30 min after treatment with vehicle or senktide (top), and vehicle or U50488 (bottom). Data are presented as the mean ± SEM.

Discussion

Based on evidence from this and previous studies, we have proposed that *Kiss1* neurons in the Arc of the mouse participate in the generation and regulation of pulsatile kisspeptin release that drives ultradian GnRH release (Fig. 6). Central to this model is the coexpression of *Dyn* and *NKB* in *Kiss1* neurons of the Arc, which has been reported previously to occur in sheep (Goodman et al., 2007). Here, we show that this is also the case in the mouse, suggesting that the presence of *Kiss1*/*Dyn*/*NKB* neurons of the Arc may be common across diverse species. Furthermore, because we found that most of the cells containing *Dyn* and/or *NKB* in the Arc are *Kiss1* neurons, we can reasonably infer that previous descriptions of the distribution of kisspeptin-, Dyn-, and NKB-containing fibers in the Arc also describe the distribution of fibers from *Kiss1/Dyn/NKB* neurons, which should contain all three neuropeptides. Using immunohistochemistry coupled with confocal microscopy, Burke et al. (2006) found that a plexus of axons and terminals containing Dyn and NKB surround and abut Dyn/NKB cell bodies in the Arc of the rat. These observations suggest that *Kiss1/Dyn/NKB* neurons are interconnected via recurrent collaterals, as shown in Figure 6. In addition, Dyn/NKB fibers project from the Arc to the ME in the rat, sheep, and horse (Burke et al., 2006; Foradori et al., 2006; Decourt et al., 2008), and in the monkey kisspeptin fibers have been shown to have extensive and intimate associations with GnRH axons in the ME (Ramaswamy et al., 2008). Thus, we deduce that axons from *Kiss1/Dyn/NKB* soma in the Arc of the mouse project to one another and to the ME in the vicinity of GnRH fibers and terminals.

Presumably, kisspeptin, Dyn, and NKB are all available to act as cotransmitters or neuromodulators at the targets of projections from *Kiss1/Dyn/NKB* soma in the Arc, and any specific action would occur as a function of the presence (or absence) of Kiss1r, KOR, and NK3 in those potential targets. Remarkably, the targets of the recurrent collaterals appear to be *Kiss1/Dyn/NKB* cells themselves. Dyn cells in the Arc express NK3 (Burke et al., 2006),

and our results confirm that *Kiss1*-expressing cells coexpress *NK3*, implying that the recurrent collaterals could signal through an NKB/NK3 signaling pathway. In addition, *KOR* is expressed in *Kiss1* neurons (albeit at low levels), so it is plausible that Dyn/KOR signaling is also involved in communication through these same recurrent collaterals (or conceivably through interneurons). *Kiss1r* mRNA does not appear to be expressed in the Arc (d'Anglemont de Tassigny et al., 2008); thus, *Kiss1/Dyn/NKB* cell bodies in this region are unlikely to be targets for the direct action of kisspeptin. However, the kisspeptin receptor itself (as opposed to its mRNA) is concentrated in the Arc/ME region—most likely in GnRH terminals—based on the observation that kisspeptin can elicit GnRH secretion from explants of the mediobasal hypothalamus, which contains few, if any, GnRH cells bodies (Irwig et al., 2004; d'Anglemont de Tassigny et al., 2008). Thus, it seems reasonable to infer that Kiss1r appears in the ME through axonal transport, originating from sites of production in GnRH cell bodies in the medial preoptic area (Irwig et al., 2004). GnRH fibers and terminals in the ME also contain NK3 (Burke et al., 2006), suggesting that GnRH fibers and terminals are sites of action for both kisspeptin and NKB, but not dynorphin, because GnRH neurons apparently do not express KOR (Mitchell et al., 1997; Sannella and Petersen, 1997).

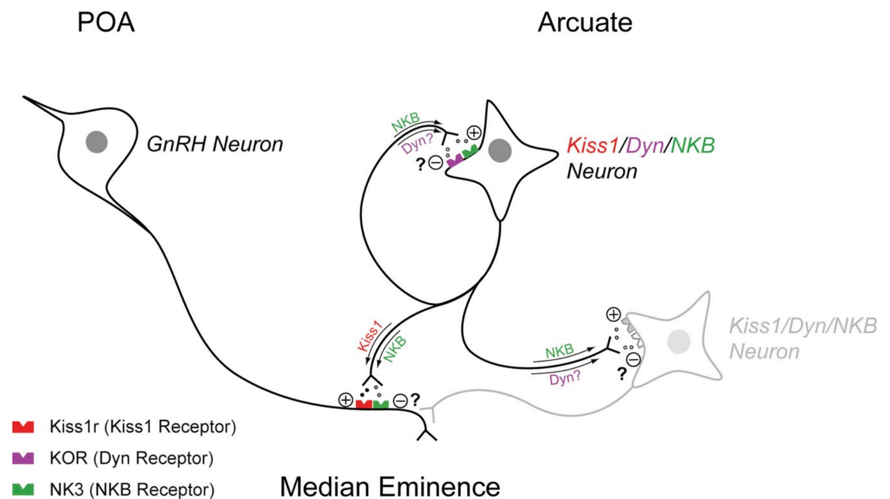

Figure 6. Schematic representation of the role of *Kiss1/Dyn/NKB* neurons in the generation of the GnRH pulses. According to this model, *Kiss1/Dyn/NKB* neurons receive autosynaptic input from NKB and Dyn and target GnRH fibers in the ME, which are responsive to kisspeptin and NKB (through Kiss1r and NK3, respectively). When E_2 levels decline, *Kiss1/Dyn/NKB* neurons become spontaneously active. This activity would be amplified by positive autosynaptic feedback through NKB/NK3 signaling, which would also propagate by collaterals to trigger synchronized firing in the ensemble of *Kiss1/DYN/NKB* neurons in the Arc (as represented by the faded neuron in the diagram). DYN, which would be released along with NKB, would act on *Kiss1/DYN/NKB* neurons (or interneurons that express KOR) with a small phase lag to clamp further discharge from *Kiss1/Dyn/NKB* neurons and thus extinguish their activity. In the absence of additional activity, Dyn release would cease, and eventually the inhibitory effect of Dyn would wane, causing the *Kiss1/Dyn/NKB* neurons to reactivate and initiate another cycle of regenerative activity, followed by inhibition. Each time *Kiss1/Dyn/NKB* neurons would undergo a burst of activity, a "pulse" of kisspeptin, Dyn, and NKB would be released in the ME, where kisspeptin and NKB would act directly on GnRH fibers or terminals. It is unlikely that Dyn acts directly on GnRH neurons, because GnRH neurons apparently do not express KOR. Kisspeptin would evoke prolonged activation of GnRH fibers or terminals, which, if unchecked, would last for hours. Thus, some mechanism must subsequently inactivate GnRH neurons so that a discrete pulse of GnRH can be delivered into the portal circulation. We postulate that NKB acts via NK3 on GnRH fibers or terminals to accomplish this task. Although this model is consistent with the results presented here, as well as the published observations of others, its validity requires additional critical investigation.

Based on what is known about kisspeptin, Dyn, and NKB signaling, we can make some predictions about their roles in this model. Assuming that NKB stimulates and Dyn inhibits *Kiss1/Dyn/NKB* neurons, the recurrent collaterals constitute a potentially oscillatory feedback loop. Any spontaneous activity in the *Kiss1/Dyn/NKB* cells would be amplified through regenerative feedback, involving NKB/NK3 signaling. Through interconnections between *Kiss1/Dyn/NKB* cells, regenerative activity would propagate throughout the entire population of cells, and, as a result, a burst of kisspeptin, Dyn, and NKB would be delivered to the GnRH terminals in the ME. This would be followed by a delayed inhibition of *Kiss1/Dyn/NKB* cells, mediated by Dyn acting through KOR. As this subsides, the cells would become active, restarting the entire process. The kisspeptin released near GnRH terminals would bind to Kiss1r and initiate prolonged volleys of action potentials, and thus produce sustained GnRH secretion (Han et al., 2005; Pielecka-Fortuna et al., 2008; Zhang et al., 2008). To limit the release of GnRH to a discrete pulse, the activity induced by kisspeptin must somehow be extinguished. Although this could be accomplished by an unidentified inhibitory neurotransmitter, it could also occur through NKB binding to NK3 and activating a delayed inhibitory signaling pathway, as indicated in Figure 6.

If *Kiss1/Dyn/NKB* neurons participate in the negative feedback regulation of GnRH/LH secretion, it would seem (at first glance) that E_2 should induce *Dyn* expression, because Dyn is an inhibitory factor. Nevertheless, we found that E_2 suppressed levels of *Dyn* mRNA, consistent with previous results (Spampinato et al., 1995). This paradoxical observation can be readily explained by the model in Figure 6. Accordingly, the GnRH pulse generator is most active when E_2 levels are low (to drive GnRH and LH secretion). Under these conditions, there is a demand for all three neurotransmitters (kisspeptin, Dyn, and NKB) and their receptors, as reflected by increases in the expression of their respective mRNAs. Not only was the expression of *Dyn*, *KOR*, and *NK3* in the Arc high when E_2 levels were low, but so too was the expression of *NKB* and *Kiss1*, confirming previous reports (Rance and Bruce, 1994; Dellovade and Merchenthaler, 2004; Smith et al., 2005b).

The model would also predict that exogenously administered Dyn or a Dyn agonist would suppress *Kiss1/Dny/NKB* neurons in the Arc, thus reducing drive to GnRH and LH secretion. In accord with this prediction, we found that the Dyn agonist U50488 inhibited LH levels, confirming previous observations in rats (Schulz et al., 1981; Kinoshita et al., 1982). We would also forecast that a permanent disruption of Dyn/KOR signaling would make the *Kiss1/Dyn/NKB* neurons continuously active, causing a desensitization of the kisspeptin → GnRH → LH cascade, resulting in low LH levels. Indeed, we observed reduced levels of LH in both *Dyn* KO and *KOR* KO mice, whereas *Kiss1* expression remained unaltered. Furthermore, the model predicts that NKB, acting through NK3, activates *Kiss1/Dyn/NKB* cells in the Arc but inhibits GnRH fibers and terminals in the ME. Thus, the administration of the NKB agonist senktide would be expected to activate *Kiss1/Dyn/NKB* neurons but also block GnRH (and thus LH) secretion. Consistent with this expectation, senktide inhib-

ited LH release in our mice, as it has been shown previously to do in rats (Kalra et al., 1992; Sandoval-Guzmán and Rance, 2004).

The results of this study accentuate the phenotypic differences between the two major *Kiss1*-expressing populations in brain located in the Arc and the AVPV. We have shown previously that, although both populations express ERα (Smith et al., 2005b), E_2 acts via an estrogen response element (ERE)-independent mechanism to inhibit *Kiss1* expression in the Arc and through an ERE-dependent mechanism to induce *Kiss1* expression in the AVPV (Gottsch et al., 2009). Here, we have shown that *Kiss1* neurons in the AVPV express virtually no *NKB* and much less *Dyn* compared with those in the Arc. These phenotypic differences likely reflect the different physiological roles these neurons play in the regulation of gonadotropin secretion. In the Arc, *Kiss1* cells stimulate basal GnRH/LH release and are negatively regulated by E_2, whereas in the AVPV, they are induced by E_2 and participate in generating the preovulatory GnRH/LH surge.

In conclusion, we report that kisspeptin-expressing neurons in the Arc of the mouse coexpress *Dyn* and *NKB*, as well as *KOR* and *NK3*, and that these neuropeptides and their receptors are targets for regulation by E_2. We also demonstrate that pharmacological activation of Dyn and NKB signaling disrupts LH secretion and that animals bearing genetically targeted deletions of *Dyn* and *KOR* have compromised abilities to regulate GnRH secretion. We present a model whereby kisspeptin, Dyn, and NKB act autosynaptically on kisspeptin neurons in the Arc to shape the pulsatile secretion of kisspeptin and hence GnRH release. This model is consistent with the present observations and with previous reports that bursts of multiple unit activity (MUA) in the Arc of monkeys, rats, and goats are coincident with LH pulses (for review, see Nishihara et al., 1991). Nevertheless, direct testing of this model with regard to GnRH/LH pulse generation remains to be performed in a species better suited for serial blood sampling and the measurement of MUA. Details of this model will evolve as we learn more about the specific actions of Dyn and NKB on *Kiss1/Dyn/NKB* neurons in the Arc and GnRH fibers and terminals in the ME.

References

Burke MC, Letts PA, Krajewski SJ, Rance NE (2006) Coexpression of dynorphin and neurokinin B immunoreactivity in the rat hypothalamus: morphologic evidence of interrelated function within the arcuate nucleus. J Comp Neurol 498:712–726.

Chavkin C, James IF, Goldstein A (1982) Dynorphin is a specific endogenous ligand of the kappa opioid receptor. Science 215:413–415.

Chowen JA, Argente J, Vician L, Clifton DK, Steiner RA (1990) Proopiomelanocortin messenger RNA in hypothalamic neurons is increased by testosterone through aromatization to estradiol. Neuroendocrinology 52:581–588.

Clarke S, Czyzyk T, Ansonoff M, Nitsche JF, Hsu MS, Nilsson L, Larsson K, Borsodi A, Toth G, Hill R, Kitchen I, Pintar JE (2002) Autoradiography of opioid and ORL1 ligands in opioid receptor triple knockout mice. Eur J Neurosci 16:1705–1712.

Cunningham MJ, Scarlett JM, Steiner RA (2002) Cloning and distribution of galanin-like peptide mRNA in the hypothalamus and pituitary of the macaque. Endocrinology 143:755–763.

d'Anglemont de Tassigny X, Fagg LA, Carlton MB, Colledge WH (2008) Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. Endocrinology 149:3926–3932.

Danzer SC, Price RO, McMullen NT, Rance NE (1999) Sex steroid modulation of neurokinin B gene expression in the arcuate nucleus of adult male rats. Brain Res Mol Brain Res 66:200–204.

Decourt C, Tillet Y, Caraty A, Franceschini I, Briant C (2008) Kisspeptin immunoreactive neurons in the equine hypothalamus Interactions with GnRH neuronal system. J Chem Neuroanat 36:131–137.

Dellovade TL, Merchenthaler I (2004) Estrogen regulation of neurokinin B gene expression in the mouse arcuate nucleus is mediated by estrogen receptor alpha. Endocrinology 145:736–742.

Dungan HM, Gottsch ML, Zeng H, Gragerov A, Bergmann JE, Vassilatis DK, Clifton DK, Steiner RA (2007) The role of kisspeptin-GPR54 signaling in the tonic regulation and surge release of gonadotropin-releasing hormone/luteinizing hormone. J Neurosci 27:12088–12095.

Foradori CD, Amstalden M, Goodman RL, Lehman MN (2006) Colocalisation of dynorphin a and neurokinin B immunoreactivity in the arcuate nucleus and median eminence of the sheep. J Neuroendocrinol 18:534–541.

Freeman ME (2005) The neuroendocrine control of the ovarian cycle of the rat. In: Physiology of reproduction (Knobil E, Neill JD, eds). New York: Raven.

Glidewell-Kenney C, Hurley LA, Pfaff L, Weiss J, Levine JE, Jameson JL (2007) Nonclassical estrogen receptor alpha signaling mediates negative feedback in the female mouse reproductive axis. Proc Natl Acad Sci U S A 104:8173–8177.

Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CV, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ (2007) Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology 148:5752–5760.

Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA (2004) A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145:4073–4077.

Gottsch ML, Navarro VM, Zhao Z, Glidewell-Kenney C, Weiss J, Jameson JL, Clifton DK, Levine JE, Steiner RA (2009) Regulation of Kiss1 and Dynorphin gene expression in the brain by classical and nonclassical estrogen receptor pathways. J Neurosci 29:9390–9395.

Goubillon ML, Forsdike RA, Robinson JE, Ciofi P, Caraty A, Herbison AE (2000) Identification of neurokinin B-expressing neurons as an highly estrogen-receptive, sexually dimorphic cell group in the ovine arcuate nucleus. Endocrinology 141:4218–4225.

Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, Clifton DK, Steiner RA, Herbison AE (2005) Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci 25:11349–11356.

Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA (2004) Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 80:264–272.

Kalra PS, Sahu A, Bonavera JJ, Kalra SP (1992) Diverse effects of tachykinins on luteinizing hormone release in male rats: mechanism of action. Endocrinology 131:1195–1201.

Keen KL, Wegner FH, Bloom SR, Ghatei MA, Terasawa E (2008) An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys in vivo. Endocrinology 149:4151–4157.

Kinoshita F, Nakai Y, Katakami H, Imura H (1982) Suppressive effect of dynorphin-(1–13) on luteinizing hormone release in conscious castrated rats. Life Sci 30:1915–1919.

Krajewski SJ, Anderson MJ, Iles-Shih L, Chen KJ, Urbanski HF, Rance NE (2005) Morphologic evidence that neurokinin B modulates gonadotropin-releasing hormone secretion via neurokinin 3 receptors in the rat median eminence. J Comp Neurol 489:372–386.

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O (1993) Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A 90:11162–11166.

McLaughlin JP, Marton-Popovici M, Chavkin C (2003) κ opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. J Neurosci 23:5674–5683.

Miller GM, Silverman AJ, Rogers MC, Gibson MJ (1995) Neuromodulation of transplanted gonadotropin-releasing hormone neurons in male and female hypogonadal mice with preoptic area brain grafts. Biol Reprod 52:572–583.

Mitchell V, Prevot V, Jennes L, Aubert JP, Croix D, Beauvillain JC (1997) Presence of mu and kappa opioid receptor mRNAs in galanin but not in GnRH neurons in the female rat. Neuroreport 8:3167–3172.

Nishihara M, Hiruma H, Kimura F (1991) Interactions between the noradrenergic and opioid peptidergic systems in controlling the electrical activ-

ity of luteinizing hormone-releasing hormone pulse generator in ovariectomized rats. Neuroendocrinology 54:321–326.

Paxinos G, Franklin K (2001) The mouse brain in stereotaxic coordinates. San Diego: Academic.

Pielecka-Fortuna J, Chu Z, Moenter SM (2008) Kisspeptin acts directly and indirectly to increase gonadotropin-releasing hormone neuron activity and its effects are modulated by estradiol. Endocrinology 149:1979–1986.

Pillon D, Caraty A, Fabre-Nys C, Bruneau G (2003) Short-term effect of oestradiol on neurokinin B mRNA expression in the infundibular nucleus of ewes. J Neuroendocrinol 15:749–753.

Popa SM, Clifton DK, Steiner RA (2008) The role of kisspeptins and GPR54 in the neuroendocrine regulation of reproduction. Annu Rev Physiol 70:213–238.

Ramaswamy S, Guerriero KA, Gibbs RB, Plant TM (2008) Structural interactions between kisspeptin and GnRH neurons in the mediobasal hypothalamus of the male rhesus monkey (*Macaca mulatta*) as revealed by double immunofluorescence and confocal microscopy. Endocrinology 149:4387–4395.

Rance NE, Bruce TR (1994) Neurokinin B gene expression is increased in the arcuate nucleus of ovariectomized rats. Neuroendocrinology 60:337–345.

Sandoval-Guzmán T, Rance NE (2004) Central injection of senktide, an NK3 receptor agonist, or neuropeptide Y inhibits LH secretion and induces different patterns of Fos expression in the rat hypothalamus. Brain Res 1026:307–312.

Sannella MI, Petersen SL (1997) Dual label in situ hybridization studies provide evidence that luteinizing hormone-releasing hormone neurons do not synthesize messenger ribonucleic acid for mu, kappa, or delta opiate receptors. Endocrinology 138:1667–1672.

Schulz R, Wilhelm A, Pirke KM, Gramsch C, Herz A (1981) Beta-endorphin and dynorphin control serum luteinizing hormone level in immature female rats. Nature 294:757–759.

Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, Clifton DK, Steiner RA (2005a) Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology 146:2976–2984.

Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA (2005b) Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146:3686–3692.

Spampinato S, Canossa M, Campana G, Carboni L, Bachetti T (1995) Estrogen regulation of prodynorphin gene expression in the rat adenohypophysis: effect of the antiestrogen tamoxifen. Endocrinology 136: 1589–1594.

Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, Serin A, Mungan NO, Cook JR, Ozbek MN, Imamoglu S, Akalin NS, Yuksel B, O'Rahilly S, Semple RK (2009) TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. Nat Genet 41:354–358.

Zhang C, Roepke TA, Kelly MJ, Rønnekleiv OK (2008) Kisspeptin depolarizes gonadotropin-releasing hormone neurons through activation of TRPC-like cationic channels. J Neurosci 28:4423–4434.