Kisspeptin neurons in the mediobasal hypothalamus (MBH) are critical targets of ovarian estrogen feedback regulating mammalian fertility. To reveal molecular mechanisms underlying this signaling, we thoroughly characterized the estrogen-regulated transcriptome of kisspeptin cells from ovariectomized transgenic mice substituted with 17β-estradiol or vehicle. MBH kisspeptin neurons were harvested using laser-capture microdissection, pooled, and subjected to RNA sequencing. Estrogen treatment significantly (p.adj. < 0.05) up-regulated 1,190 and down-regulated 1,139 transcripts, including transcription factors, neuropeptides, ribosomal and mitochondrial proteins, ion channels, transporters, receptors, and regulatory RNAs. Reduced expression of the excitatory serotonin receptor-4 transcript (Htr4) diminished kisspeptin neuron responsiveness to serotonergic stimulation. Many estrogen-regulated transcripts have been implicated in puberty/fertility disorders. Patients (n = 337) with congenital hypogonadotropic hypogonadism (CHH) showed enrichment of rare variants in putative CHH-candidate genes (e.g., LRP1B, CACNA1G, FNDC3A). Comprehensive characterization of the estrogen-dependent kisspeptin neuron transcriptome sheds light on the molecular mechanisms of ovary–brain communication and informs genetic research on human fertility disorders.

Endocrine homeostasis depends on the complex interplay between the hypothalamus and the pituitary and peripheral endocrine organs. Gonadotropin-releasing hormone (GnRH)-synthesizing neurons constitute the final output conduit from the hypothalamus for the control of reproduction (1, 2). The neurosecretory axons of these neurons terminate in the external zone of the median eminence. Episodic release of GnRH at this site into the hypothalamic-hypophysial portal circulation system evokes pulsatile luteinizing hormone (LH) and follicle-stimulating hormone secretion from the anterior pituitary, which, in turn, stimulates gametogenesis and sex steroid synthesis in the male and female gonads. In both sexes, gonadal steroids inhibit the hypothalamic-pituitary-gonadal axis via homeostatic negative feedback to the hypothalamus and the anterior pituitary. In females, rising levels of ovarian estrogen hormones at the late follicular phase of the reproductive cycle cause a switch from negative to positive feedback. This rise is a key signal for the midcycle GnRH/LH surge, which triggers ovulation (1, 2).

Hypothalamic peptidergic neurons synthesizing kisspeptin (KP) express estrogen receptor-α (ERα) and play crucial roles in mediating the positive and negative estrogen feedback to GnRH neurons via KP/KP receptor signaling. In rodents, KP neurons located in the rostral periventricular area of the third ventricle (RP3V; also referred to as the KP neuron population of the anteroventral periventricular nucleus) are critically involved in the induction of preovulatory GnRH/LH surges during positive feedback. The arcuate nucleus (ARC) in the mediobasal hypothalamus (MBH) contains an additional large KP neuron population. This anatomical region has long been known as a critical feedback site in the communication between the ovary and the hypothalamus. In postmenopausal women, absence of estrogen feedback causes profound morphofunctional changes within this region, characterized by neuronal hypertrophy (3) and increased neurokinin B (NKB) (4, 5), KP (5, 6), and substance P (4, 7) biosynthesis. In various mammals, KP neurons of the ARC (aka KNDy neurons) coexpress KP, NKB, and dynorphin. Growing evidence suggests that ARC KNDy neurons are key players in negative estrogen feedback (2, 8), and their KP output also regulates the pattern of pulsatile GnRH/LH secretion (9).
A recent RNA sequencing (RNA-seq) study successfully used the RiboTag technology to selectively isolate actively translated messenger RNAs (mRNAs) from RP3 KP neurons. In this elegant work, hundreds of transcripts showed differential expression between ovariectomized (OVX) mice with and without 17β-estradiol (E2) substitution; these transcripts may play important roles in estrogen positive feedback (10).

Detailed information about the estrogen-dependent transcripts and biological processes of the ARC and its KP (KNDy) neuron population involved in negative feedback is currently unavailable.

In this study, we characterize the estrogen-dependent transcriptome of the mouse ARC and its KP neurons with RNA-seq. Using tissue samples isolated with laser-capture microdissection (LCM) from OVX mice subjected to a chronic 4-d E2 treatment, we identify thousands of genes regulated by estrogen in the ARC and, specifically, in its KP cell population. We confirm with slice electrophysiology that the E2-induced transcriptional changes of serotonin receptor isoforms influence KP neuron responsiveness to serotonin. Based on the observation that many E2-regulated transcripts we identified were previously implicated in human puberty/fertility disorders, we propose a strategy to discover disease genes using the list of the most heavily E2-dependent KP neuron transcripts. Indeed, burden analysis of such genes can reveal significant enrichment of rare transcript variants in patients with congenital hypogonadotropic hypogonadism (CHH).

Results

**E2 Treatment of OVX Mice Results in High Physiological Serum E2 Levels and Robust Uterine Hypertrophy**. Estrogen-regulated transcripts were investigated in surgically OVX mice receiving a 4-d E2 substitution (OVX+E2) from a subcutaneous capsule implant (SI Appendix, Fig. S1A). This treatment produced a 7.59 ± 0.7 pg/mL (mean ± SEM) serum E2 concentration in test animals (n = 4), which falls within the physiological rage in cycling female mice (~6 pg/mL in diestrus and ~8 pg/mL in proestrus) (11) and exerts strong negative feedback on the neuroendocrine hypothalamus. The biological response of the OVX mice to the 4-d E2 treatment was confirmed by a robust increase in uterine weight (136.5 ± 11.8 mg of OVX+E2 animals; n = 6 vs. 18.0 ± 0.4 mg of OVX+Veh controls; n = 6; P = 6.3E−07).

**The Laser-Microdissected ARC Provides High-Quality RNA for Deep Transcriptome Profiling**. An optimized protocol developed for deep transcriptome profiling of the ARC is shown in SI Appendix, Fig. S1A. This hypothalamic nucleus was readily recognized in Nissl-stained coronal sections. Laser-microdissected tissues collected and pooled proportionally from the rostral, middle, and caudal ARC of OVX+E2 (n = 6) and OVX+Veh (n = 5) mice provided sources for well-preserved RNA (RNA integrity number: 7.1-8.2). Sequencing of the RNA-seq libraries with the Illumina NexSeq 500/550 High Output (v2.5) kit generated 22,6 M reads as an average from the 11 cDNA libraries. Reads were mapped to the mouse reference genome with a mean overall alignment rate of 86.8%. Gene level quantification of read counts was performed by featureCounts, and 89.1% of mapped reads were assigned to genes.

**Bioinformatic Analysis Identifies 1,157 Up-Regulated and 960 Down-Regulated Transcripts in the ARC of Chronically E2-Treated OVX Mice**. The 2,117 estrogen-regulated transcripts (p.adj. < 0.05) showed conspicuous sample homogeneity among animals of the same treatment group. The ARC of OVX+E2 mice contained 1,157 up-regulated (fold change [FC] > 1) and 960 down-regulated (FC < 1) transcripts (SI Appendix, Fig. S1B). In total, 235 transcripts exhibited a |log2FC| above 1.0. The top changes in each direction are shown in SI Appendix, Fig. S2A. The full list of the 2,117 estrogen-regulated transcripts and their functional categories defined based on multiple online databases and methods are provided in Dataset S1. Categorized transcripts were related to autophagy (n = 115), ubiquitination (n = 37) and encoded neuropeptides or neuropeptide-associated proteins (n = 20), miscellaneous ion channels (n = 42), transporters (n = 83), classical receptors (n = 30), other receptors (n = 136), ribosomal proteins (n = 21), transcription factors ([TFs] n = 104), long noncoding RNAs (lncRNAs; n = 40), and microRNAs (miRNAs; n = 9). The list also included predicted protein coding genes (n = 17) and other predicted genes (n = 83) with unknown functions. Genes regulated by E2 in the ARC were known to be expressed in multiple cell types (e.g., Egr1, Ar, Pgr, Ghst), unknown cell types (e.g., Agrp, Sst, Carpt, Slc6a3, Nts), in addition to a large set found enriched in KP neurons in earlier Drop-seq analyses (e.g., Tarc2, Nhlh2, Pdyn, Pitr, Nr5a2, Tmem35a, Rad1, Nraa2, Tacr3, Sox14, Oprk1, Kiss1) (SI Appendix, Fig. S2B) (12, 13).

**ORA Detects 47 Functional Pathways Altered by E2 Treatment in the ARC**. Overrepresentation analysis (ORA) identified 47 functional KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways that were altered significantly in response to E2. The highest number of changing transcripts (n = 56) occurred in the neuroactive ligand-receptor interaction KEGG pathway, with changes related to both classic and pepsidgeric (e.g., orexin [OX], neuropeptide Y [NPY], somatotatin [SST]) neurotransmission (SI Appendix, Fig. S2 C and D and Dataset S1). Given that ORA does not consider the functional connectivity of regulated transcripts, signaling pathway impact analysis (SPIA) was used additionally to predict net changes in selected neurotransmitter functions. Results of SPIA indicated the overall activation of the GAABergic and serotonergic pathways and inhibition of the glutamatergic, cholinergic, and dopaminergic synapse pathways (SI Appendix, Fig. S2D).

**RNA-Seq Studies of Fluorescent Neurons Isolated with LCM Provide Insight into the Estrogen-Dependent KP Neuron Transcriptome**. Although the ARC hosts multiple estrogen-receptive cell types (12, 13), a large number of genes critical for pubertal development, GnRH/LH pulsatility, and estrogen negative feedback are expressed in KP neurons of this region (e.g., Kiss1, Tac2, Tac3, and Egr1). To shed light specifically on the estrogenic regulation of ARC KP cells, in a second RNA-seq experiment, we compared the transcriptomes of these neurons from OVX+Veh (n = 3) and OVX+E2 (n = 3) mice. Collection and pooling of 300 fluorescently tagged KP neurons from the ARC of each mouse were performed with LCM from sections of formaldehyde-fixed brains. Total RNA samples were isolated (RIN 4.8 to 6.9), and RNA-seq libraries were prepared with the TruSeq Stranded Total RNA Library Prep Gold kit, as recommended for partly degraded RNA falling into the nanogram range (14). RNA-seq generated 20.5 M reads/sample, as an average. Reads were mapped to the mouse reference genome using the RNA-seq aligner software STAR (Spliced Transcripts Alignment to a Reference; v.2.7.3a), with an average overall alignment rate of 74.9% (SD = 3.5%). Gene level
Bioinformatic Analysis Identifies 1,190 Up-Regulated and 1,139 Down-Regulated KP Neuron Transcripts in OVX Mice Substituted with E2. The number of KP neuron transcripts that changed significantly (p.adj. < 0.05) in response to the 4-d E2 treatment was 2,329. Genes with the highest \(\log_{2}(FC)\) values shown in Fig. 1A included many low-to-medium abundance transcripts, which were undetected in earlier Drop-seq studies of hypothalamic cell types (12, 13). In total, 1,442 changes (774 up- and 668 down-regulations) within KP cells exceeded \(\log_{2}(FC)\) 1.0 (Fig. 1B), unlike during the analysis of the whole ARC, where the magnitude of most changes was much smaller (SI Appendix, Fig. S2B).

ORA Detects 83 Estrogen-Regulated Functional Pathways in KP Neurons. ORA identified 83 significantly altered KEGG pathways, with the highest numbers of changing genes in the ribosome (n = 57) and the neuroactive ligand-receptor interaction (n = 57) functional pathways (Fig. 1 C and D and Dataset S2). Use of SPIA indicated overall activation of the GABAergic and glutamatic synapse pathways and overall inhibition of the cholinergic, dopaminergic, and serotonergic synapse pathways in response to the E2 treatment. Changes in neurotransmitter and neurotransmitter receptor transcripts are presented graphically in Fig. 1D.

Estrogen-Responsive Transcripts of KP Neurons Fall into Various Functional Categories. As in the case of the ARC transcriptome, we used several online databases for the functional classification of estrogen-regulated transcripts. Many E2-regulated genes were related to autophagy (n = 156) or ubiquitination (n = 45). Others encoded neuropeptides (n = 15), miscellaneous ion channels (n = 46), transporters (n = 72), classical neurotransmitter receptors (n = 26), other receptor types (n = 110), ribosomal (n = 60) and mitochondrial (n = 37) proteins, TFs (n = 109), IncRNAs (n = 60), and miRNAs (n = 5). They included predicted protein coding genes (n = 31) and a large number of other predicted genes (n = 192), in addition to transcripts left un categorized. Members of selected gene categories are listed in Fig. 2, and all of the significant estrogen-induced changes (n = 2,329) are provided in Dataset S2.

The large number of regulated TFs (n = 109) likely contributed to secondary transcriptional changes. E2 suppressed mRNA expression of nuclear hormone receptors (Ar, Esr1), AP-1 TFs (Fos, Fosb, JunB, JunB), early growth response proteins (Egr1, 3, 4), the neuroactive-liquidation-dependent TF (NPY4R), orphan nuclear receptors (Nra4A1, Nra4A3), steroidogenesis regulators (Crem), and many zinc finger proteins. Estrogen inhibited the prohormone convertase PC1/3 transcriptional regulator Creb3l1 and the enzyme (Pck1) as well. Furthermore, E2 activated mRNA expression of some hormone receptors (Pgr, Thrb), transcriptional mediator of progesterone synthesis (Cebp), and TFs SOX (Sox11, Sox18) and STAT (Stat5a, Stat5b).

Estrogen Regulates Postsynaptic Receptors for Classic and Neuropeptide Neurotransmitters Innervating KP Neurons. A number of neurotransmitter receptors responded to the 4-d E2 treatment. These included the Htr2a, Htr4, Htr5a, and Htr7 serotonin receptors; the Npy1r, Npy4r, and Npy5r NPY receptors; the Sstr2 and Sstr3 SST receptors; and the Orx2 OX receptor, among others (Figs. 1 D and 2). These changes suggested that the receptor ligands can exert estrogen-dependent afferent control on KP neurons. To provide anatomical support to this notion, a series of dual-immunofluorescence experiments were carried out on ARC sections of OVX+Eveh and OVX+E2 KP-Cre/ZsGreen mice. Confocal microscopic analysis demonstrated the presence of the 5-HT marker serotonin transporter (SERT; Fig. 3A), OX B (Fig. 3B), NPY (Fig. 3C), and SST (Fig. 3D) in axosomatic and axodendritic afferents to ARC KP neurons, thus suggesting the potential contribution of these transmitters to the estrogen-dependent neuronal control of KP cells. As clear differences in innervation patterns were not noticed between the two animal models, the number of contacts was not analyzed quantitatively. To confirm that SSTergic fibers provide synaptic input to KP neurons, dual-label immuno-electron microscopy was used. This study established that SST-immunoreactive fibers establish synaptic contacts on KP dendrites (Fig. 3E) and cell bodies (Fig. 3F). Similar ultrastructural evidence for serotonin, NPY, and OX containing synapses onto KP cells is currently unavailable. Antibodies used in anatomical studies are specified in SI Appendix, Table S1.

Estrogenic Regulation of Serotonergic Receptors Correlates with Reduced Excitatory Responses of KP Neurons to Low Doses of 5-HT and the 5-HT4R AG Cisapride. The serotonergic system has emerged as an important mediator of leptin (15) and estrogen (16) effects on reproduction. Four distinct serotonin receptors showed robust estrogen regulation within KP neurons; the inhibitory Htr5a was up-regulated, and the excitatory Htr2a, Htr4, and Htr7 isoforms were all down-regulated in E2-treated mice (Fig. 4A and Dataset S2). We hypothesized that these changes cause reduced KP neuron responsiveness to 5-HT stimulation in OVX+E2 mice. Whole-cell patch-clamp electrophysiology was carried out in ARC KP neurons from brain slices of OVX+Eveh and OVX+E2 KP-Cre/ZsGreen mice (Fig. 4B). A holding current of +10 pA was used to evoke action potentials in KP neurons, most of which (~94%) are silent in OVX mice (17). Notably, 1,000 nM 5-HT increased the mean firing rate of KP neurons similarly in the OVX+E2 (266.1 ± 14.06% of ctrl) and the OVX+Eveh (267.9 ± 12.69% of ctrl) models. The medium 5-HT dose that we used (200 nM) produced a significantly higher response in OVX+Eveh mice (210.4 ± 10.34% of ctrl) than in OVX+E2 mice (157.9 ± 20.76% of ctrl), whereas the lowest applied dose (100 nM) increased the mean firing frequency in OVX+Eveh (163.6 ± 6.10% of ctrl) but not in OVX+E2 (99.5 ± 6.60% of ctrl) animals (Fig. 4 C and D). Preincubation of slices with the selective 5-HT4R antagonist (ANT) SB204070 (13 μM) blunted this response (123.4 ± 4.14% of OVX+Eveh ctrl), indicating that the 5-HT4R receptor isoform has a major contribution to the effect of 5-HT (Fig. 4D). The highest 100-μM dose of the selective 5-HT4R agonist (AG) Cisapride stimulated KP neurons equally in the OVX+E2 (388.6 ± 17.12% of ctrl) and OVX+Eveh (394.7 ± 20.64% of ctrl) models. The magnitude of response to a medium dose (10 μM) was significantly higher in OVX+Eveh (277.2 ± 13.18% of ctrl) than in OVX+E2 (175.6 ± 22.95% of ctrl) mice, whereas 1 μM Cisapride (the lowest dose having an effect in pilot studies) raised the mean firing frequency of KP cells in the OVX+Eveh (184.1 ± 13.64% of ctrl) but not in the OVX+E2 (116.0 ± 10.06% of ctrl) model (Fig. 4 E and F). Cisapride acted selectively on 5-HT4R because its effect was eliminated in the presence of 13 μM SB204070 (Fig. 4F). The action was direct because 1) it was maintained in the
presence of the ionotropic glutamate and GABAA receptor inhibitors kynurenic acid (KYN; 2 mM) and picrotoxin (PIC; 100 μM), respectively, and 2) it was absent if the intracellular solution was supplemented with the membrane impermeable G protein inhibitor guanosine 50-β,β-thio] diphosphate (GDP-β-S; 2mM) (Fig. 4F). The higher responsiveness of the OVX+Veh model to low doses of 5-HT and Cisapride supported the notion that estrogenic regulation of the 5-HT receptor isoforms reduces the sensitivity of KP cells to the stimulatory serotonergic input in OVX+E2 mice.
Estrogen-regulated KP neuron transcripts fall into diverse functional categories. E2-regulated transcripts of ARC KP neurons were categorized based on function using online databases. Many of these transcripts are related to ubiquitylation (n = 156) and ubiquitylation (n = 45). They encode neuropeptides (n = 15), miscellaneous ion channels (n = 46), transporters (n = 72), classical neurotransmitter receptors (n = 26), other receptor types (n = 110), ribosomal (n = 60) and mitochondrial (n = 37) proteins, Tfs (n = 109), lncRNAs (n = 60), and miRNAs (n = 5). They include predicted protein coding genes (n = 31) and a large number of other predicted genes (n = 192). Dataset S2 contains the full list of E2-regulated KP neuron transcripts and a more detailed functional categorization. Genes that also show significant E2-dependent regulation in the ARC are underlined. Asterisks indicate inverse regulatory changes in the ARC region and in KP neurons, whereas all other genes changed significantly (p < 0.05) only in KP neurons. Numbers in red reflect transcript abundances in CPM units. Dot areas change in proportion to transcriptional responses, with the larger dot representing 100%. For a more complete listing of regulated genes, see Dataset S2.
The Estrogen-Regulated Transcriptome of ARC KP Neurons May Shed Light on the Pathomechanisms of Fertility Disorders and Designates Disease Gene Candidates. We hypothesized that similarly to Kiss1, Tac2, and Tac3, other transcripts heavily involved in reproductive regulation and disorders are 1) enriched in KP neurons versus their ARC environment, 2) expressed abundantly in KP cells (high counts per million [CPM]), and 3) regulated robustly (high log2FC) by E2. Searches of mouse (http://www.informatics.jax.org/vocab/mp_ontology) and human (https://www.ebi.ac.uk/ols/ontologies/hp) disease phenotype databases revealed that 7.1% (n = 166) and 1.8% (n = 42), respectively, of the 2,329 E2-regulated KP neuron transcripts can be linked to pubertal and reproductive disorders, including CHH (Fig. 5A). In total, 125 transcripts were expressed at mean CPM ≥ 100, showed at least a threefold enrichment (in CPM) in KP neurons versus the ARC region, and showed at least a twofold up- or down-regulation by E2. The incidences of mouse and human disease genes increased to 20% (n = 25) and 8% (n = 12), respectively, by applying the above stringent selection criteria (color symbols in Fig. 5A). The list of the 125 candidate protein-coding genes was used for a gene-based burden analysis comparing the abundance of rare, likely pathogenic variants in a large cohort of CHH patients (n = 337) versus gnomAD control database (Fig. 5B). As expected, we observed an enrichment in known CHH genes such as TAC3 (P = 6.61E−08), TACR3 (P = 1.01E−03), and KISS1 (P = 6.15E−03). Moreover, this analysis highlighted putative CHH-candidate genes like the low-density lipoprotein receptor-related protein 1B (LRP1B, P = 3.14E−05) and the voltage-gated calcium channel subunit alpha1 G (CACNA1G, P = 1.84E−04), a gene previously associated with spinocerebellar ataxia. Interestingly, the voltage-gated calcium channel auxiliary subunit alpha2delta 1 (CACNA2D1, P = 1.49E−03) was also found among the enriched genes, suggesting an important role of L-type and T-type calcium channels in human reproduction. The emerging role of these candidate genes in the estrogen-dependent regulation of KP neurons and in CHH pathophysiology requires further clarification by basic research and clinical genetic studies.

Discussion

Technical Considerations. LCM is a high-precision approach to collect defined anatomical areas or subpopulations of tissue cells from histological sections under direct microscopic visualization. The regions of interest are selected and then dissected out with the aid of a laser beam for downstream applications, including transcriptomics. RNA-seq library preparation includes a DNase digestion step to eliminate nuclear DNA prior to reverse transcription of RNA. In this study, we developed two optimized protocols for tissue processing. LCM-based sample collection, RNA isolation, library preparation, and RNA-seq to achieve deep transcriptome profiling of the ARC and ARC KP (KNDy) neurons (SI Appendix, Fig. S1A). In both methods, the use of LCM allowed us to achieve a very precise histological sampling. This was reflected in 1) the homogeneous color blocks within heat maps and 2) the high number of transcripts that differed statistically (p.adj. < 0.05) between the OVX+Veh and OVX+E2 treatment groups. Manually microdissected ARC samples in a recent report (18) exhibited 132 estrogen-regulated transcripts at FC > 1.5 and P < 0.05, whereas 844 transcripts would correspond to the same cutoff criteria in our LCM-isolated ARC samples. We note that the two studies also differed in other technical
Fig. 4. Transcriptional changes of serotonin receptor isoforms cause reduced excitatory serotonergic neurotransmission to KP neurons in E2-treated OVX mice. (A) The 4-d E2 treatment of OVX mice (OVX+E2) causes up-regulation of the inhibitory Htr5a and down-regulation of the excitatory Htr2a, Htr4, and Htr7 serotonin (5-HT) receptor isoforms in ARC KP neurons, leading to a prediction that 5-HT might stimulate KP neurons more efficiently in the OVX+E2 model. (B) Electrophysiological studies address the differential responsiveness of KP neurons from OVX+E2 versus OVX+E2 and OVX+E2 mice to decreasing doses of 5-HT or the selective 5-HT4R agonist (AG) Cisapride. (C–F) The highest doses of 5-HT (1,000 nM) and Cisapride (100 μM) elicit similar increases in the mean firing frequency of KP neurons in OVX+E2 and OVX+E2 mice. In contrast, the OVX+E2 mouse model responds more to the medium and low doses (D vs. C; F vs. E). #P < 0.05 in OVX+E2 versus OVX+E2 model with ANOVA. (C and D) The 100-nM 5-HT, which has no effect in the OVX+E2 model (C), increases the mean firing frequency of KP cells in OVX+E2 mice to 163.6 ± 6.10 of the ctrl rate (D). Preincubation of slices with the selective 5-HT4R receptor antagonist (ANT) SB204070 (13 μM) blunts this response (D), indicating the contribution of 5-HT4R isoform to the effect of 5-HT. (E and F) The 1-μM dose of the selective 5-HT4R AG Cisapride has no significant effect on the mean firing rate of KP neurons in OVX+E2 mice (E; 116.6 ± 10.06% of ctrl; P = 0.13) but efficiently stimulates KP neuron firing in the OVX+E2 model (F; 184.1 ± 13.64% of ctrl; P = 0.0002). Cisapride acts selectively on 5-HT4R and, therefore, is ineffective in the presence of 13 μM SB204070 (second trace in F). The effect on KP neurons is direct because it is preserved in the presence of the ionotropic glutamate and GABAA receptor inhibitors KYN (2 mM) and PIC (100 μM), respectively (third trace in F). Further, Cisapride is ineffective if the intracellular solution is supplemented with the G protein inhibitor GDP-β-S (2 mM; fourth trace in F). Scatter dot plots summarize the results obtained with different blockers. See also Dataset S3. *P < 0.05 versus ctrl with Student’s t test. **P < 0.05 with ANOVA.
new genetic causes of reproductive disturbances, including CHH. Note that 125 out of the 2,329 regulated transcripts (in yellow circle) are enriched at least 3 times in ARC KP neurons versus the ARC region, are expressed abundantly in KP cells (mean CPM = 20,000), and respond to E2 at log2FC ≥ 1.0. Gene names contain basic data of putative CHH-candidate genes. Asterisks indicate enrichment p-value is less than 0.05. Fig. 5. The E2-regulated KP neuron transcriptome may serve as a resource to discover new genes causing fertility disorders. (A) The 2,329 E2-regulated genes of ARC KP neurons overlap with the lists of genes proposed to cause reproductive disturbances in mice and/or humans, according to the Jackson Mammalian Phenotype Ontology (green circle) and the Human Phenotype Ontology (blue circle) databases. In total, 17 disease genes are shared between mice and humans in the Venn diagram. Information about the E2-dependent transcripts of ARC KP neurons may serve as an important resource to identify new genetic causes of reproductive disturbances. (B) Manhattan plot illustrates the results of gene-based burden analysis of the top 125 disease genes in CHH patients versus gnomAD controls. Dotted lines indicate the threshold for statistical significance after correction for multiple testing (red: exome wide Bonferroni correction, n = 20,000, P < 2.5E–06; green: 5% false discovery rate [FDR] threshold; blue: 10% FDR threshold). Table contains basic data of putative CHH-candidate genes. Asterisks indicate enrichment p-value below the 0.05% FDR threshold.
parameters, including steroid treatments, which could also contribute to partly different outcomes. A further methodological consideration relates to the choice of using LCM-isolated and pooled neurons in our study, as opposed to dispersed single cells in recent Drop-seq experiments on rodent hypothalamis (12, 13). These two sampling methods differ basically in their goals as well as in advantages and drawbacks. The LCM-based sample collection from pooled KP neurons ensured tens of thousands of identified transcripts, whereas reaching the same sequencing depth is beyond the goal of single-cell (e.g., Drop-seq) experiments in which tens of thousands of living cells are dispersed and then analyzed in the same sequencing run to identify clusters with identical gene expression properties (19). Accordingly, earlier Drop-seq studies of the hypothalamus (13) and its ARC region (12) were able to identify basic cell-type-specific transcriptional features of KNDy neurons, with intrinsic compromises in depth of sequencing. A critical advantage of LCM is the snapshot from a given functional status when the mice are killed, whereas the lengthy cell isolation process of Drop-seq and other single-cell techniques (19) may allow unwanted transcriptional changes to occur. This problem persists if living fluorescent neurons are isolated via fluorescence-activated cell sorting for sequencing. The RiboTag technique (20), which allows the selective isolation of actively translated mRNAs from a given cell population, offers an elegant alternative to our LCM-based cell harvesting approach, and it was used recently to identify estrogen-regulated transcripts of the preoptic (RP3V) KP neuron population (10). It has to be recognized that harvesting KP neurons with LCM has potential disadvantages as well. The transcriptomic information obtained from pooled neurons characterizes a mixed-cell population (~300 neurons in our study) instead of individual isolated cells, leaving possible heterogeneity of KP neurons unexplored. Another technical pitfall is some unavoidable sample contamination from surrounding tissues, which can be reduced using thin sections (12 μm in our study). Although some low-CPM transcripts identified in KP neurons may still represent false detection, we argue that the strict statistical criteria (p.adj. < 0.05) we applied ensure that the majority of changes reflect E2-dependent regulation within KP neurons.

Animal Models. In our study, we aimed to identify the E2-dependent transcripts of the ARC and its KP neurons. Our decision to use nonphysiological animal models to this goal relies on three main arguments. 1) Milder transcriptional effects of physiological E2 fluctuations across the estrus cycle would be much more difficult to identify as significant. 2) Transcriptomic snapshots from normal cycling animals would be impossible to interpret because of the different expression dynamics of E2-dependent transcripts. For example, some transcriptional responses to the high proestrus E2 levels might persist through estrus when serum E2 levels are at their nadir, whereas others could have already waned. Furthermore, ovarian progesterone dynamics would complicate the interpretation of estrogen effects based on the gene expression profile of intact females. 3) Surgical OVX and the subsequent treatment of OVX mice with exogenous E2 or vehicle generate two easily reproducible animal groups with low biological variations and high group homogeneity. Young female mice from mixed cycle phases contain 2.7 pg/mL serum E2 (Diestrus: ~6 pg/mL; Proestrus: ~8 pg/mL; Estrus: <0.3 pg/mL; Metestrus: ~0.5 pg/mL, as determined by gas chromatography-tandem mass spectrometry [GC-MS/MS]) (11). Thus, the 7.59 ± 0.74 pg/mL serum E2 cc. we detected in OVX+E2 test mice falls in the physiological range, which characterizes cycling female mice at times of strong negative feedback. We note that the real serum E2 concentration in this OVX+E2 model might be somewhat lower due to the previously noted serum matrix effect (21) that we also detected. Accordingly, the 1.73 ± 0.34 pg/mL E2 concentration this kit detected in our OVX+E2h mice is likely an overestimate because OVX mice are known to contain E2 below the 0.3 pg/mL detection threshold of GC-MS/MS (11). Despite these technical issues, a systematic comparison of nine commercial enzyme immunoassay kits showed that the CalBiotech Kit ES180S-100 kit is still the only one available currently that shows good E2 parallelism to the standard curve as well as accuracy versus the gold standard GC-MS/MS technique (21).

It is reasonable to speculate that the estrogen-dependent ARC and KP neuron transcriptomes identified in this study show physiological fluctuations in intact female mice according to cyclic changes in E2 negative feedback. However, the expression level of individual transcripts in our OVX+E2 model may not necessarily reflect the transcriptional landscape of intact diestrus/proestrus animals. We propose that the extended 4-d E2 treatment of OVX mice may be clinically relevant to chronic estrogen exposure with relative progesterone deficiency, which is a common perimenopausal pathology, whereas transcriptomes of the OVX+E2h model may mimic the gene expression status of the ARC and its KP neurons following the loss of estrogens in postmenopausal women.

Comparison with Earlier Studies of the ARC. Earlier RNA-seq experiments have already addressed the estrogen responsiveness of the ARC transcriptome (18). This study used OVX wild-type mice, ERα knockout mice, and mice containing ERα with a mutated DNA binding domain to distinguish among ERα-mediated, estrogen response element (ERE)-dependent, and ERα-independent E2 signaling, respectively. The number of significant changes (at P < 0.05 and FC > 1.5) occurred in 132, 35, and 22 transcripts, respectively, in these three cases. The ARC of OVX+E2 mice in our study reproduced many (e.g., Stc1, Mfges, Pcdh20, Creb1, Wnt4, Pouf3f1, Pror88), but not all (e.g., Mgp, Erp27, Max1, Pouf2f1, Olig2, Max1, Glapf), of the above 132 transcriptional changes. In addition, the ARC transcriptome of our OVX+E2 mice exhibited significant changes not reported by these investigators. Altogether, we found 4,039 transcripts at P < 0.05 and 2,117 transcripts at p.adj. < 0.05 that were expressed differentially in the two treatment groups. Out of these, 844 and 483 changed more than 1.5 times at P < 0.05 and p.adj. < 0.05, respectively. The much higher number of regulated transcripts identified in our study may partly reflect the use of different methods, including the very precise LCM-based tissue collection. Differences in the estrogen treatment of OVX mice could also contribute to the partly different transcriptional responses and gene regulation dynamics; our study used a chronic 4-d E2 supplementation of OVX mice via silastic capsule implants, whereas Yang and colleagues (18) applied two estradiol benzoate injections in oil to OVX mice 24 and 48 h before sacrifice, respectively. While our studies focused on the regulation of KNDy neurons, the comparative analysis of the E2-dependent ARC and KP neuron transcriptome databases will be very useful in future studies to identify transcripts that are regulated specifically within KP neurons. Among these, newly identified peptide cotransmitters of KNDy neurons include Vgf and Nms, and regulated receptors expressed at higher levels (CPMs) in KP neurons than in the ARC include Npy4r, Ghr, Lepr, and Pdhr, among others. It
is important to note that KP neurons are not the only estrogen-dependent cell type in the ARC, as revealed by the altered expression of neurotensin (Nts), SST (Sst), agouti-related peptide (Agpr), prepronociceptin (Pnoc), preproenkephalin (Penk), and CART prepropeptide (Carpp) in the ARC of our OVX+E2 model. The above and similar E2-dependent transcripts that are not regulated and enriched within KP cells contribute to our understanding of the estrogenic control of non-KP neurons, potentially including currently unknown ARC neuron populations.

Differential Regulation of the Two KP Neuron Populations by E2.

This comprehensive study identifies the estrogen-sensitive transcripts of ARC KP neurons with deep transcriptome profiling. A handful of transcriptional changes including the estrogenic down-regulation of Kiss1, Tac2, and Tac3 were previously known from in situ hybridization experiments (22), whereas the majority of the 2,329 regulated transcripts represent discoveries. While we did not address the estrogenic regulation of RP3V KP neurons implicated in positive E2 feedback, a recent RNA-seq study based on the RiboTag technology managed to identify 683 E2-dependent changes in the transcriptome of RP3V KP neurons at \( P < 0.05 \) (10). Although it is not clear how many of these changes satisfy the more stringent statistical criteria of \( p_{adj} < 0.05 \), the relatively small number of responsive transcripts raises the possibility that the transcriptome of RP3V KP neurons is less responsive to E2 treatment than the ARC KP neuron transcriptome, the latter of which exhibited 2,329 changes at \( p_{adj} < 0.05 \) and 4,391 changes at \( P < 0.05 \). Not too surprisingly, the neuroactive ligand-receptor interaction KEGG pathway was altered significantly in both KP cell types, with 57 changes in ARC and 32 reported changes in RP3V KP neurons. Comparison of the E2-dependent genes identified in the two studies reveals interesting regulatory similarities as well as differences between the two KP neuron populations. Transcripts regulated in the same direction in RP3V and ARC KP neurons include Pgr, Pdyn, Vglut2, and Npy4r. Many interesting examples also exist for an inverse regulation within the two KP cell groups (e.g., Kiss1, Vgf, Gat3, and Pitrk). Comparative analysis of regulatory patterns in the ARC and the RP3V KP neuron population may provide important insight into the regulatory mechanisms underlying negative and positive estrogen feedback. Immunohistochemical studies in the future will be used to investigate further the presence and estrogen-dependent regulation of neuropeptide cotransmitters (neuromedin S, VGF, galanin) in ARC KP neurons. Although our present study focused on the 2,329 E2-dependent transcripts of ARC KP neurons, we note that the full transcriptome contained 19,796 different transcripts at a mean CPM > 1 and 11,164 transcripts at a mean CPM > 10. Many of these transcripts, if not shown here to be regulated by E2, might fulfill critical roles in KP neuron physiology.

Mechanisms Underlying the Transcriptional Changes.

Multiple mechanisms that account for the altered gene expression profile of E2-treated OVX mice (23–25) are inseparable in our study. Classical genomic actions of E2 involve ligand binding to cytoplasmic ERs (ER\( \alpha \) and ER\( \beta \)), which induces receptor dimerization and entry into the nucleus. The homo- or heterodimer complex binds to ER\( \alpha \)s in the promoter region of target genes and induces or represses transcription. It was estimated that the mouse and human genomes contain \( \sim \)70,000 such ER\( \alpha \)s (26). Primary responsive genes may also lack any ERE-like sequence and require a DNA-binding transcription cofactor. In this indirect (tethered) genomic signaling mechanism, ERs participate in protein-protein interactions to modulate the function of other response elements and TFs, many of which were expressed in KP neurons (e.g., Sp1, Nfkb1, Cebpb, Stat5a, and Stat5b). Unknown fractions of classic ER\( \alpha \)s may be membrane bound to mediate rapid estrogen effects. This pool in the proximity of the plasma membrane can be associated with the scaffold protein caveolin-1 or Src, Ras, and PI3 kinases. Upon binding E2, various signaling cascades can be activated to generate calcium currents, cyclic adenosine monophosphate, or inositol phosphate or to stimulate protein kinases (25). Estrogens may also act via the G protein-coupled ER 1 (Gper1, a.k.a. Gpr30) (27). However, Gper1 was expressed only at extremely low levels in the ARC and in KP neurons (mean CPM = 2.2 and 2.5, respectively), suggesting that its contribution to the observed transcriptional changes is negligible. There is a large body of evidence that estrogens regulate mitochondrial functions (28).

In addition to the above direct effects, many changes we observed in this study could be consequences of indirect estrogen actions on neuronal, glial, or endothelial cell types communicating with the ARC and with KP neurons. Detailed analysis of mechanisms regulating individual genes would not be possible within the limits of this article.

Estrogenic Regulation of TFs. RNA-seq results revealed that Esr1 is one of the most abundant TFs in KP neurons conveying robust estrogen dependence to these neurons. Strikingly, E2 treatment of OVX mice altered mRNA expression of 109 TFs. The transcription of Ar, Egr1, Fox, Fosb, Junb, Nr4a1, and Sox5 was suppressed by E2, whereas the well-known estrogen target Pgr showed a strong stimulatory response. Transcriptional regulation of TFs could contribute significantly to secondary changes in the expression profile and functions of KP cells.

Regulatory RNAs in ARC KP Neurons. In addition to five miRNAs, at least 60 lncRNAs were regulated by E2 in KP neurons. These transcripts are longer than 200 nucleotides, with no or only limited protein-coding capability, and they are categorized as long intronic ncRNAs, long intergenic ncRNAs, circular RNAs, and natural antisense transcripts. Most lncRNAs act in the cell nucleus as molecular scaffolds to assist in alternative splicing, chromatin structure modification, and other biological processes. In the cytoplasm, lncRNAs regulate translation, promote or inhibit mRNA degradation, and function as miRNA sponges (29, 30). The critical role of lncRNAs is increasingly recognized in a variety of biological processes and in the pathophysiology of cancer initiation, development, and progression (31). These lncRNAs are also emerging as important determinants of nuclear hormone receptor action (29, 30). For example, MALAT1, which was expressed abundantly but not regulated in KP cells, associates with both ER\( \alpha \) and ER\( \beta \) at the chromatin level, and E2 treatment of prostate organotypic slice cultures decreases MALAT1 recruitment to chromatin (32). MIAT was regulated robustly by E2 in KP cells, similarly to previous observations obtained in ER-positive breast cancer cells (33). The crosstalk among lncRNAs, estrogens, and ERs may have wider implications in sex steroid signaling, as indicated by the estrogenic regulation of 60 lncRNAs within KP neurons. The importance of these changes in the regulation of sex steroid feedback to the neuroendocrine hypothalamus requires clarification.

Functional Consequences of E2 Treatment on Neurotransmitter Signaling to ARC KP Neurons. ORA unveiled the significant alteration of 83 KEGG pathways, including the mitogen-activated protein kinase-, cyclic adenosine monophosphate-, PI3K-Akt-,
Calcium-, cyclic guanosine monophosphate-dependent protein kinase-, and 5′ adenosine monophosphate-activated protein kinase-signaling pathways. Future research will need to investigate the functional consequences of these changes, which would be impossible to discuss thoroughly in this manuscript. In our study, we paid special attention to the neuroactive ligand-receptor interaction pathway with 57 E2-regulated transcripts, which indicated that E2 treatment profoundly alters neuroepithelial, GABAergic, glutamatergic, dopaminergic, cholinergic, and serotonergic signaling to ARC KP neurons. Using neuroanatomical approaches, we confirmed that various neuromodulator receptor ligands (OX B, SST, NPY) and serotonin are present in neuronal afferents to KP neurons. In the case of SST, it would be difficult to speculate about the physiological consequences of the opposite regulation of Sstr2 and Sstr3 (induction of Sstr2 and inhibition of Sstr3 by E2), both of which encode receptors that inhibit adenylyl cyclases. Similar problems hinder the physiological interpretation of regulatory changes shown by the three distinct Npyr transcripts (down-regulation of Npyr1r and Npyr5r vs. up-regulation of Npyr4r), in which case the encoded receptors can even have multiple physiological ligands (NPY/PYY/PP). In contrast, changes of all four regulated serotonin receptors (estrogenic up-regulation of the inhibitory Htr5a and down-regulation of the excitatory Htr2a, Htr7, and Htr4) allowed us to hypothesize reduced serotonergic stimulation of KP neurons in the OVX+E2 model, also in accordance with the reduced function of the serotonergic synapse pathway predicted by SPAR. Our experiments using slice electrophysiology confirmed the hypothesis that serotonin exerts reduced excitatory action on KP neurons in the OVX+E2 mouse model, and this reduction is partly due to decreased excitatory signaling on serotonin receptor 4. A large body of evidence indicates the involvement of serotonergic mechanisms in the estrogen-dependent control of the pulsatile and surge modes of LH release (for a review, see ref. 16). The high rate of LH release is inhibited by serotonin in OVX rats, whereas LH secretion is stimulated in rats pretreated with E2. Counterintuitively, ARC KP neurons in our electrophysiological studies gave higher responses to serotonin in slices of OVX+Eveh mice, which suggests multiple hypothalamic sites and mechanisms for the in vivo reproductive actions of serotonin. OVX female rats were reported to show a morning/afternoon oscillatory pattern in serotonin metabolism in several brain areas associated with the control of LH secretion. This pattern is modulated by E2 in a way that the peak serotonergic activity coincides with the induced afternoon LH surge (16). ARC KP neurons contribute to the reproductive effects of serotonin. In male rats, intracerebroventricular serotonin injection increases hypothalamic Kiss1 expression encoding KP (34). Disregulation of NKB secretion from ARC KP neurons plays an important role in postmenopausal hot flushes (35). Interestingly, selective serotonin reuptake inhibitors are among the most efficient non-hormonal treatment options for hot flushes (36).

**Putative Involvement of Estrogen-Regulated Genes in Human Fertility Disorders.** Several E2-regulated transcripts enriched in KP neurons are known for their role in the control of reproduction. These include Kiss1, Tac2, Tac3r, and Esr1, among others that could also be found in the Jackson Mammalian Phenotype Ontology and the Human Phenotype Ontology databases. We propose that such genes can be critical regulators of human fertility, and their mutations may underlie currently unexplained cases of CHH. To facilitate future genetic screening of patient databases, we have generated a list rich in putative disease genes using stringent selection criteria (at least threefold enrichment in KP neurons vs. the ARC region, at least twofold change (log2FC ≥ 1.0) in response to E2, and mean CPM ≥ 100 within KP neurons). This strategy enhanced the incidence of known murine disease genes from 7.1 to 20.0% and the incidence of known human disease genes from 1.8 to 8.0% in the list. This enrichment made it very likely that the incidences of currently unknown disease genes were also increased. This hypothesis is supported by the gene-based burden analysis of the top 125 candidate genes from our expression study comparing a large cohort of CHH patients versus gnomAD controls. Indeed, patients showed enrichments in rare transcript variants of several known CHH genes such as TAC3, TACR3, and KISS1 together with putative CHH-candidate genes, including two members of the voltage-gated calcium channel families. Although results of this preliminary burden analysis do not necessarily indicate a causal relationship between the rare variants and CHH, they suggest that the list of the heavily estrogen-dependent KP neuron transcripts may become an important resource for the discovery of new genes responsible for human reproductive phenotypes.

**Conclusions.** According to the prevailing view, ARC KP (KNDy) neurons are key regulators of pulsatile GnRH secretion via KP/KP receptor signaling on the preterminal “dendrons” of hypophysiotropic GnRH processes. This signaling is critically influenced by the negative feedback effects of the ovarian sex steroid E2 (1, 2). Molecular mechanisms underlying this feedback are only poorly understood. E2 uses direct and indirect pathways to alter the gene expression profile of KP neurons. RNA-seq studies of the ARC identified changes in neuropeptide genes that are enriched in non-KP cells. Altered Npyr, Ns, and Sst expression may act transsynaptically to influence KP neurons that express Me3r, Nstr2, and Sstr2/Sstr3 receptors, respectively, according to results of our KP neuron transcriptomics. Further actions of E2 outside the ARC may also alter the gene expression profile of KP neurons via indirect transsynaptic actions, whereas our RNA-seq studies found no solid evidence for a putative glial/endothelial involvement in indirect E2 actions upon KP cells. Transcriptional changes within KP neurons include the reduced expression of nearly all neuropeptide cotransmitters (e.g., Kiss1, Tac2, Pdyn, Gal, Vgf, and secretory proteins (e.g., Chga, Chlb, Scg3)) colocalized with KP. In contrast, the level of the Sst17a6 transcript, which encodes VGLUT2 and confers a glutamatergic phenotype onto KNDy neurons (37), increased considerably. E2 changed the expression of various nuclear (e.g., Efr1, Ar, Pgr) and membrane (e.g., Prlr, Ghsr, Lepr) hormone receptors, receptors for classic (e.g., Adra1a, Htr4) and neuropeptide (e.g., Htr2, Npy1r, Tac3r) transmitters, and ion channels and regulated a variety of biological processes, including transcription, mitochondrial and ribosomal functions, ubiquitination, autophagy, and synaptic plasticity. The surprisingly large number of E2-regulated IncRNAs opens an area in sex steroid research. The comprehensive characterization of the estrogen-dependent KP neuron transcriptome in mice also has important clinical implications for the estrogenic regulation of the human hypothalamus during the menstrual cycle and for the putative molecular consequences of postmenopausal estrogen deficiency.

**Materials and Methods**

A detailed description of methodologies—including the use of transgenic mouse strains, estrogen treatment, transcriptomic studies, immunohistochemical experiments, confocal and electron microscopic studies, slice electrophysiology, and gene-based burden analysis—is provided in SI Appendix, Materials and Methods.
1. S. M. Moenter, M. A. Silvesia, L. Wang, C. Adams, Central aspects of systemic oestriol-negative and positive-feedback on the reproductive neuroendocrine system. J. Neuroendocrinol. 32, e1727A (2020).

2. A. E. Hershson, A simple model of etsrus cycle negative and positive feedback regulation of GnRH secretion. Front. Neuroendocrinol. 57, 700837 (2020).

3. N. E. Rance, N. T. McMullen, J. E. Smalke, D. L. Price, W. S. Young III, Postmenopausal hypothy泰国 of neurons expressing the estrogen receptor gene in the human hypothalamus. J. Clin. Endocrinol. Metab. 71, 97–85 (1990).

4. N. E. Rance, W. S. Young III, Hypothyroidism and increased expression of neuron containing neuronin-s and substance P messenger ribnucleic acids in the hypothalami of postmenopausal women. Endocrinology 128, 2239–2247 (1991).

5. E. Hrabovszky et al., Sexual dimorphism of kisspeptin and neurokinin B immunoreactive neurons in the infrundibular nucleus of aged men and women. Front. Endocrinol. (Lausanne) 2, 80 (2011).

6. A. M. Romoot, S. J. Krajewski, M. L. Voytko, N. E. Rance, Hypothyroidism and increased kisspeptin gene expression in the hypothalamic infrundibular nucleus of postmenopausal women and ovariectomized monkeys. J. Clin. Endocrinol. Metab. 92, 2744–2750 (2007).

7. E. Hrabovszky et al., Substance P immunoreactivity exhibits frequent colocalization with kisspeptin and neurokinin B in the human infrundibular nucleus. PLoS One 8, e27379 (2013).

8. M. A. Mittelman-Smith et al., Acute kisspeptin/neurokinin B (NKB) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. Endocrinology 153, 2808–2812 (2012).

9. X. Liu et al., Highly redundant neuropeptide volume co-transmission underlying episodic activation of the GnRH neuron dendrite. eLife 10, e62455 (2021).

10. A. B. Z. Stephens, A. S. Kaufman, Estrogen regulation of the molecular phenotype and active trastor state of RIVP kisspeptin neurons. Endocrinology 162, hqbb080 (2021).

11. M. E. Nilsson et al., Measurement of a comprehensive sex steroid profile in cord serum by high-sensitivity gas chromatography-tandem mass spectrometry. Endocrinology 156, 2492–2502 (2015).

12. J. N. Campbell et al., A molecular census of arcuate hypothalamus and median eminence cell types. Nat. Neurosci. 20, 484–496 (2017).

13. R. Chen, X. Lu, L. Jiang, Y. Zhang, Single-cell RNA-seq reveals hypothalamic cell diversity. Cell Rep. 18, 3227–3241 (2017).

14. S. Schuierer et al., A comprehensive assessment of RNA-seq protocols for degraded and low-quantity samples. BMC Genomics 18, 642 (2017).

15. S. D. Sullivan, L. C. Howard, A. H. Clayton, S. M. Moenter, Serotonergic activation rescues reproductive function in fasted mice: Does serotonin mediate the metabolic effects of leptin on reproduction? Biol. Reprod. 66, 1702–1706 (2002).

16. M. L. Vitala, S. R. Chiocchio, Serotonin, a neurotransmitter involved in the regulation of luteinizing hormone release. Endocr. Rev. 14, 480–493 (1993).

17. S. de Croft et al., Spontaneous kisspeptin neuron firing in the adult mouse reveals masked sex and brain region differences but no support for a direct role in negative feedback. Endocrinology 153, 5384–5393 (2012).

18. J. A. Yang, H. Stres, W. J. Belden, T. A. Roepke, The acute estrogen-regulated transcription: Estrogen response element-dependent and -independent signaling of ERα in female mice. Endocrinology 158, 612–626 (2017).

19. B. Veith, J. Pareek, C. Liebenow, W. E. Hagen, A systematic evaluation of single cell RNA-seq analysis pipelines. Nat. Commun. 10, 4667 (2019).

20. E. E. S. Z. et al., Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. Proc. Natl. Acad. Sci. U.S.A. 106, 13939–13944 (2009).

Author affiliations: “Laboratory of Reproductive Neurobiology, Institute of Experimental Medicine, Budapest 1083, Hungary; School of Medicine, University of Limerick, Limerick, Ireland; and “Laboratory of Endocrine Neuroendocrinology, Institute of Experimental Medicine, Budapest 1083, Hungary; Department of Cellular and Network Neurobiology, Institute of Experimental Medicine, Budapest 1083, Hungary; “Laboratory of Integrative Neuroendocrinology, Institute of Experimental Medicine, Budapest 1083, Hungary; “Centre for Bioinformatics, University of Veterinary Medicine, Budapest 1078, Hungary; “Department of Physics of Complex Systems, Eötvös Loránd University, Budapest 1117, Hungary; “Department of Biochemistry and Molecular Biology, Faculty of Medical University of Debrecen, Debrecen 4032, Hungary; “Department of Animal Biotechnology, Institute of Genetics and Biotechnology, Hungarian University of Agriculture and Life Sciences, Gödöllő 2100, Hungary; “Service of Endocrinology, Diabetology, and Metabolism, Lausanne University Hospital, Lausanne 1011, Switzerland; “Faculty of Biology and Medicine, University of Lausanne, Lausanne 1005, Switzerland; “Centre for Neuroendocrinology, University of Pretoria, Pretoria 0031, South Africa; “Translational & Clinical Research Institute, University of Newcastle-upon-Tyne, Newcastle upon Tyne NE1 4EP, United Kingdom; “Department of Endocrinology, Diabetes & Metabolism, Newcastle-upon-Tyne Hospitals, Newcastle upon Tyne NE1 4LP, United Kingdom; and “Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3EJ, United Kingdom.

Author contributions: B.G., E.R., M.S., K.S., S.T., I.F., S.P., Z.S., L.B., Y.Z., A.M., N.P., W.H.C., and E.H. designed research; B.G., E.R., M.S., K.S., S.T., I.F., V.C., S.H.T., Z.B., Y.R., N.S., S.P., Z.S., L.B., Y.Z., Z.S., N.M., P., S.M.M., and E.H. performed research; R.C.A., R.P.M., and R.Q. contributed new reagents/analytic tools; B.G., E.R., M.S., K.S., I.F., V.C., N.S., S.P., Z.S., L.B., Y.Z., A.M., N.P., and E.H. analyzed data; and B.G., E.R., M.S., K.S., I.F., S.P., Z.S., L.B., Y.Z., A.M., N.P., S.M.M., W.H.C., and E.H. wrote the paper.