Additional methods:

HEK293A and C7 cell cultures

HEK293A cells were cultured in high glucose (4.500 mg/L) Dulbecco’s modified Eagle’s medium (DMEM) with GlutaMAX™ I (Invitrogen, Cergy Pontoise, France), supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 25 mM Hepes (Invitrogen). The C7 cell line stably expressing KISS1R tagged with hemagglutinin (HA) was cultured in the same way but with 200 µg/mL geneticin (Invitrogen). The cells were incubated in a humidified 95% air/5% CO₂ controlled atmosphere at 37°C. The medium was changed every 3 or 4 days. Passages were performed once a week, using 1X trypsin/EDTA (Invitrogen).

Establishment of a stable HEK293A cell line expressing KISS1R

Twenty-four hours before transfection, HEK293A cells were seeded in 35-mm dishes at a density of 300 000 cells/dish without antibiotics. The cells were transfected using Lipofectamine (Invitrogen) with a pCDNA3.1 vector encoding for KISS1R and having an HA tag at its N-terminal end (1). After 3 days, the cells were put in culture medium containing 1 mg/mL geneticin for selection. Selected clones were tested by immunocytochemistry (ICC) using an anti-tag antibody.

Membrane protein extraction and Western blot

HEK293A and C7 cells were cultured to confluence in dishes 10 cm in diameter then scraped off into phosphate buffered saline (PBS) containing a protease inhibitor cocktail (Sigmafast, Sigma-Aldrich, Saint Quentin Fallavier, France) and EDTA. Membrane proteins were extracted as previously described (2). Briefly, after 5 minutes’ centrifugation at 4°C, the cell pellet was resuspended in lysis buffer (1 mM Tris pH 8, 1 mM EDTA, and Sigmafast
protease inhibitor cocktail). After two liquid nitrogen/37°C cycles, the cells were passed through a 26G syringe three times. After 5 minutes’ centrifugation at 400 g at 4°C, the supernatant was collected and centrifuged at 21 000 g at 4°C for 30 minutes. The pellet was then resuspended in solubilized buffer (50 mM Tris pH 8, 150 mM NaCl, 10% glycerol, and 1% triton-X100). After 1 hour of incubation at 4°C under agitation, the tubes were centrifuged at 21 000 g at 4°C for 15 minutes. The supernatant containing the solubilized membrane proteins was kept at -20°C.

Protein concentrations were determined using the BCA protein assay kit (Interchim, Montluçon, France). After 30 minutes’ denaturation at 55°C, 15 µg of protein was resolved in 8% SDS-PAGE gel and electroblotted for 1 hour at 100 V onto Hybon-P membranes (GE Healthcare, Vélizy, France). Membranes were blocked at room temperature using TBS-0.1% Tween 20 (TBS-T) containing 5% bovine serum albumin (BSA) and incubated overnight at 4°C with the primary antibody, which was either 3F10 or gp74 diluted in TBS-T-1% BSA. After intensive washes with TBS-T, the membrane was incubated for 1 hour with the peroxidase-coupled secondary antibody, which was either goat anti-rat antibody (Jackson ImmunoResearch, Suffolk, UK) or goat anti-rabbit antibody (Santa Cruz Biotechnologies, Heidelberg, Germany). Bound antibodies were revealed by chemiluminescence with the Immun-Star™ WesternC™ Chemiluminescence Kit (Biorad, Marne-la-Coquette, France) using ChemiDocTM XRS (Biorad).

To evaluate glycosylation of KISS1R, 45 µg of proteins were denatured in a final volume of 50 µL containing 5 µL denaturing buffer 10X (New England Biolabs, Evry, France) for 45 minutes at 50°C. Denatured proteins were then incubated with PGNaseF (New England Biolabs) for 1 hour. The deglycosylation reaction was stopped by adding the loading buffer. Protein sizes of deglycosylated proteins were determined by immunoblotted as described above.
LH and FSH pituitary contents.

LH and FSH pituitary contents were quantified by counting pituitary cells expressing LH and FSH in five microscopic fields on three consecutive pituitary sections. The ratio of LH-expressing (LH+) or FSH-expressing (FSH+) cells over total cells per microscopic field was established. The results are reported as the percentages of LH+ and FSH+ cells at each gestational age. Data are described as mean±SEM.

Immunocytochemistry (ICC)

HEK293A cells were plated in 8-well Lab-Tek® chamber slides previously coated with 100 µg/mL poly-D-lysine (Sigma-Aldrich). After 24 hours, the cells were transiently transfected with Fugene HD (Roche Diagnostics) using a plasmid encoding KISS1R-HA. After 48 hours, the cells were washed with PBS, fixed with 2% paraformaldehyde, permeabilized with 0.2% Triton-X100, and blocked with PBS supplemented with 2% BSA (Sigma-Aldrich). The cells were incubated with monoclonal rat anti-HA antibody (3F10, Roche Diagnostics) and gp74 in PBS-1% BSA. After three washes with PBS, the cells were incubated in PBS-1% BSA with the secondary antibody, which was either goat anti-rat Cy3-coupled antibody (Jackson ImmunoResearch) or goat anti-rabbit alexa488-coupled antibody (Invitrogen). The nuclei were labeled for 15 minutes using TO-PRO3R (Invitrogen) diluted 1/500 in PBS. The slides were mounted using Fluoromount (Dako, Trappes, France) then examined using a Zeiss Axio Observer inverted microscope equipped with a laser-scanning microscope 5 Exciter confocal scanning system (Carl Zeiss, Jena, Germany) and a x40 oil-immersion objective.


Figure legends

Figure S1: Levels of hypothalamic sections to determine the distribution of Kisspeptin and KISS1R neurons in fetus.

Figure S2: KISS1 and KISS1R immunostaining in the ARC and Pa throughout the human fetal hypothalamus at 15-16, 21-23, 30-31, and 38-40 WG. Arrows and arrowheads indicate perykaria and fibers respectively. Note at 38-40 WG, the declined expression of KISS1 and KISS1R in the ARC compared to the Pa. Scale bar: 100 μm.