Murine Arcuate Nucleus Kisspeptin Neurons Communicate with GnRH Neurons *In Utero*

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Puberty is a transition period of reproductive development from juvenile stages to adulthood and depends upon the activity of gonadotropin-releasing hormone (GnRH) neurons. GnRH neurons are initially activated *in utero* but remain quiescent throughout the juvenile period. Premature reactivation of GnRH neurons results in precocious puberty in mice and humans, but the mechanisms underlying developmental control of GnRH neuron activity remain unknown. The neuropeptide kisspeptin, a potent activator of GnRH neurons that is implicated as a critical permissive signal triggering puberty and a major regulator of the adult female hypothalamus–pituitary–gonadal axis, is paradoxically produced by neurons in the developing brain well before puberty onset. Thus, the neural circuits controlling the timing of reproductive maturation remain elusive. Here, we delineate the underlying neural circuitry using conditional genetic transsynaptic tracing in female mouse embryos. We find that kisspeptin-producing neurons in the arcuate nucleus (ARC) already communicate with a specific subset of GnRH neurons *in utero*. We show that ARC kisspeptin neurons are upstream of GnRH neurons, and that GnRH neuron connectivity to ARC kisspeptin neurons does not depend on their spatial position in the brain. Furthermore, we demonstrate that the neural circuits between ARC kisspeptin and GnRH neurons are fully established and operative before birth. Finally, we find that most GnRH neurons express the kisspeptin receptor GPR54 upon circuit formation, suggesting that the signaling system implicated in gatekeeping puberty becomes operative in the embryo.

Key words: Cre recombinase; gene targeting; genetic transneuronal tracing; GPR54; kisspeptin; puberty

Introduction

Gonadotropin-releasing hormone (GnRH) neurons originate from the olfactory placode and migrate into the brain during embryonic maturation (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). After initial activation in the embryo (Aubert et al., 1985; Terasawa and Fernandez, 2001; Sisk and Foster, 2004; Wen et al., 2010), GnRH neurons are restrained and remain quiescent throughout the juvenile period until the onset of puberty (Bronson, 1981; Teles et al., 2008; Styne and Grumbach, 2008; Mayer et al., 2010; Abreu et al., 2013). Mice and humans harboring inactivating mutations in the genes encoding the neuropeptide kisspeptin (d'Anglemont de Tassigny et al., 2007; Topaloglu et al., 2012) or its G-protein-coupled receptor GPR54 (Seminara et al., 2003; de Roux et al., 2003) fail to initiate puberty and remain infertile. Therefore, kisspeptin/GPR54 signaling has been

implicated as a gatekeeper of puberty (Seminara and Crowley, 2008; Clarkson et al., 2010). Consistent with this, kisspeptin receptor antagonists delay puberty in rodents (Pineda et al., 2010), while kisspeptin administration itself advances puberty onset (Matsui et al., 2004; Navarro et al., 2004). In addition, kisspeptin has been implicated as a major regulator of the adult female hypothalamus–pituitary–gonadal (hpg) axis (Castellano et al., 2006; Smith et al., 2006; Clarkson, 2013). GPR54 is expressed in most GnRH neurons in adults (Irwig et al., 2004). In fact, kisspeptin is the most potent activator of GnRH neurons known to date (Han et al., 2005). Consistent with an important role of kisspeptin neurons in the regulation of reproductive physiology, adult female mice stop cycling upon acute toxin-mediated kisspeptin neuron ablation (Mayer and Boehm, 2011).

Paradoxically however, both kisspeptin and GPR54 are expressed in the developing brain long before puberty onset (Han et al., 2005; Fiorini and Jasoni, 2010; Cao and Patisaul, 2011; Desroziers et al., 2012; Brock and Bakker, 2013; Knoll et al., 2013). Whether kisspeptin/GPR54 signaling plays a functional role in the developing brain before puberty is not known. There are mainly two anatomically segregated populations of neurons that express kisspeptin in the female hypothalamus, one in the anteroventral periventricular nucleus (AVPe; Oakley et al., 2009) and the other in the arcuate nucleus (ARC) of the hypothalamus (Clarkson et al., 2009b). Distinct roles have been proposed for ARC and AVPe kisspeptin neurons during female reproductive

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maturation (Mayer et al., 2010). Despite their pivotal role in the regulation of reproductive physiology, little is known about the development and connectivity of kisspeptin neurons. Furthermore, previous attempts to find connections between GnRH and kisspeptin neurons before puberty failed (Clarkson and Herbison, 2006; Clarkson et al., 2010), leaving the neural circuits underlying temporal control of GnRH neurons during reproductive development elusive. Due to their scattered distribution within the ARC and AVPe, kisspeptin neural circuitry has been difficult to analyze using classical tracer injection methods (Yeo and Herbison, 2011). Here we generated two novel mouse strains for Cre-dependent genetic transsynaptic tracing to study the formation and activity of kisspeptin/GnRH neural circuitry in the developing female mouse embryo.

Materials and Methods

Generation of ROSA26-BL-IRES- τ lacZ and ROSA26-GFP-TTC mice. To generate the ROSA26-BL(barley lectin)-IRES (internal ribosomal entry site)- τ lacZ targeting vector, we inserted a cDNA coding for BL (Horowitz et al., 1999; provided by Linda Buck, Howard Hughes Medical Institute, Seattle, WA) followed by an IRES- τ lacZ cassette (Rodríguez et al., 1999; provided by Peter Mombaerts, Max Planck Institute of Biophysics, Frankfurt, Germany) into the pROSA26STOP* τ lMOG plasmid (Buch et al., 2005; provided by Ari Waisman, University of Mainz, Mainz, Germany) via AscI and FseI sites. In a complementary approach, we inserted a cDNA coding for tetanus toxin fragment C fused to GFP (GFP-TTC; Maskos et al., 2002; provided by Takeshi Sakurai, Japan Science and Technology Corporation, Tokyo, Japan) into the pROSA26STOP* τ lMOG targeting vector via AscI and FseI sites to generate the ROSA26-GFP-TTC targeting vector. The final targeting vectors were composed as follows: 5' ROSA26 genomic sequence, adenoviral splice acceptor, loxP site, 2X SV40 polyadenylation signal, flippase recombinase target (FRT)-flanked *pgk* promoter-driven neomycin resistance cassette, another transcriptional STOP cassette, a second *loxP* site in the same orientation as the first, BL-IRES- τ lacZ or GFP-TTC, and 3' ROSA26 genomic sequence. Both targeting vectors were linearized using BbvCI and electroporated into R1 mouse embryonic stem (ES) cells, and resistant clones were analyzed by Southern blotting using EcoRI and an external 600 bp probe. Correctly targeted ES cells were injected into C57BL/6J blastocysts to generate chimeras that were backcrossed to C57BL/6J animals to give ROSA26-BL-IRES- τ lacZ (R26-BIZ) and R26-GFP-TTC (R26-GTT) mice. Genomic DNA obtained from tail biopsy specimens was prepared by proteinase K (Roche) digestion and subsequent purification with DNA Isolation Reagent for genomic DNA (AppliChem) for genotyping.

Mice. Animal care and experimental procedures were performed in accordance with the guidelines established by the animal welfare committee of the University of Hamburg and the University of Saarland. Mice were kept under standard light/dark cycle with food and water *ad libitum*. Kisspeptin-IRES-Cre (KissIC; Mayer et al., 2010), GPR54-IRES-Cre (GPIC; Mayer and Boehm, 2011), R26-CAGS- τ GFP (eR26- τ GFP; Wen et al., 2011), R26-BIZ, and R26-GTT mice were all kept in a mixed (129/SvJ and C57BL/6J) background. All animals analyzed in this study were females heterozygous for the respective *Cre* and *R26* alleles. Littermates carrying one *Cre* or one *R26* allele, respectively, were used as controls. KissIC and GPIC animals were genotyped using CRE-forward 5'-aacgttgatgccgttgacgtgc-3' and CRE-reverse 5'-taactatcccacgtcagtagc-3' primers. eR26- τ GFP mice were genotyped with YFP-forward 5'-cctgagtgccgttggtggcg-3' and YFP-reverse 5'-cgcgcccaacggcactcagg-3' primers.

Tissue preparation. Pregnant KissIC/eR26- τ GFP, GPIC/eR26- τ GFP, KissIC/R26-BIZ, KissIC/R26-GTT, and KissIC/R26-BIZ/R26-GTT mice were anesthetized and killed by decapitation. Embryos were removed, washed in ice-cold 1× PBS, soaked in 4% paraformaldehyde (PFA; fixative) on ice for 1.5–4.5 h, and then transferred to 30% sucrose for 24 h. The embryos were frozen in tissue-freezing medium optimal cutting temperature (OCT) compound (Leica) and cut into 14 μ m sagittal sections with a cryostat. For gender determination, embryo tail biopsy spec-

Figure 1. *De novo* expression and maturation of the kisspeptin system in female mice. **A**, Genetic strategy to analyze the embryonic development of kisspeptin neurons. In KissIC/eR26- τ GFP embryos, kisspeptin and τ GFP expression are genetically coupled, providing a fluorescent readout for *Kiss1* promoter activity. **B**, Immunofluorescence for τ GFP (green) of a sagittal section through the head of female KissIC/eR26- τ GFP embryos at E12.5, E13.5, and E16.5. Kisspeptin neurons (green) are first found at E13.5 in the ARC. **C**, Double immunofluorescence for GnRH (red) and τ GFP (green) of a sagittal section through the head of a female KissIC/eR26- τ GFP mouse at E16.5. Note that kisspeptin expression is restricted to the ARC, whereas GnRH neurons are scattered throughout the POA. **D**, Double immunofluorescence for kisspeptin (red) and τ GFP (green) demonstrates faithful activation of the τ GFP reporter in kisspeptin neurons. **E**, τ GFP⁺ fibers project to the POA at E16.5. **F**, Quantification of kisspeptin neurons during embryonic maturation. *** p < 0.001. RLRP, Residual lumen of Rathke's pouch; OB, olfactory bulb; OE, olfactory epithelium; 3V, third ventricle. Scale bars: **B**, **D**, **E**, 50 μ m; **C**, 200 μ m.

imens were digested in lysis buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 0.2% NP40, 0.2% Tween, 1 mM EDTA, 0.1 mg ml⁻¹ proteinase K) at 55°C and genotyped by PCR (Agulnik et al., 1997). At postnatal day 2 (P2; day of birth, P0), mice were transcardially perfused with fixative under ketamine/xylazine (Bayer) anesthesia, brains were removed, postfixed in 4% PFA for 2 h on ice, transferred to 30% sucrose for 24 h, frozen in OCT compound, and cut into 14 μ m coronal sections with a cryostat (Boehm et al., 2005).

Immunofluorescence. The 14 μ m sagittal embryo head and coronal P2 brain sections from KissIC/eR26- τ GFP and GPIC/eR26- τ GFP mice were incubated in TNB blocking solution (PerkinElmer) for 1 h at 20–25°C, followed by overnight incubation at 4°C with primary antisera in TNB blocking solution. The primary antisera used were (1) rabbit antibody to GnRH (1:1000; catalog #PA1-121, Affinity BioReagents), (2) rabbit antibody to estrogen receptor α (ER α ; 1:1000; catalog #06-935, Millipore), (3) rabbit antibody to kisspeptin (1:500; catalog #AB9754, Millipore), and (4) Alexa Fluor 488 rabbit antibody to GFP (1:1000; catalog #A21311, Invitrogen). In the case of using two primary antibodies from rabbit, sections were first stained with either rabbit antibody to ER α , to GnRH, or to Kisspeptin, followed by monovalent Cy3-donkey antibody

to rabbit IgG (1:500; catalog #711-487-003, The Jackson Laboratory) for 2 h at 20–25°C, and then blocked with 1% ChromPure rabbit IgG (catalog #011-000-008, The Jackson Laboratory) for 2 h followed by Alexa Fluor 488 rabbit antibody to GFP (1:1000; Invitrogen) for 2 h at 20–25°C. To visualize the transneuronal tracers BL and GFP-TTC, 14 μ m sagittal embryo head and coronal P2 brain sections from KissIC/R26-BIZ, KissIC/R26-GTT, and KissIC/R26GTT/R26BIZ mice were treated with goat anti-serum against wheat germ agglutinin [1:1000; catalog #AS-2024, Vector Laboratories (which recognizes BL)] in TNB blocking solution at 4°C followed by biotinylated horse anti-goat IgG (catalog #BA-9500, Vector Laboratories) at 1:500 for 1 h at 20–25°C or with rabbit anti-GFP (catalog #A11122, Invitrogen) at 1:15,000 in TNB blocking solution at 4°C overnight followed by biotinylated goat antiserum against rabbit IgG (1:5000; catalog #BA-1000, Vector Labs) for 1 h at 20–25°C, and then treated with components of the TSA kit (BL; PerkinElmer) or the TSA + kit (GFP; PerkinElmer) according to the manufacturer's instruction. Sections were then incubated with Alexa Fluor 546-streptavidin (1:500; catalog #S11225, Invitrogen) in TNB blocking solution for 30 min at 20–25°C followed by rabbit anti-GnRH (1:1000) at 4°C overnight and Dylight488-donkey antiserum against rabbit IgG (catalog #SA5-10038, Thermo Scientific) for 2 h at 20–25°C. For nuclear staining, sections were incubated with 5 μ g ml⁻¹ Hoechst 33258 dye (Sigma) in 0.1 M PBS for 10 min and mounted with Fluoromount-G (Southern Biotech). Appropriate controls omitting primary antibodies were performed and did not yield any staining. Sections were analyzed on an Axioskop2 microscope equipped with AxioVision software (Zeiss).

LacZ staining. Embryo sections were fixed with 0.2% glutaraldehyde (Boehringer Ingelheim), 2 mM MgCl₂, 5 mM EGTA, 1× sodium phosphate buffer, pH 7.3, for 2 min at 20–25°C, followed by a 30 min wash with wash buffer A (2 mM MgCl₂, 5 mM EGTA in 1× sodium phosphate buffer, pH 7.3) and a 10 min wash with wash buffer B (2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% Nonidet P40, 1× sodium phosphate buffer, pH 7.3). Sections were then incubated overnight in 1× sodium phosphate buffer, pH 7.3, containing 0.5 mg ml⁻¹ X-gal (40 mg ml⁻¹ stock solution in dimethylformamide), 0.5 mg ml⁻¹ NBT (50 mg ml⁻¹ stock solution in 70% dimethylformamide), and 2 mM MgCl₂ at 30°C, followed by a wash with 1× sodium phosphate buffer, pH 7.3, and then mounted with Mayer's glycine gelatin. Sections were analyzed on a Zeiss Axiophot light microscope equipped with a differential interference contrast imaging setup. Brain structures were identified using a prenatal mouse brain atlas (Schambra, 2008) and an atlas of mouse development (Kaufmann, 2001).

Quantification of neurons. Neurons were manually counted in every fifth section extending through the entire embryonic head (sagittal sections) and postnatal brain (coronal sections), respectively, using the ImageJ Cell Counter plugin. All sections were counterstained with Hoechst 33258 dye before analysis to visualize nuclei. Note that the τ GFP fusion protein is excluded from the nucleus due its size (Wen et al., 2011). Kisspeptin, GPR54, and GnRH neurons were counted based on positively stained cytoplasm, and ER α neurons were counted based on nuclear signal. Counted numbers were multiplied by 2.5 to give estimates of the total number of neurons per brain (Boehm et al., 2005).

Figure 2. *De novo* expression and maturation of the GPR54 system in female mice. **A**, Genetic strategy to analyze the embryonic development of GPR54 neurons. GPR54 neurons in GPIC/eR26- τ GFP double-heterozygous mice express τ GFP from the recombinant ROSA26 allele. **B–G**, Double immunofluorescence for GnRH (red) and τ GFP (green) of a sagittal section through the head of a GPIC/eR26- τ GFP female at E12.5 (**B**, **E**), E13.5 (**C**, **F**), and E16.5 (**D**, **G**). Note that GPR54 expression starts at E13.5 and is restricted to GnRH neurons throughout embryonic development. **H**, Quantification of GnRH/GPR54 neurons throughout embryonic maturation. *** p < 0.001, ** p < 0.01. Nuclei were stained with Hoechst 33258 (blue). OE, Olfactory epithelium; fb, forebrain; LV, lateral ventricle; CB, cribriform plate; ME, median eminence. Scale bars: **B–D**, 200 μ m; **E–G**, 50 μ m.

Statistical methods. All data are presented as the mean $\pm$ SEM. Two-tailed unpaired Student's *t* tests were used to determine statistical significance in all experiments. In addition, one-way ANOVA with Tukey's multiple-comparison test was used to determine statistical significance when making comparisons between different age groups.

Results

Orchestrated onset of kisspeptin and GPR54 expression in female mouse embryos

We first investigated the *de novo* expression of kisspeptin in female KissIC/eR26- τ GFP embryos, which report *Kiss1* promoter activity (Mayer et al., 2010). KissIC (Kisspeptin-IRES-Cre) mice carry a targeted insertion of an IRES followed by a Cre recombinase cDNA downstream of the kisspeptin coding sequence (Fig. 1A). Transcription of the recombinant *Kiss1* allele yields a bicistronic mRNA from which kisspeptin and Cre recombinase are independently translated. The eR26- τ GFP (ROSA26-CAGS- τ GFP) reporter strain expresses a fusion of the microtubule-associated protein (τ GFP) under control of the β -actin promoter/CMV enhancer (CAGS) in the ROSA26 locus after Cre-mediated excision of a transcriptional stop signal (Wen

Figure 3. Targeted integration of a Cre-dependent BL-IRES- τ lacZ transneuronal tracer into the *R26* locus. **A**, Schematic representation of the targeting strategy. From top to bottom: the targeting vector, the wild-type *R26* allele, and the targeted *R26* allele before and after Cre-mediated recombination are shown. The targeting vector contains a *loxP*-flanked transcriptional stop cassette with an *FRT*-flanked phosphoglycerate kinase promoter-driven neomycin resistance gene (PGK-neo), and the coding sequence for BL-IRES- τ lacZ. *LoxP* and *FRT* sites are indicated by solid arrowheads and ellipses, respectively. SA, Adenovirus splice acceptor sequence. **B**, Southern blot analysis of genomic ES cell DNA. The expected fragment sizes detected by the probe are indicated (wild-type, 15.6 kb; mutant, 6.6 kb). **C**, Germline transmission of the *R26-BIZ* allele was confirmed by Southern blot analysis of tail DNA. **D**, Histochemical analysis of β -galactosidase activity in a female KissIC/*R26-BIZ* brain at E18.5. The section is adjacent to the one shown in **E**. **E**, Antibodies against kisspeptin demonstrate faithful activation of BL in kisspeptin neurons. Scale bars: **D**, 20 μ m; **E**, 50 μ m. Wt, Wild type; Mut, mutant.

et al., 2011). Kisspeptin neurons in KissIC/eR26- τ GFP double-heterozygous mice express τ GFP from the recombined *ROSA26* allele and are thus identifiable by fluorescence. While we did not detect any kisspeptin expression at embryonic day 12.5 (E12.5; $n = 3$ mice; Fig. 1B), we observed 116 ± 22 kisspeptin neurons ($n = 4$ mice) at E13.5 in the ARC of the hypothalamus (Fig. 1B,F). At E14.5, we found 145 ± 34 kisspeptin neurons ($n = 3$ mice), which increased to 907 ± 63 ($n = 3$ mice) at E16.5 ($p < 0.001$) and remained restricted to the ARC (Fig. 1B,C,F). We immunostained for kisspeptin and found that $91 \pm 3\%$ of the

τ GFP⁺ cells (304 ± 46 kisspeptin⁺/ τ GFP⁺ of 333 ± 44 τ GFP⁺; $n = 3$ mice) express kisspeptin at E16.5, demonstrating faithful activation of the τ GFP reporter in kisspeptin neurons (Fig. 1D). At this stage, kisspeptin neurons send projections to the preoptic area (POA; Fig. 1E).

Next, we questioned when GnRH neurons start to express the kisspeptin receptor GPR54, and thus may become responsive to kisspeptin. To analyze this, we used female GPIC/eR26- τ GFP mice (Mayer and Boehm, 2011), which report *GPR54* promoter activity (Fig. 2A). While we did not detect any *GPR54* expression at E12.5 (Fig. 2B,E), $\sim 3\%$ of GnRH neurons expressed *GPR54* at E13.5 (7 ± 0.8 τ GFP⁺/GnRH⁺ of 215 ± 21 GnRH⁺, $n = 3$ mice; Fig. 2C,F,H). At this embryonic age, we observed GnRH neurons in the nasal septum, at the junction between the nasal septum and the anterior forebrain, and in the anterior forebrain itself (Fig. 2C). The percentage of GnRH neurons expressing *GPR54* increased to $\sim 8\%$ at E14.5 (21 ± 0.5 τ GFP⁺/GnRH⁺ of 265 ± 14 GnRH⁺, $n = 3$ mice; $p < 0.01$, E13.5 vs E14.5). At E16.5, the majority of GnRH neurons were found in the anterior forebrain (mainly in the septum, in the organum vasculosum of the lamina terminalis, and in the preoptic area; Fig. 2D). At this age, $\sim 50\%$ of the GnRH neuron population expressed *GPR54* (116 ± 12 τ GFP⁺/GnRH⁺ of 249 ± 18 GnRH⁺, $n = 3$ mice; $p < 0.001$, E14.5 vs E16.5) to reach $>81\%$ at P2 (165 ± 12 τ GFP⁺/GnRH⁺ of 203 ± 10 GnRH⁺, $n = 3$ mice; $p < 0.01$, E16.5 vs P2). Throughout embryonic development, *GPR54* expression was exclusively restricted to GnRH neurons (Fig. 2B–D). *GPR54* expression in GnRH neurons did not depend on GnRH spatial position as GnRH⁺/GPR54⁺ and GnRH⁺/GPR54[−] neurons were found in all areas containing GnRH neurons (Fig. 2). Together, these data demonstrate orchestrated onset of kisspeptin and *GPR54* expression in the embryo at E13.5, raising the possibility that the signaling system implicated in gatekeeping puberty becomes operative *in utero*.

ARC kisspeptin neurons communicate with a subset of GnRH neurons *in utero*

Therefore, we next asked whether ARC kisspeptin neurons become connected to and communicate with GnRH neurons during embryonic maturation. Despite recent technical advances in conditional transneuronal tracing using genetically engineered neurotrophic viruses, however (Lo and Anderson, 2011), studying the formation of neural circuits during embryonic development remains especially challenging.

To overcome this, we generated two new mouse strains allowing conditional transsynaptic tracing after Cre-mediated recombination. We first inserted a Cre-dependent expression cassette consisting of the transneuronal tracer BL (Horowitz et al., 1999) followed by an IRES and a fusion of the microtubule-associated protein τ with lacZ (i.e., BL-IRES- τ lacZ) into the R26 locus (R26-BIZ) locus (Fig. 3A–E). After Cre-mediated excision of transcriptional stop signals, BL is transferred across synapses in both retrograde and anterograde directions, labeling neurons upstream and downstream of the Cre-producing cells, whereas the axonal marker τ lacZ is restricted to the producing cells (Fig. 4A). We then prepared sections through the brain of female KissIC/R26-BIZ double-knock-in embryos and immunostained these with antiserum against BL (Fig. 4). Whereas τ lacZ was restricted to the ARC (Fig. 4B), we detected BL⁺ vesicles in the POA of the hypothalamus first at E16.5 (Fig. 4D), suggesting transsynaptic transfer of the BL tracer. At E18.5, we immunostained for GnRH and found that the BL⁺ vesicles in the POA were exclusively restricted to GnRH neurons (Fig. 4E). Not all GnRH neurons contained the BL tracer, suggesting functional specialization within the GnRH neuron population (Fig. 4F). A total of 59% of GnRH neurons (145 $\pm$ 13 BL⁺/GnRH⁺ of 246 $\pm$ 17 GnRH⁺ neurons; n = 3 mice) contained the BL tracer at this stage (Fig. 4G), demonstrating that kisspeptin neurons are already synaptically connected to a subset of GnRH neurons in the embryo.

ARC kisspeptin neurons are upstream of GnRH neurons

To determine whether GnRH neurons are upstream or downstream of kisspeptin neurons, we then generated—in a complementary genetic approach—a second recombinant R26 allele containing GFP-TTC (Maskos et al., 2002; Fig. 5A–D, R26-GTT). Upon activation, GFP-TTC is selectively transferred across synapses in a retrograde manner (Maskos et al., 2002), thus exclusively labeling neurons upstream of the Cre-producing cells (Fig. 6A). We then prepared sections through the brain of female KissIC/R26-BIZ/R26-GTT triple-knock-in embryos, and immunostained these with antisera against BL and GFP-TTC. While BL was clearly detected in GnRH neurons as early as E16.5, we did not find the GFP-TTC tracer in these cells at any age analyzed (E16.5, E18.5, and P2; Fig. 6G–I), demonstrating that kisspeptin neurons are upstream of GnRH neurons. Transsynaptic transfer of the GFP-TTC tracer was, however, obvious in other areas of the brain at E18.5, an important control (Fig. 6J–M). GFP-TTC-positive cell bodies were found in the ventromedial, lateral (Fig.

Figure 4. Arcuate nucleus kisspeptin neurons communicate with a subset of GnRH neurons *in utero*. **A**, In KissIC/R26-BIZ mice, τ lacZ is confined to kisspeptin neurons, but BL is transferred to presynaptic and postsynaptic neurons. **B**, **C**, β -galactosidase activity (β -gal) is restricted to the ARC in a female KissIC/R26-BIZ brain at E18.5. **D**, BL-filled vesicles are found in the POA at E16.5. **E**, **F**, Immunofluorescence for GnRH (green) and BL (red) demonstrates BL transfer to some (filled arrowhead) but not all (open arrowhead) GnRH neurons. **G**, Quantification of GnRH neurons communicating with kisspeptin neurons during reproductive maturation. Note that the number of GnRH neurons connected to kisspeptin neurons does not increase between E18.5 and P2, suggesting that connectivity to kisspeptin neurons is established before birth. ** p < 0.01. Scale bars: **C**, **F**, 20 μ m; **D**, **E**, 50 μ m; **B**, 200 μ m.

6K), and posterior hypothalamus. Interestingly we did not detect any GFP-TTC-positive cells outside of the hypothalamus at this developmental stage (Fig. 6G,J). These data demonstrate that GFP-TTC is transsynaptically transferred to neurons upstream of kisspeptin neurons in KissIC/R26-BIZ/R26-GTT animals.

Connectivity to ARC kisspeptin neurons does not depend on GnRH neuron spatial position

We observed GnRH neurons with and without the BL tracer immediately adjacent to each other (Figs. 4E,F, Fig. 6D–F) in all

Figure 5. Targeted integration of a Cre-dependent GFP-TTC transneuronal tracer into the *R26* locus. **A**, Schematic representation of the targeting strategy. From top to bottom: the targeting vector, the wild-type *R26* allele, and the targeted *R26* allele before and after Cre-mediated recombination are shown. The targeting vector contains a loxP-flanked transcriptional stop cassette with a *FRT*-flanked phosphoglycerate kinase promoter-driven neomycin resistance gene (*PGK-neo*), and the coding sequence for GFP-TTC. *LoxP* and *FRT* sites are indicated by solid arrowheads and ellipses, respectively. SA, Adenovirus splice acceptor sequence. **B**, Southern blot analysis of genomic ES cell DNA. The expected fragment sizes detected by the probe are indicated (wild-type, 15.6 kb; mutant, 6.6 kb). **C**, Germline transmission of the *R26-GTT* allele was confirmed by Southern blot analysis of tail DNA. **D**, Antibodies against kisspeptin demonstrate faithful activation of GFP-TTC in kisspeptin neurons. Scale bar, 50 μ m. Kiss, Kisspeptin; Wt, wild type; Mut, mutant.

hypothalamic nuclei containing GnRH neurons (Fig. 7), demonstrating that connectivity to kisspeptin neurons depends neither on their spatial position within an area nor on their spatial position in the brain. Together, our data reveal that the first formation of neural circuits between kisspeptin and GnRH neurons occurs *in utero*.

Neural circuits between ARC kisspeptin and GnRH neurons are fully established and operative before birth

Previous studies have indicated that transfer of BL across synapses depends on synaptic activity (Tabuchi et al., 2000; Nakashiba et al., 2012), raising the possibility that arcuate nucleus kisspeptin neurons communicate with GnRH neurons *in utero*. We therefore next analyzed communication between kisspeptin and GnRH neurons at different developmental time points using BL transfer as a marker for synaptic activity. Of note, the expression of the transneuronal tracer BL in kisspeptin neurons is independent of *Kiss1* promoter activity after Cre-mediated activation (Fig. 3A). The number of GnRH neurons communicating with kisspeptin neurons initially increased from 38% (111 ± 8 BL⁺/GnRH⁺ of 290 ± 2 GnRH⁺; $n = 3$ mice) at E16.5 to 59% at E18.5, reaching a plateau, which then remained constant after birth [118 ± 9 BL⁺/GnRH⁺ of 210 ± 11.5

GnRH⁺ (56%) at P2; $n = 3$ mice; Fig. 4G]. These data suggest that the neural circuits between ARC kisspeptin and GnRH neurons are fully established and operative before birth.

ER α immunoreactivity within the embryonic female brain delineates the birthplace of kisspeptin neurons

We have recently shown that GnRH neuron activity before puberty depends on ER α signaling in kisspeptin neurons, as female mice lacking ER α in these cells show premature activation of GnRH neurons resulting in precocious puberty onset (Mayer et al., 2010). We therefore asked when embryonic ARC kisspeptin neurons might become estrogen sensitive. Immunofluorescence analysis using antibodies against ER α showed that ER α immunoreactivity is essentially restricted to one area within the developing female mouse brain at E13.5 (Fig. 8A). Strikingly, we found that the ER α -positive area delineates the birthplace of kisspeptin neurons in the arcuate nucleus (Fig. 8B). Consistent with a functional role of ER α signaling in embryonic ARC kisspeptin neurons, we observed that the vast majority of these cells express ER α during embryonic maturation (Fig. 8C–E).

Discussion

Kisspeptin neurons play an important role in the regulation of puberty onset and adult reproductive physiology in vertebrates, but little is known about their function and connectivity during embryonic and early postnatal development. Characterization of kisspeptin neural circuitry has been difficult due to their scattered distribution in the ARC and AVPe.

Using complementary binary genetic strategies, we have started to characterize the development and connectivity of ARC kisspeptin neurons in the developing female mouse brain. Our genetic analyses show that ARC kisspeptin neurons are born between E12.5 and E13.5 in the female mouse brain, consistent with previous studies showing the presence of *Kiss1* mRNA at E13.5 (Fiorini and Jasoni, 2010; Knoll et al., 2013). GPR54 expression is also first observed at E13.5, consistent with earlier reports demonstrating the presence of *GPR54* mRNA at this stage (Constantin et al., 2009a; Fiorini and Jasoni, 2010; Knoll et al., 2013). At E16.5, ~50% of the adult ARC kisspeptin neuron population (Mayer et al., 2010) is already in place. Previous genetic analyses in mice have shown that embryonic gonadotropin-releasing hormone signaling is necessary for maturation of the reproductive axis (Wen et al., 2010). It is therefore tempting to speculate that fetal ARC kisspeptin neurons might act on fetal GnRH neurons and influence their activity, possibly via kisspeptin/GPR54 signaling. ARC kisspeptin neurons might indeed regulate embryonic GnRH secretion by stimulating GPR54, which would explain why GPR54 KO mice have significantly lower serum gonadotropin (luteinizing hormone and follicle-stimulating

Figure 6. GnRH neurons are downstream of kisspeptin neurons. **A**, While BL is transferred to presynaptic and postsynaptic neurons in Kiss1C/R26-BIZ/R26-GTT mice, GFP-TTC transfer is restricted to presynaptic neurons. **B**, **C**, Histochemical analysis of β -galactosidase (β -gal) activity in a sagittal section through the whole head of a female Kiss1C/R26-BIZ/R26-GTT triple-heterozygous mouse at E18.5. Note that β -gal activity is restricted to the ARC. **D–F**, Immunofluorescence for BL (red) and GnRH (green) on the section adjacent to the one shown in **B**. Note that some, but not all, GnRH neurons contain BL. **G–I**, Immunofluorescence for GFP-TTC (red) and GnRH (green) on the section adjacent to the one shown in **B**. Note that none of the GnRH neurons contain GFP-TTC. **J**, **K**, Immunofluorescence for GFP-TTC (red) on a section prepared from a Kiss1C/R26-BIZ/R26-GTT mouse. GFP-TTC is found in cells outside of the preoptic area. **L**, **M**, Lack of β -gal activity in section adjacent to the one shown in **J** and **K** suggests transsynaptic transfer of the GFP-TTC tracer. Nuclei were stained with Hoechst 33258 dye (blue). Scale bars: **B–D**, **G**, **J**, **L**, 200 μ m; **E**, **F**, **H**, **I**, **K**, **M**, 50 μ m.

Figure 7. Connectivity and communication between ARC kisspeptin and GnRH neurons *in utero* is independent of their spatial position. Immunofluorescence for BL (red) and GnRH (green) is shown on a sagittal section through the head of a female KissIC/R26-BIZ mouse at E18.5. BL⁺/GnRH⁺ neurons are present in the medial septum (MS), the POA, the horizontal diagonal band of Broca (HDB), and the organum vasculosum of lamina terminalis (OVLT). Note that each area also contains GnRH neurons that do not communicate with kisspeptin neurons (BL[−]/GnRH⁺). Scale bars, 50 μm.

hormone) levels compared with wild-type mice at P1 (Poling and Kauffman, 2012). Reduced serum gonadotropin levels are apparently due to lack of GnRH secretion, as serum gonadotropins are also significantly lower in *hpg* mice lacking a functional *GnRH* gene (Poling and Kauffman, 2012). Furthermore, earlier reports have demonstrated that GnRH neurons in nasal explants can respond to kisspeptin application in a dose-dependent manner similar to responses of GnRH in acute adult brain slice preparations (Constantin et al., 2009a,b).

Importantly, GPR54 expression is exclusively restricted to GnRH neurons during embryonic and early postnatal development. This is in contrast to adult GPR54 expression, which is found in many additional areas of the brain (Herbison et al., 2010; Mayer and Boehm, 2011), suggesting that the potential functional role of kisspeptin during early development is indeed—unlike in adult animals—restricted to GnRH neurons.

The neural circuitry of kisspeptin neurons has been difficult to analyze due to their scattered distribution within the ARC and AVPe (Yeo and Herbison, 2011). We developed two complementary mouse strains allowing Cre-dependent genetic transneuronal tracing to investigate the connectivity of kisspeptin neurons in the embryonic female mouse brain. One major limitation of other available tracing techniques is that most tracers must be stereotactically injected into the brain, which is technically challenging, especially in developing mouse brains *in utero*. Our strategy to express two complementary transsynaptic tracers and a stationary marker labeling the producing cell as transgenes from two knock-in alleles overcomes this limitation. This not only allowed us to visualize the connectivity between embryonic ARC kisspeptin neurons and GnRH neurons but also enabled us to

compare the maturation of this connectivity at different time points (E16.5–P2). In contrast, comparative analyses of neural circuits over extended periods of time using neurotrophic viruses is usually not possible due to their cytotoxicity (Norgren and Lehman, 1998; Callaway, 2008). Therefore, the novel R26-BIZ and R26-GTT mouse strains should be useful to study neural circuits in addition to those underlying reproductive maturation. We chose to express the tracers under control of the constitutive and ubiquitous R26 promoter rather than of the heavily regulated *Kiss1* promoter. This is an important point because it uncouples synaptic activity analysis from the upregulation or downregulation of one neuropeptide expressed by these cells. Therefore, transsynaptic BL and GTT transfer in KissIC/R26-BIZ/R26-GTT mice reflects synaptic communication between two neuronal populations independent of steroid hormone levels or other factors known to modulate kisspeptin expression (Clarkson et al., 2009a; Clarkson, 2013; Poling and Kauffman, 2013).

Cre-dependent genetic transneuronal tracing from kisspeptin neurons revealed intense synaptic connectivity between ARC kisspeptin and GnRH neurons early in development. Remarkably, these neural circuits are established and operative before birth. Not all perinatal GnRH neurons are BL positive, suggesting functional subpopulations of GnRH neurons at this developmental time point. This is consistent with studies showing that only subsets of GnRH neurons in adult mice respond to kisspeptin (Dumalska et al., 2008; Constantin et al., 2009a,b). Differential onset of kisspeptin expression in the ARC and AVPe allowed us to selectively study neural connectivity of the ARC kisspeptin neuron population using this approach. While kisspeptin expression in the AVPe begins between P10 and P15 (Semaan et al., 2010; Losa et al., 2011), the majority of this second major population of kisspeptin neurons in the female rodent brain start to express kisspeptin mainly during puberty (Clarkson and Herbison, 2006; Oakley et al., 2009; Mayer et al., 2010; Knoll et al., 2013). Consistent with this, τ GFP reporter expression in KissIC/eR26- τ GFP brains remained restricted to the ARC at all embryonic ages analyzed (E12.5, E13.5, E14.5, and E16.5). Furthermore, β -galactosidase activity was only found in the ARC in female KissIC/R26-BIZ brains at E18.5. Analysis of older KissIC/R26-BIZ/R26-GTT females should also provide additional information about the neural circuitry of AVPe kisspeptin neurons. While we focused on the analysis of the female brain in this study, it will be interesting to compare kisspeptin neural circuits in male and female mice at different time points. Furthermore, transneuronal transfer of BL and GTT in KissIC/R26-BIZ/R26-GTT females is not restricted to GnRH neurons at later embryonic and perinatal stages (Fig. 6J,K), and comparative analysis of BL- and/or GTT-labeled cells throughout the brain of these animals should provide detailed information about kisspeptin neural circuitry independent of GnRH neurons.

Our data provide substantial new insight into functional organization of the circuitry underlying reproductive maturation in the female brain. Communication between kisspeptin and GnRH neurons is established with remarkable specificity arguing against an overabundance of connections needed for the formation of reproductive circuitry in the developing brain (Low and Cheng, 2006). We observed similar numbers of BL⁺/GnRH⁺ and BL[−]/GnRH⁺ neurons, not only in different regions of the brain but also in different animals (Fig. 4G), raising the possibility that connectivity between kisspeptin and GnRH neurons is stereotyped.

We have recently shown that an ER α -dependent mechanism—acting like a “brake”—is operating in arcuate kisspeptin-

producing neurons during pubertal development of female mice to gate GnRH release. Conditional inactivation of the *Esr1* gene in kisspeptin neurons results in a dramatic advancement of puberty onset in female mice (Mayer et al., 2010). These data suggest that an ER α -dependent mechanism is acting in ARC kisspeptin neurons during female reproductive maturation. Consistent with this, the birthplace of ARC kisspeptin neurons in the developing hypothalamus is delineated by estrogen-sensitive cells, and most ARC kisspeptin neurons already express ER α *in utero*. While the role of estrogens during female reproductive maturation remains controversial (Bakker and Baum, 2008), it seems interesting to note that the first appearance of ER α immunoreactivity in the fetal female mouse brain coincides—both temporally and spatially—with the first activity of the *Kiss1* promoter. These data may suggest a role for ER α signaling in fetal ARC kisspeptin neurons. Where could the fetal estrogen potentially come from? The fetal mouse ovaries are an unlikely source, as they do not express some of the enzymes necessary for estrogen production early in development (Greco and Payne, 1994). One alternative source might be local estrogen production in the brain. Future studies will be directed at determining the source of estrogen in the fetal female mouse brain and at analyzing its effect on the fetal ARC kisspeptin neuron population.

Together, we have started to delineate the neural circuits controlling the timing of reproductive maturation using conditional genetic transsynaptic tracing in female mouse embryos. Our genetic analyses show that kisspeptin is first expressed at E13.5 in the arcuate nucleus of the hypothalamus. We demonstrate that arcuate kisspeptin neurons already communicate with a specific subset of GnRH neurons *in utero*. We also show that arcuate kisspeptin neurons are upstream of GnRH neurons and that GnRH neuron connectivity to arcuate kisspeptin neurons does not depend on their spatial position in the brain. Furthermore, our data indicate that the neural circuits between ARC kisspeptin and GnRH neurons are fully established and operative before birth. Finally, our data show that most GnRH neurons express the kisspeptin receptor GPR54 upon circuit formation, raising the possibility that the signaling system implicated in gatekeeping puberty becomes operative in the embryo.

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Figure 8. Fetal ARC kisspeptin neurons express ER α . **A–D**, Immunofluorescence for ER α (red) and τ GFP (green) of sagittal sections through the head of female Kiss1Cre/τGFP embryos at E13.5 (**A**, **B**) and E16.5 (**C**, **D**). Note that ER α immunoreactivity is restricted to the mediobasal hypothalamus at E13.5. Most ARC kisspeptin neurons express ER α at E16.5. Merged images also display nuclear counterstaining (Hoechst 33258 stain, blue). Please note that the ER α immunoreactivity in kisspeptin neurons seems to be localized to the nucleus (inset). **E**, Quantification of kisspeptin/ER α neurons during embryonic maturation. No ER α expression was found at E12.5; at E13.5, nearly 70% of kisspeptin neurons were ER α positive (43 $\pm$ 7 ER α ⁺/τGFP⁺ of 61 $\pm$ 10 τGFP⁺, n = 3 mice), increasing to 90% at E16.5 (340 $\pm$ 10 ER α ⁺/τGFP⁺ of 378 $\pm$ 6 τGFP⁺, n = 3 mice, ** p < 0.01). 3V, Third ventricle; RLRP, residual lumen of Rathke's pouch. Scale bars: **A**, **C**, 200 μ m; **B**, **D**, 50 μ m.

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