Primary cilia enhance kisspeptin receptor signaling on gonadotropin-releasing hormone neurons

Andrew I. Koemer-Coixa, Thomas W. Sherwoodb, Jill A. Greenb, Robert A. Steinerb, Nicolas F. Berbarid, Bradley K. Yoderd, Alexander S. Kauffmana, Paula C. Monsmaa, Anthony Brownb, Candice C. Askwithb,1 and Kirk Mykytyna,1

aDepartment of Pharmacology, College of Medicine, The Ohio State University, Columbus, OH 43210; bDepartment of Neuroscience, College of Medicine, The Ohio State University, Columbus, OH 43210; cDepartment of Physiology and Biophysics and Obstetrics and Gynecology, University of Washington, Seattle, WA 98195; dDepartment of Cell, Development, and Integrative Biology, University of Alabama at Birmingham Medical School, Birmingham, AL 35294; and eDepartment of Reproductive Medicine, University of California at San Diego, La Jolla, CA 92039

Edited by Richard D. Palmiter, University of Washington, Seattle, WA, and approved May 28, 2014 (received for review February 21, 2014)

www.pnas.org/cgi/doi/10.1073/pnas.1403286111 PNAS

P

GPR54 | neuronal primary cilia | electrophysiology

Primary cilia are typically solitary nonmotile appendages that project from nearly every cell type in the mammalian body (1). They are specialized sensory organelles that incorporate a myriad of extracellular stimuli into signal transduction pathways to modulate cell physiology (2–4). Consequently, ciliary dysfunction can result in numerous human diseases, termed ciliopathies, which impact many organ systems (5). Ciliopathies are associated with certain neuropathologies, including structural malformations, hyperphagia-induced obesity, intellectual disability, and hypogonadism, thereby highlighting the importance of cilia for proper CNS development and function (3).

Most adult neurons in the mammalian brain possess a primary cilium that projects from its cell body. Specific signaling proteins are selectively targeted to and retained within neuronal cilia, which are restricted compartments and regulate entry and exit of proteins through multiple mechanisms (6, 7). These signaling proteins include type 3 adenyl cyclase (AC3) (8), which converts ATP to cAMP, and the GPCRs, somatostatin receptor 3 (Sstr3) (9), serotonin receptor 6 (10, 11), melanin-concentrating hormone receptor 1 (Mchr1) (12, 13), dopamine receptor 1 (14), (Sstr3) (9), serotonin receptor 6 (10, 11), melanin-concentrating hormone receptor 1 (Mchr1) (12, 13), dopamine receptor 1 (14), and neuropeptide Y receptors 2 and 5 (15). The functions of primary cilia are determined by the proteins that are enriched within them, thus, it is likely that neuronal cilia sense neuromodulators in the extracellular milieu and initiate signaling cascades. However, the precise roles of neuronal cilia remain unknown.

Identification of signaling proteins that are selectively targeted to neuronal cilia is a critical step in elucidating the functions of these organelles. We previously identified ciliary localization sequences in the third intracellular loop and carboxy tail of ciliary GPCRs and used these sequences to predict novel ciliary GPCRs (12, 14). One of these, the kisspeptin receptor (Kiss1r, also known as GPR54), was considered a strong candidate ciliary GPCR given that loss of Kiss1r leads to hypogonadotropic hypogonadism in humans and mice (16, 17) and hypogonadotropic hypogonadism is a feature of the ciliopathy Bardet-Biedl syndrome (18). Kiss1r is expressed in a large proportion of gonadotropin-releasing hormone (GnRH) neurons (19), a population of hypothalamic neurons that are central effectors driving the neuroendocrine reproductive axis. Treatment of Kiss1r-expressing GnRH neurons with kisspeptin increases the firing rate of the GnRH neurons and augments GnRH secretion. GnRH stimulates luteinizing hormone and follicle-stimulating hormone secretion, which in turn initiates puberty and supports adult sexual function.

Here, we show that Kiss1r is enriched in primary cilia on GnRH neurons in the mouse. Notably, GnRH neurons can possess multiple Kiss1r-positive cilia and the proportion of multiciliated GnRH neurons increases in parallel with sexual maturation. We also show that cilia are required for proper Kiss1r-mediated signaling on GnRH neurons. These results provide insight into the mechanism...
of Kiss1r signaling and demonstrate that loss of cilia on central neurons impairs neuronal signaling.

**Results**

**Kiss1r Localizes to Cilia.** To test whether Kiss1r localizes to cilia, a construct encoding mouse Kiss1r fused at the carboxy-terminus to EGFP was expressed in inner medulla collecting duct (IMCD) cells. We have previously shown that ciliary GPCRs selectively localize to cilia when expressed in IMCD cells (12, 14). Notably, Kiss1r localized to cilia on IMCD cells (Fig. 1 A–C). We then tested whether Kiss1r localizes to neuronal cilia in tissue by labeling mouse brain slices with a custom antibody generated against Kiss1r. The specificity of the antibody was confirmed by immunofluorescence and immunoblotting (Fig. S1). In the mouse brain there are only ~800 GnRH neurons, which are widely dispersed across the medial hypothalamus and basal forebrain (20). To facilitate identification of GnRH neurons we labeled brain slices from adult male and female transgenic mice expressing GFP (27). Sstr3::EGFP is efficiently targeted to cilia across cell types and CiliaGFP mice provide a powerful tool for visualizing cilia in vivo or in vitro. Labeling sections from the medial hypothalamus of CiliaGFP brains with an antibody against GnRH revealed the presence of GnRH neurons projecting one or more GFP-positive cilia (Fig. 2 A–C). Moreover, labeling of corresponding sections with the Kiss1r antibody confirmed a subset of Sstr3::EGFP expressing cilia were positive for Kiss1r (Fig. 2 D–F). Multiple cilia were not detected on any surrounding non-GnRH neurons (Table S1), which is consistent with previous studies of neuronal cilia frequency (28–31) and suggests the presence of multiple cilia on central neurons is rare. Together, our results verify that GnRH neurons can be multiciliated and demonstrate that Kiss1r is selectively targeted to cilia on GnRH neurons.

**Kiss1r Multiciliation Increases Over Development.** Given the important role of Kiss1r in sexual development, we tested whether Kiss1r ciliary localization is developmentally regulated. Brain slices of male and female GnRH::GFP mice from P0 to P60 were labeled for Kiss1r and the percentage of GnRH neurons possessing Kiss1r-positive cilia was quantified. The percentage of GnRH neurons displaying at least one Kiss1r-positive cilium was ~75% in both sexes at birth and did not change significantly during postnatal development (Fig. 3A). However, quantifying the percentage of GnRH neurons possessing more than one Kiss1r-positive cilium revealed that the frequency of multiciliated GnRH neurons significantly increased during postnatal development (Fig. 3B). At birth, ~10% of ciliated GnRH neurons in male and female mice possessed more than one Kiss1r-positive cilium but by P60 the percentages in male and female mice were 42% and 35%, respectively. This result indicates that the frequency of GnRH neurons possessing multiple Kiss1r-positive cilia increases during postnatal development in parallel with sexual maturation.

**GnRH Neuronal Cilia are not Essential for Sexual Maturation.** As Kiss1r is enriched in cilia and the proportion of neurons with multiple Kiss1r-positive cilia increases during development, we next asked whether GnRH neuronal cilia are required for proper sexual maturation in mice. To selectively ablate cilia on GnRH neurons, mice carrying a conditional allele of Ift88 (32), which encodes an intraflagellar transport protein and is required for the formation and maintenance of neuronal cilia (32–34), were

---

**Fig. 1.** Kiss1r localizes to primary cilia in vitro and in vivo. (A–C) Representative image of transiently transfected IMCD cells expressing Kiss1r fused at the C terminus to EGFP. (A) EGFP fluorescence (green) shows expression of Kiss1r. (B) Acetylated α-tubulin (AcTub; red) marks the cilia. (C) Merged image. (D–F) Representative image of the medial hypothalamus in adult GnRH::GFP mice. (D) GFP fluorescence (green) indicates a GnRH neuron. (E) Labeling for Kiss1r (red) shows the presence of multiple Kiss1r-positive cilia. (F) Merged image. (G) 3D rendering of the same neuron confirms the cilia project from the same cell. Nuclei are stained with DRAQ5 (blue). (Scale bars: 10 μm.)

**Fig. 2.** GnRH neurons possess cilia that are positive for Kiss1r. (A–C) Representative image of the medial hypothalamus in adult CiliaGFP mice. (A) EGFP fluorescence (green) shows Sstr3 expression and ciliary localization. (B) Labeling for GnRH (red) indicates GnRH neurons. (C) Merged image confirms GnRH neurons are ciliated. (D–F) Representative image of the medial hypothalamus in adult CiliaGFP mice. (D) EGFP fluorescence (green) shows Sstr3 expression and ciliary localization. (E) Labeling for Kiss1r (red) confirms Kiss1r ciliary localization. (F) Merged image. Nuclei are stained with DRAQ5 (blue). (Scale bars: 10 μm.)
crossed with transgenic mice expressing Cre recombinase under the control of the GnRH promoter (35). These mice also carried the 
GnRH::GFP transgene to allow identification and analysis of 
GnRH neurons. GnRH expression begins during embryonic 
development and is detected as early as E11.5 (36). Control ani-
mals possessed an 
exception of P0, = n

3). Note the proportion of 
multiciliated GnRH neurons increases significantly between P0 and P60. 1 Significantly different from P0 
(P < 0.01). 2Significantly different from P5 (P < 0.01). 3Significantly different from P5 (P < 0.001). (C) Percentage 
of GnRH neurons with one or more Kiss1r-positive cilia in the medial 
hypothalamus of P0-P60 male and female GnRH::GFP mice (n = 4 animals of each sex at all ages, with the 
exception of P0, = n = 3). Note the proportion of 
multiciliated GnRH neurons increases significantly between P0 and P60. 1 Significantly different from P0 
(P < 0.01). 2Significantly different from P0 (P < 0.01). 3Significantly different from P5 (P < 0.001). (C) Percentage 
of GnRH neurons with one or more Kiss1r-positive cilia in the medial 
hypothalamus of P0 male GnRH::GFP, GnRH<sup>il</sup> mice, and 
GnRH<sup>il</sup> mice (n = 3-4 animals for each genotype). Note that there is no difference in the percentage 
of Kiss1r-positive cilia between GnRH::GFP and GnRH<sup>il</sup> mice, but Kiss1r-positive cilia are completely 
lacking in GnRH<sup>il</sup> mice. (D) Number of GnRH neurons 
throughout the medial septum (MS), rostral preoptic area (rPOA), and anterior 
hypothalamic area (AHA) of P60 male GnRH::GFP and 
GnRH<sup>il</sup> mice (n = 3 animals for each genotype). Note there is no significant difference in the number of 
GnRH neurons in any region between GnRH<sup>il</sup> and GnRH<sup>il</sup> mice. Rates are expressed as mean ± SEM.

To test whether loss of Kiss1r-positive cilia on GnRH neurons 
causes defects in sexual maturation in mice, we quantified sex 
organ weights and day of vaginal opening, a measure of puberty 
onset in female mice. None of these measures was significantly 
different between GnRH<sup>il</sup> and GnRH<sup>il</sup> animals (Table S2). 
Furthermore, histological analysis revealed mature sperm in the 
testes and seminiferous tubules of adult GnRH<sup>il</sup> mice (Fig. 4 
A and B) and follicles in all stages of development in the ovaries 
of adult GnRH<sup>il</sup> females (Fig. 4 C and D). Estrous cycle 
tracking in GnRH<sup>il</sup> and GnRH<sup>il</sup> females revealed no 
obvious differences in cycling between the two groups (Fig. 4E). 
Finally, male and female GnRH<sup>il</sup> mice were fertile and 
generated normal numbers and litter sizes. These results indicate 
that cilia on GnRH neurons are not required for reproductive 
function in mice.

**GnRH Cilia are Required for Proper Kiss1r-Mediated Signaling.** 
Because Kiss1r is normally targeted to and enriched within 
GnRH cilia, we then asked whether loss of cilia affects Kiss1r signaling. 
Application of the Kiss1r ligand, kisspeptin, increases the action 
potential firing rate of GnRH neurons (38) and this response is 
absent in GnRH neurons lacking Kiss1r (39). Thus, we 
performed extracellular loose-cell attached patch-clamp recording 
on acute sagittal brain slices from adult male and female 
GnRH<sup>il</sup> and GnRH<sup>il</sup> animals. The female mice were overanesthetized to reduce potential variability from the estrous cycle. There was no difference in the baseline firing rate of GnRH neurons between 
male or female GnRH<sup>il</sup> and GnRH<sup>il</sup> mice (Fig. 5 A and B) 
and application of 100 nM kisspeptin resulted in an increase in 
firing rate in the vast majority of GnRH neurons, consistent with 
previous results (38). Interestingly, GnRH neurons from male 
GnRH<sup>il</sup> mice showed a significantly reduced kisspeptin-mediated 
increase in firing rate than GnRH neurons in GnRH<sup>il</sup> animals (Fig. 5C). The kisspeptin-mediated increase in firing rate 
was also reduced in GnRH neurons of female GnRH<sup>il</sup> mice 
compared with GnRH<sup>il</sup> mice, but this difference was not 
statistically significant (Fig. 5D). To confirm that the decreased 
responsiveness to kisspeptin in male GnRH<sup>il</sup> mice was not 
attributable to a decrease in Kiss1r expression, we performed 
real-time PCR analysis of hypothalamic RNA from adult male 
GnRH<sup>il</sup> and GnRH<sup>il</sup> mice and found that Kiss1r was ex-
pressed at equivalent levels in both genotypes (Fig. 5E). We 
also observed an equivalent kisspeptin-induced induction of Fos 
expression, a response that is mediated by Kiss1r (40), in GnRH<sup>il</sup> 
and GnRH<sup>il</sup> male mice (Fig. 5E). Together, our results suggest 
that in the absence of cilia Kiss1r is present and functional on the 

![Fig. 3.](image-url) Quantification of GnRH cilia. (A) Percentage of 
GnRH neurons with one or more Kiss1r-positive cilia in the medial 
hypothalamus of P0-P60 male and female GnRH::GFP mice (n = 4 animals of each sex at all ages, with the 
exception of P0, = n = 3). Note the percentages do not vary significantly be-
tween ages or sexes. (B) Percentage of ciliated GnRH neurons with one 
or more Kiss1r-positive cilia in the medial hypothalamus of P0-P60 male 
and female GnRH::GFP mice (n = 4 animals of each sex at all ages, with the 
exception of P0, = n = 3). Note the proportion of 
multiciliated GnRH neurons increases significantly between P0 and P60. 1 Significantly different from P0 
(P < 0.01). 2Significantly different from P0 (P < 0.01). 3Significantly different from P5 (P < 0.001). (C) Percentage 
of GnRH neurons with one or more Kiss1r-positive cilia in the medial 
hypothalamus of P0 male GnRH::GFP, GnRH<sup>il</sup>, and 
GnRH<sup>il</sup> mice (n = 3-4 animals for each genotype). Note there is no difference in the percentage 
of Kiss1r-positive cilia between GnRH::GFP and GnRH<sup>il</sup> mice, but Kiss1r-positive cilia are completely 
lacking in GnRH<sup>il</sup> mice. (D) Number of GnRH neurons 
throughout the medial septum (MS), rostral preoptic area (rPOA), and anterior 
hypothalamic area (AHA) of P60 male GnRH::GFP and 
GnRH<sup>il</sup> mice (n = 3 animals for each genotype). Note there is no significant difference in the number of 
GnRH neurons in any region between GnRH<sup>il</sup> and GnRH<sup>il</sup> mice. Values are expressed as mean ± SEM.

![Fig. 4.](image-url) GnRH cilia are dispensable for sexual maturation in male and female 
mice. (A and B) Representative tests (Left) and seminiferous tubule (Right) 
sections from P60 GnRH<sup>il</sup> (A) and GnRH<sup>il</sup> (B) mice (n = 3 animals for 
each genotype) shows the presence of mature sperm in both genotypes, 
(C and D) Representative ovary section from P60 GnRH<sup>il</sup> (C) and 
GnRH<sup>il</sup> (D) mice (n = 3 animals for each genotype) shows the presence of follicles 
at all stages of development. (Scale bars: 100 μm.) (E) Vaginal cytology of P60 
GnRH<sup>il</sup> and GnRH<sup>il</sup> mice show all stages of estrous cyclicity, C, cornified 
(estrous); L, leukocytic (mestrous and diestrous); N, nucleated (proestrus).
GnRH cilia are required for proper Kiss1r signaling. Basal firing rates of GnRH neurons from adult male (A) and ovariectomized female (B) GnRH<sup>Wild</sup> and GnRH<sup>Null</sup> mice. Note there is no significant difference in the basal firing rates between GnRH<sup>Wild</sup> and GnRH<sup>Null</sup> mice. Percentage increase in the firing rate of GnRH neurons after kisspeptin treatment in adult male (C) and ovariectomized female (D) GnRH<sup>Wild</sup> and GnRH<sup>Null</sup> mice. Note the increase in firing rate is significantly lower in male GnRH<sup>Null</sup> mice compared with GnRH<sup>Wild</sup> mice. Values are expressed as mean ± SEM. For males n = 10–13 neurons from 4 to 7 animals of each genotype. For females n = 9 neurons from 4 to 5 animals of each genotype. *Significantly different from GnRH<sup>Wild</sup> percentage (P = 0.02).

**Discussion**

Our results show that GnRH neurons possess cilia, which are enriched for Kiss1r. There are several theories regarding the possible functions of cilia on adult central neurons. Because cilia are enriched for signaling proteins, they may well function as sensory organelles. However, a direct role for cilia in neuronal signaling has not been observed. Here, we demonstrate that loss of Kiss1r-positive cilia on GnRH neurons causes a reduction in the response of GnRH neurons to kisspeptin in male mice, indicating that cilia enhance Kiss1r signaling. To our knowledge, this is the first demonstration that cilia play a role in GPCR signaling in central neurons. What is the role of cilia in Kiss1r signaling? One possibility is that Kiss1r normally signals on the ciliary membrane and ciliary localization concentrates the receptors to optimize ligand binding and signal propagation. There is evidence for both synaptic (41, 42) and volume (43) transmission of kisspeptin to GnRH neurons. Ciliary response to ligand should be involved in volume transmission as cilia project from the cell body and synaptic contacts have never been observed on neuronal cilia. Enriching and concentrating receptors in multiple long cilia protruding from the same neuron could thus increase the efficacy of kisspeptin binding in the extracellular space and enhance volume transmission. This model would be analogous to olfactory sensory neurons that project multiple primary cilia into the olfactory epithelium, which increases the sensory surface and the ability to detect and respond to odors (44). Another possibility is that the signal generated by Kiss1r on the ciliary membrane is qualitatively different from the signal generated on the plasma membrane. The expression of Kiss1r on cilia may generate a unique signal based on coupling to different signaling transduction pathways and/or regulation of these signaling cascades. Loss of this signal may affect the overall response to kisspeptin. Alternatively, Kiss1r may not directly signal on the ciliary membrane, but rather, the cilium may act as a reservoir for the receptor and facilitate signaling. Regardless of the mechanisms, our results show that cilia are necessary for normal Kiss1r signaling on GnRH neurons.

We also demonstrate that GnRH neurons possess multiple Kiss1r-positive cilia and the proportion of multiciliated GnRH neurons increases over pubertal maturation. Because GnRH neurons become more responsive to the effects of kisspeptin over development (45), we suggest that the increase in Kiss1r-positive cilia per neuron may contribute to this process. Our results also show that ablation of cilia on GnRH neurons affects neither puberty nor adult reproductive function in mice; this is perhaps not surprising, because there is incredible redundancy in the Kiss1r signaling pathway in the brain, which supports reproductive function with only minute quantities of Kiss1 activity (46). In addition, there is evidence for Kiss1r signaling at the nerve terminals (47), which could overcome the loss of Kiss1r ciliary signaling. Given that GnRH release is a function of firing rate, we suggest that ablation of cilia at the cell body leads to a reduction in GnRH release at the nerve terminals but not below the threshold for sexual maturation.

We also demonstrate that cilia are not required for GnRH neuron migration. GnRH neurons originate in the olfactory placode/vomeronasal organ and migrate into the hypothalamus. This migration and targeting is a highly orchestrated process requiring a variety of factors and disruption of GnRH neuron migration is associated with deficits in reproductive function (48). Cilia have recently been shown to influence migration of interneurons of the developing cerebral cortex (49) and neurons migrating from the medial ganglionic eminence (50). We did not observe any alterations in either the number or location of GnRH neurons in newborn or adult GnRH<sup>Wild</sup> mice, suggesting that cilia are not necessary for developmental migration of these cells.

Conditional disruption of Ift88 is a well-established method for ablating cilia in a cell-type-specific manner (51) and has been effectively used to disrupt cilia on central neurons (32–34). Although we saw a complete lack of Kiss1r-positive cilia in adult GnRH<sup>Wild</sup> mice, the fact that GnRH neurons are not positive for any canonical ciliary markers prevents us from validating the loss of cilia structure. Thus, it is feasible that disrupting Ift88 in GnRH neurons prevents Kiss1r ciliary localization without affecting cilia structure. Regardless, our results suggest that Kiss1r ciliary localization is necessary for proper kisspeptin-mediated signaling in male mice. It is also possible that loss of Ift88 has additional functional consequences that impact GnRH neuronal firing. However, it should be noted that the baseline firing rate was the same in GnRH<sup>Wild</sup> and GnRH<sup>Null</sup> neurons.

There are other possible links between cilia function and reproductive maturation. Hypogonadotropic hypogonadism is a typical clinical feature of Bardet-Biedl syndrome, a human ciliopathy. Another putative link is suggested by the finding that WDR11, which is mutated in patients with hypogonadotropic hypogonadism (52), is present within primary cilia (53). However, our results indicate that disruption of GnRH neuronal cilia structure is not sufficient to prevent normal sexual maturation in mice. It is conceivable that differences among species account for the discordant results; however, another possibility is that hypogonadotropic hypogonadism in humans with BBS reflects ciliary dysfunction on either multiple neuronal subtypes or other nonneuronal cells within the reproductive circuit. Alternatively, BBS and WDR11 proteins may have additional nonciliary functions that are required for reproductive maturation and function.

In summary, we have shown that Kiss1r is enriched in cilia on GnRH neurons, thereby implicating neuronal cilia in kisspeptin signaling and reproduction. We also demonstrate that disruption of cilia on GnRH neurons reduces Kiss1r signaling, indicating that cilia enhance Kiss1r signaling. Thus, cilia are likely to play a broad, hitherto unappreciated, role in the function of the brain.
Materials and Methods

Plasmid Construction. The coding sequence for Kiss1r was amplified from cDNA generated from reverse-transcribed mouse whole brain RNA using the SuperScript First-Strand Synthesis RT-PCR kit (Invitrogen). The coding sequence was then cloned into the TA cloning vector pSTBlue-1 (Novagen), with primers at the C- and N-terminal regions designed for directional cloning. The Kiss1r construct was then subcloned into pEGFP-N (Clontech). All DNA constructs were sequence verified at the Nucleic Acid Shared Resource at Ohio State’s Comprehensive Cancer Center.

Cell Culture and Transient Transfections. IMCD-3 cells (ATCC) were maintained in DMEM:F12 media supplemented with 10% (vol/vol) FBS, 1.2 g/L of sodium bicarbonate, and 0.5 mM sodium pyruvate (Invitrogen). Cells (n = 5 × 10^6) were electroporated with 10 μg DNA and plated at high density on glass coverslips. Cells were re-fed 16–18 h after transfection and harvested at 48 h after transfection by fixation in 4% (wt/vol) paraformaldehyde.

Mice and Tissue Preparation. All animal procedures described are in accordance with institutional guidelines based on National Institutes of Health Standards, and were performed with Institutional Animal Care and Use Committee approval at the Ohio State University, University of California at San Diego, and the University of Alabama at Birmingham. All animals were maintained in a temperature and humidity controlled vivarium with 12 h light/dark cycle and given access to food and water ad libitum. Littermates were group housed by sex, no more than five to a cage, after weaning. CiliaKoemeter-Cox et al. PNAS | July 15, 2014 | vol. 111 | no. 28 | 10339

neuron was centered under the objective, and the filter set was switched to the 546 wavelength. If a Kiss1r-positive cilium emanated from the same area on the GFP+ cell body, it was considered a ciliated cell. If more than one cilium emanated from a single GFP+ cell body, the neuron was considered multiciliated. The number of ciliated GFP+ neurons was counted using a manual cell counter. The percentage of ciliated GnRH neurons was calculated by dividing the number of GFP+ neurons possessing one or more cilium by the total number of GFP+ neurons and multiplying by 100. The percentage of multiciliated GnRH neurons was calculated by dividing the number of GFP+ neurons possessing multiple cilia by the number of ciliated GFP+ neurons and multiplying by 100. Statistical analysis was performed using Graphpad Prism (Graphpad Software). Analysis of the percentages of GnRH neurons possessing Kiss1r-positive cilia across age and sex was performed using ANOVA with post hoc tukey test for multiple comparisons. Analysis of the percentages of GnRH neurons possessing Kiss1r-positive cilia between genotypes was performed using Student t test. A P value < 0.05 was considered significant.

Histology. Hematoxylin/eosin staining of testes and ovaries sections was performed according to the manufacturer’s instructions (BBC Biochemical) kit directions. Stained sections were mounted with Permount (Fisher Scientific) and imaged on a Zeiss LSM 510 laser scanning confocal microscope using an Axiocam and Axiocam software.

Vaginal Opening and Estrous Cycle Determination. Time of vaginal opening was determined by daily inspection of mice between 9 and 11:00 AM from P20 onward. Estrous cycle samples were collected daily between 9 and 11:00 AM from group housed animals via vaginal flush with 1× PBS. Samples were deposited on SuperFrost Plus slides, allowed to dry and stained with a Kwikk-Diff kit (Thermo Scientific) according to the manufacturer’s instructions. Slides were scanned to dry and examined on a Zeiss Axioskop 2 MOT microscope by an individual blinded to the genotypes of the animals. Criteria for the different phases of the estrous cycle have been described previously (57).

Slice Preparation for Electrophysiology. Procedures were adapted from a previous study (58). Briefly, adult mice (age 6–7 wk) were euthanized by decapitation, and whole brains were removed and immediately submerged in ice cold high-sucrose extracellular solution for 3 min [208 mM sucrose, 2 mM KCl, 2.6 mM NaH2PO4, 2 mM MgCl2, 2 mM CaCl2, 124 mM NaCl, 5 mM Hepes, 10 mM glucose, oxygenated (95% O2, 5% CO2)] at 25 °C for 1.5–3.5 h before use in electrophysiology experiments [aCSF: 124 mM NaCl, 5 mM KCl, 2.6 g/L NaHPO4, 2 mM MgCl2, 2 mM CaCl2, 26 mM NaHCO3, 10 mM Hepes, 10 mM glucose, oxygenated (95% O2, 5% CO2), pH 7.4]. Cerebral tissue was then removed, and 250 micron sagittal slices were cut from the tissue block containing the diagonal band of the preoptical area (DB-POA) using a manual advance vibroslicer (World Precision Instruments) in ice cold high-sucrose extracellular solution (oxygenated). Slices were immediately transferred upon isolation to an auxiliary chamber containing artificial CSF (aCSF) at 25 °C for 1.5–3.5 h before use in electrophysiology experiments [aCSF: 124 mM NaCl, 5 mM KCl, 2.6 g/L NaHPO4, 2 mM MgCl2, 2 mM CaCl2, 26 mM NaHCO3, 10 mM Hepes, 10 mM glucose, oxygenated (95% O2, 5% CO2), pH 7.4].

Electrophysiology Recording and Data Analysis. Individual GnRH neurons in tissue slices were visually selected for recording by positive detection of GFP expression (Nikon eclipse 80iFBN epifluorescence microscope with 40x water-immersed objective). Loose cell-attached patch configuration in current-clamp mode (i = 0) was used to record extracellular action potential firing of the selected GnRH neurons. Recording pipettes with 2.5–4.0 MΩ (bath resistance) were filled with aCSF. At time of recording, seal resistance was between 18–100 MΩ. Recordings were obtained using an EPC10 amplifier (HEKA) with Patchmaster software (HEKA); the sampling frequency was 5 kHz. Intracellular CSD (25 ± 5% of Csh) was oxygenated with 95% O2 /5% CO2 and perfused over slices at rate of 1.4 ml/min with a peristaltic pump (Peri-Star Pro World Precision Instruments). Basal firing rate was recorded for at least 3 min before treatment with 100 nM KISS (112-121) Amide (Phoenix Pharmaceuticals) in aCSF for 4.5 min. Data were analyzed with Clampfit 9.2 software (Axon), and average firing rate was determined from the action potential events per time for each condition. “% Change in Firing Rate” was determined from the difference in average firing rates with peptide normalized to the average basal firing rate for each cell. Statistical significance was assessed with a two-tailed T test (unpaired data), a P value < 0.05 was considered significant.

ACKNOWLEDGMENTS. We thank Jackie Domire for contributions to this project, Tiansen Li [National Institutes of Health (NIH)/National Eye Institute] for the rootletin antibody, and the University of Alabama at Birmingham Hepatorenal Fibrocytic Kidney Disease Core Center (UAB Institute) for the Nucleoside Shared Resource at Ohio State’s Comprehensive Cancer Center.
RPKDC. P30 DK074038 for providing the CiliaGFP mice. This work was supported by NIH/National Institute of General Medical Science research Project Grant R01 GM083120 (to K.M.), NIH/Eunice Kennedy Shriver National Institute of Child Health and Human Development research Project Grant R01 HD065856 (to A.S.K.), NIH/Office of Research Infrastructure Programs Shared Instrumentation Grant S10 OD010383 (to A.B.), and NIH/ National Institute of Neurological Disorders and Stroke Center Core Research Project P30 NS045758.

Supporting Information

Koemeter-Cox et al. 10.1073/pnas.1403286111

SI Materials and Methods

**Protein Isolation and Immunoblotting.** HEK293T cells (ATCC) were maintained in DMEM supplemented with 10% (vol/vol) FBS and 1.5 g/l of sodium bicarbonate (Invitrogen). pcDNA3.1 encoding Kiss1r::myc or nothing was transfected by electroporation into HEK293T cells. After 48 h, proteins were isolated and immunoblotted, as described (1).

**Immunofluorescence.** The Kiss1r knockout (KO) mice have been described (2). To label sections from Kiss1r WT and KO mice with rabbit anti-GnRH (gonadotropin-releasing hormone) and anti-Kiss1r simultaneously, we used a modified double labeling protocol. Following tissue blocking and permeabilization, sections were incubated with anti-Kiss1r at 1:5,000 overnight at 4 °C, washed with PBS, and incubated with Alexa Fluor 546-conjugated goat anti-rabbit IgG for 1 h at room temperature. Sections were washed with PBS and incubated with 5% (vol/vol) normal rabbit serum in PBS for 1 h at room temperature to saturate open binding sites on the first secondary, followed by washing with PBS and incubation with 20 μg/mL goat anti-rabbit Fab fragments (Jackson ImmunoResearch) in PBS for 2 h at room temperature to block any exposed rabbit IgG binding sites. Sections were then washed with PBS and incubated with anti-GnRH at 1:1,000 in PBS overnight at 4 °C. Sections were washed with PBS, incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG in PBS, washed with PBS again, and mounted. Rabbit anti-adenyl cyclase III (C-20; Santa Cruz Biotechnology) was used at 1:350. GnRH::GFP tissue was labeled with chicken anti-rootletin (3) at 1:1,000 and anti-Kiss1r at 1:5,000. Secondary antibodies were donkey anti-chicken Cy3 (Jackson ImmunoResearch) and Alexa Fluor 647-conjugated donkey anti-rabbit IgG (Invitrogen).

**Quantification of Cilia.** In sections from Kiss1r WT and KO mice (n = 3 of each genotype), GnRH neurons were identified and examined for the presence of a Kiss1r-positive cilium. The total number of GnRH neurons with Kiss1r-positive cilia was divided by the total number of GnRH neurons counted and multiplied by 100 to obtain the percentage of GnRH neurons possessing Kiss1r-positive cilia. At least 35 GnRH neurons were counted across sections from each genotype. Sections from Cilia GFP mouse brains were labeled with an antibody against GnRH and images of non-GnRH neurons expressing GFP+ cilia were taken. The non-GnRH+ nuclei in each image were counted and then examined for GFP+ cilia. At least 40 nuclei were counted in each section.

**Quantification of GnRH Neurons.** For P0 neuron migration analysis, sections for P0 animals were matched to plate 13 of the coronal gestation day (GD) 18 atlas from the Schambra, Lauder and Silver prenatal mouse brain atlas (4). For each animal, four sections containing the paraventricular hypothalamic nucleus (pHV) were analyzed. Reductions in the number of GnRH neurons present in this caudal region at P0 is an indication of aberrant GnRH neuronal migration (5). The number of GFP+ neurons in this particular region was determined by examination under the 20× objective. The numbers counted in the four sections were added together to obtain the number for each genotype. The numbers from each animal were then averaged to calculate the mean and SE by genotype.

**Real-Time PCR.** Total RNA was made from the hypothalami of adult GnRH<sup>clus+</sup> and GnRH<sup>clus−</sup> mice (n = 4 animals of each genotype) using the RNeasy Miniprep kit (Qiagen), according to manufacturer’s instructions. The extracted RNA was treated with DNase using the Ambion DNA-free Kit (Ambion) to eliminate genomic DNA contamination. Oligo(dT) <sub>12-18</sub> primed cDNA was made from purified RNA using Invitrogen SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). For each sample, 300 ng of RNA was used in the cDNA synthesis reaction. Control “No RT” reactions lacking reverse transcriptase were also performed to test for genomic DNA contamination. cDNA was quantified by qPCR, using the POWER SYBR Green Master Mix (Applied Biosystems) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The primers used for β actin were forward 5′-tagctacctacccacagc-3′ and reverse 5′-cttccagggagagcttca-3′ to produce a 121-bp product. The primers used for Kiss1r were forward: 5′-tcttacgcactctacctc-3′ and reverse: 5′-caca-taccagtcacact-3′ to produce a 138-bp product. The primers used for GnRH were forward: 5′-aactgctctatatgggttgc-3′ and reverse: 5′-cgaaagctgctcatcc-3′ to produce a 106-bp product. The efficiency of each primer pair was tested. Reactions were set up in triplicate. Per well, 20-μL reactions consisted of: 10 μL of 2x Power SYBR Green Master Mix, 7.8 μL of DEPC treated DNase, RNase free water (Invitrogen), 0.6 μL of each forward and reverse primer at 10 μM concentration (Integrated DNA Technologies), and 1 μL of diluted cDNA. Data were normalized to mouse β actin expression and was analyzed to determine ΔCt, of Kiss1r and GnRH expression. Cycle threshold was taken at 0.2 ΔRn. Melt curve analysis indicated single products.

**Fos Labeling.** Gonad intact adult male GnRH<sup>clus+</sup> and GnRH<sup>clus−</sup> mice were anesthetized with isoflurane and then injected ICV with either aCSF (vehicle) or aCSF containing 200 pmol KISS121 Amide. Two hours after injection, mice were transcardially perfused with 4% (wt/vol) paraformaldehyde and brains were collected for immunofluorescence. Sections were labeled with Rabbit anti-C-Fos (sc-253; Santa Cruz) and Alexa Fluor 546-conjugated goat anti-rabbit IgG, along with DRAQ-5 as a nuclear marker. Five sections containing GFP+ neurons from each animal were evaluated. The total number of GFP+ neurons with C-Fos positive nuclei was divided by the total number of GFP+ neurons counted in each animal and multiplied by 100 to obtain the percentage of C-Fos−positive GnRH neurons in each animal. At least 50 GFP+ neurons were evaluated per animal.

---

**Fig. S1.** Kiss1r polyclonal antibody specifically recognizes mouse Kiss1r in immunofluorescent and Western blot analysis. (A–C) Representative image of transiently transfected IMCD cells expressing Kiss1r fused at the carboxy-terminus to EGFP. (A) EGFP fluorescence (GFP; green) shows expression of Kiss1r. (B) Kiss1r polyclonal antibody (Kiss1r; red) marks Kiss1r. (C) Merged images confirming anti-Kiss1r labels Kiss1r::EGFP. (Scale bar: 10 μm.) (D) Extracts from HEK293T cells expressing myc-tagged Kiss1r::myc analyzed by Western blotting (IB) with anti-Kiss1r (Left) and anti-myc (Right). Extracts from HEK293T cells expressing empty vector (EV) are included as a negative control. Note the presence of a band around 43 kDa, which is the predicted molecular weight of Kiss1r::myc, specifically in extracts from cells expressing Kiss1r::myc. (E and F) Representative images of the medial hypothalamus in adult Kiss1r WT (E) and KO (F) mice (n = 3 animals of each genotype) colabeled with anti-GnRH (green) and anti-Kiss1r (red). Corresponding Insets (a and b) show higher magnification images of the boxed regions containing GnRH-positive neurons. Note that GnRH neurons in the WT section possess Kiss1r-positive cilia, whereas GnRH neurons in the KO section do not. (Scale bars: 50 μm in main images and 10 μm in insets.) (G) The majority of GnRH neurons in sections from Kiss1r WT mice possessed Kiss1r-positive cilia (24 of 36 GnRH neurons) in Kiss1r-positive cilia were not detected (0 of 35 GnRH neurons) in Kiss1r KO sections. (H and I) Representative images of the medial hypothalamus in adult Kiss1r WT (H) and KO (I) mice (n = 3 animals of each genotype) labeled with anti-AC3 (green). Note the presence of AC3-positive cilia in both genotypes. Nuclei were stained with DRAQ5. (Scale bar: 10 μm.)
Fig. S2. GnRH neurons project multiple Kiss1r-positive cilia and cilia on non-GnRH neurons are positive for AC3. (A and B) Representative images of the medial hypothalamus in adult GnRH::GFP mice. GFP fluorescence (green) indicates a GnRH neuron. Labeling for Kiss1r (red) shows the presence of multiple Kiss1r-positive cilia. Two-dimensional projections (Left) and corresponding 3D renderings (Right) are shown. Nuclei are stained with DRAQ5 (blue). (Scale bar: 10 μm.) (C and D) Representative images of the medial hypothalamus in adult GnRH::GFP mice. GFP fluorescence (green) indicates a GnRH neuron. Labeling for Kiss1r (red) shows the presence of multiple Kiss1r-positive cilia. Labeling for Rootletin (white) marks the base of each cilium and confirms the cilia are projecting from the same neuron. The cell bodies are displayed as maximum projections and the cilia and rootlets are isosurfaced. Note in C the presence of two cilia that project from the cell in parallel and diverge at their tips (indicated by an arrow). (Scale bars: 5 μm.) (E and F) Representative images of the medial hypothalamus in adult GnRH\textsuperscript{tie+} (E) and GnRH\textsuperscript{tie−} (F) mice. EGFP fluorescence (green) indicates a GnRH neuron. Labeling for AC3 (red) confirms cilia on non-GnRH neurons are positive for AC3 in both GnRH\textsuperscript{tie+} and GnRH\textsuperscript{tie−} mice. Note the lack of AC3-positive cilia on GnRH neurons. Nuclei are stained with DRAQ5 (blue). (Scale bars: 50 μm in main images and 5 μm in Insets.)
Fig. S3.  Loss of Kiss1r-positive cilia primarily occurs prenatally in both male and female GnRH<sup>cilia+</sup> mice and does not impact GnRH neuronal migration. (A) Percentages of GnRH neurons with a Kiss1r-positive cilium in the medial hypothalamus of P0 male and female GnRH<sup>cilia+</sup> and GnRH<sup>cilia-</sup> mice (n = 3 animals for each sex and genotype). There is no significant difference between the percentage of GnRH neurons with cilia in male and female GnRH<sup>cilia+</sup> mice or male and female GnRH<sup>cilia-</sup> mice. (B and C) Representative images of the medial hypothalamus in adult GnRH<sup>cilia+</sup> (B) and GnRH<sup>cilia-</sup> (C) mice. EGFP fluorescence (green) indicates a GnRH neuron. Labeling for Kiss1r (red) shows the presence of cilia in GnRH<sup>cilia+</sup> mice (B) and the absence of cilia in GnRH<sup>cilia-</sup> mice (C). Nuclei were stained with DRAQ5. (Scale bar: 5 μm.) (D) Number of GnRH neurons in the paraventricular hypothalamic nucleus of P0 male GnRH<sup>cilia+</sup> and GnRH<sup>cilia-</sup> mice (n = 3 animals for each genotype). Note there is no significant difference in the number of GnRH neurons in this region between GnRH<sup>cilia+</sup> and GnRH<sup>cilia-</sup> mice. Values are expressed as mean ± SEM.
Fig. S4. Kiss1r is expressed at equivalent levels in GnRH+ and GnRH− mice. Real-time PCR data representing the relative level of Kiss1r (A) and GnRH (B) mRNA expression compared with β-actin (Kiss1r or GnRH mRNA/β-actin mRNA) as determined by quantitative real time PCR using the SYBR green method. Note the relative level of Kiss1r mRNA in the hypothalamus of GnRH+ mice is not significantly different from GnRH mice (P = 0.384). RNA was extracted from hypothalamic tissue from adult male GnRH+ and GnRH− mice (n = 4 animals for each genotype). Values are expressed as mean ± SEM.
Intracerebroventricular injection of kisspeptin induces Fos expression in GnRH cilia+ and GnRH cilia− mice. GnRH neurons (green) from GnRH cilia+ (A) and GnRH cilia− (B) gonad intact male mice (n = 3 animals for each genotype) treated with aCSF (vehicle; Upper) show little Fos expression (red). GnRH neurons from GnRH cilia+ and GnRH cilia− mice (n = 3 animals for each genotype) treated with 200 pmol kisspeptin (KP-10; Lower) show induction of Fos expression (red). (C) Percentages of GnRH neurons positive for Fos labeling in GnRH cilia+ and GnRH cilia− mice in response to aCSF and kisspeptin injection. The percentages of GnRH neurons positive for Fos labeling is low in response to aCSF injection but is significantly increased in response to kisspeptin injection in both GnRH cilia+ and GnRH cilia− mice. There was no significant difference in Fos expression between genotypes in response to aCSF or kisspeptin injection. Values are expressed as mean ± SEM *Significantly different from aCSF injection (P < 0.05).

Table S1. Quantification of cilia frequency on non-GnRH nuclei

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>Ciliated nuclei</th>
<th>Multiciliated nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>337</td>
<td>240</td>
<td>0</td>
</tr>
</tbody>
</table>

Frequency of cilia and multiple cilia on non-GnRH nuclei in medial hypothalamic sections (n = 2) from P60 CiliaGFP mice (n = 3).
Table S2. Physiological measures of sexual maturation in GnRH<sup>cilia+</sup> and GnRH<sup>cilia−</sup> mice

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GnRH&lt;sup&gt;cilia+&lt;/sup&gt;</td>
<td>GnRH&lt;sup&gt;cilia−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>22.6 ± 0.85</td>
<td>23.8 ± 0.087</td>
</tr>
<tr>
<td>Sex organ weight, mg</td>
<td>153.4 ± 9.1</td>
<td>146.4 ± 9.5</td>
</tr>
<tr>
<td>Time of vaginal opening, day</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Physiological measurements from GnRH<sup>cilia+</sup> and GnRH<sup>cilia−</sup> animals ages 2.5–4 mo (<i>n</i> = 8–12). Sex organ refers to both testis for each male and both ovaries for each female. Values are expressed as the mean ± SEM.

**Movie S1.** A full rotation (360 degrees) around the vertical axis of the 3D rendering presented in Fig. 1G. Movie length is 5 s with 20 frames per s.

**Movie S2.** A full rotation (360 degrees) around the horizontal axis of the 3D rendering presented in Fig. S2A. Movie length is 5 s with 20 frames per s.
Movie S3. A full rotation (360 degrees) around the vertical axis of the 3D rendering presented in Fig. S2B. Movie length is 5 s with 20 frames per s.

Movie S4. A full rotation (360 degrees) around the vertical axis of the 3D rendering presented in Fig. S2C. Movie length is 10 s with 20 frames per s.
Movie S5. A full rotation (360 degrees) around the vertical axis of the 3D rendering presented in Fig. S2D. Movie length is 10 s with 20 frames per s.