Appendix (Methods):

Synthetic peptides

- *KISS1R* peptides (H-NASDDPGSAPRPLD-C) were synthesized by Kelowna International Scientific Inc. (Taipei, Taiwan).

Preparation of antibodies

- Two Institut de Sélection Animale brown hens (40-week-old) were immunized through intramuscular injection at multiple sites on the breast. Primary immunization was performed with 400 μg of *KISS1R* peptide-KLH in 0.5 mL of saline and an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) for each hen. Three boosters with 300 μg of *KISS1R* peptide-KLH in 0.5 mL of saline and an equal volume of Freund's incomplete adjuvant were used. The first two boosters were performed at 1-week-intervals, and the third booster was performed 4 weeks after the second booster. The health status of the hens was monitored daily, their blood was tested weekly, and their laid eggs were collected daily. All samples were stored at −20°C or 4°C until further processing.

Purification of egg yolk antibodies

- To analyze the average quality of the antibodies in the weeks after immunization, the eggs laid weekly (approximately 5–7 eggs) of each hen were pooled prior to IgY extraction. Because IgY in the serum is selectively transferred to the yolk, we only retained the egg yolk. After recording the total volume of weekly yolks, the yolks were mixed with phosphate-buffered saline (PBS) that was double the yolk volume. Then, 3.5% PEG 6000 (Sigma-Aldrich) of the total volume (yolk + PBS) was added, followed by 10 min of mixing with a rolling mixer. The tubes were centrifuged at 13,000 g at 4°C for 20 min. After centrifugation, the supernatant was passed through a folded filter and was transferred to a new tube. Then, 8.5% of PEG 6000 in grams (calculation based on the new volume) was added to the tube. The tube was rolled with a rolling mixer and centrifuged as aforementioned. The pellet was dissolved in 1 mL of PBS by using a glass stick and a vortex. PBS was added to ensure a final volume of 10 mL. The solution was mixed with 12% PEG 6000 (w/v, 1.2 g), followed by rolling and centrifugation. The pellet was carefully dissolved in 2 mL of PBS buffer, and the solution was dialyzed for 24 h in PBS. Thereafter, the IgY extract was removed from the dialysis bag (Membrane Filtration Products Inc., Seguin, TX, USA) and stored at −20°C until further processing. The protein content (mg/mL) of the samples was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the quality of the antibody was analyzed through simple sodium dodecyl sulfate–polyacrylamide gel

electrophoresis (SDS-PAGE).

Enzyme-linked immunosorbent assay (ELISA)

- High-affinity microtiter plates (Costar Corning Inc., Corning, NY, USA) were coated with *KISS1R* peptides (10 mg/mL) in coating buffer (35 mM $NaHCO_3$ and 15 mM Na2CO3, pH 9.6) and incubated overnight at 4°C. Plates were washed twice with washing buffer (6.1 mM $Na_2HPO_4 \cdot 2H_2O$, 3.9 mM $NaH_2PO_4 \cdot H_2O$, and 0.1% Tween-20, pH 7.0) and blocked with blocking buffer (0.25% gelatin, 0.15 M NaCl, 0.05 M Tris-base, 6 mM EDTA, and 0.05% Tween-20, pH 8.0) overnight at 4°C. Antibodies (100 mg/mL) in weekly serum or yolk extract were diluted to 1:10,000 in assay buffer, added to the wells in duplicate, and incubated for 1 h at room temperature. After washing, plates were incubated with peroxidase-conjugated goat anti-chicken IgY antibodies (Abcam PLC, Cambridge, UK) and diluted to 1:20,000 in assay buffer for 1 h at room temperature. The color was developed by adding 3.7 mM o-phneylenediamine in 0.03% H2O2 within 5 min, and the color-presenting reaction was stopped by adding 8 N of H2SO4. The optical density was determined at 490 nm on an ELISA reader (DynaTech, Rückersdorf, Germany).

Animal and tissue collection

- The Institute of Cancer Research mice (ICR mice) have been created as a fertile mouse line. It is a strain of albino mice originating in SWISS. The mice of this strain were named ICR because they have been sent to various laboratories from the Institute of Cancer Research in the USA.
- Pregnant mares' serum gonadotropin (Sigma-Aldrich)

Primary mouse Leydig cell culture

- The testes were immediately collected and placed in an isolation buffer [10 mg collagenase and 10 mg bovine serum albumin (BSA) in Hank's balanced salt solution buffer].
- After drying, the cells were covered with a staining solution (1% BSA, 1.5 mM β-nicotinamide adenine dinucleotide, 0.25 mM nitro blue tetrazolium chloride, 0.2 mM dehydroepiandrosterone, and 80% PBS) for 8 h.
- RP-cAMPS (Enzo Life Science Inc., Farmingdale, NY, USA): The term-planar chirality used to refer to stereoisomerism resulting from the arrangement of out-of-plane groups with respect to a plane. The configuration of molecular entities possessing planar chirality is specified by the stereo-descriptors Rp and Sp. A center with a clockwise sense of rotation is an R (*rectus*) center and a center with a counterclockwise sense of rotation is an S (*sinister*) center. The names are derived from the Latin for 'right' and 'left', respectively.The reason why we used RP-cAMPs in our study is that RP-cAMPs is

a potent and specific competitive inhibitor of protein kinase A (PKA) and it can antagonize actions of cAMP. RP-cAMPs has the properties of cell permeability and complete resistance to cyclic nucleotide phosphodiesterase. Therefore, RP-cAMPs becomes a unique tool for researching cAMP-dependent signaling.

Cell line culture

- We used MA-10 mouse Leydig tumor cells as the cell model to confirm *KISS1R* expression on the Leydig cell membrane by using immunohistochemistry, and we investigated the mechanism of luteinizing hormone (LH)-dependent *Kiss1* gene expression through real-time PCR.
- These cells were maintained in Dulbecco's Modified Eagle Medium/F-12 medium supplemented with 10% FBS, 2.2 mg/mL NaHCO3, 100U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C and 5% CO2.

Immunohistochemistry

- Formalin-fixed mouse tissues were embedded in paraffin, sectioned into 5-mm thick slices, and adhered to poly-L-lysine-coated slides. Tissue sections were deparaffined in xylene, rehydrated in descending concentrations of ethanol, and washed with H_2O. Then, they were immersed in 10 mM citrate buffer (pH 6.0) with 0.05% Tween-20 and heated twice in a microwave for 10 min at 750 W at a 5-min interval. The sections were removed and allowed to cool by a brief wash in tap water and then in PBS. After quenching endogenous peroxidase activity with 1% (v/v) H_2O_2 in methanol for 30 min, the sections were rinsed three times with PBS for 5 min. Nonspecific binding sites were blocked with goat serum in PBS [3% (v/v) normal goat serum and 0.2% (v/v) Triton X-100 in PBS] for 1 h. Commercial rabbit polyclonal antibodies raised against mouse kisspeptin 145 (1:100 dilution; Abcam) were used to visualize kisspeptin. For visualizing *KISS1R*, we used our chicken anti-mouse *KISS1R* antibodies diluted at 1:5000. This antibody specificity was validated by presenting gradually obscure bands when the antibodies were preincubated with graded concentrations of antigens in the absorption test. Negative controls for antibodies were established using the blocking buffer alone. After 2 h (*KISS1R*) or 20 h (kisspeptin) of incubation at 4°C, the antibodies were visualized with a biotinylated secondary antibody directed against rabbit immunoglobulin G (for kisspeptin) or chicken immunoglobulin G and Y (for *KISS1R*) for 1 h. Slides were washed three times with PBS for 5 min at room temperature and incubated with an avidin–biotin–HRP complex from the Vectastain Universal ELITE ABC Kit (Vector Laboratories, Burlington, ON, Canada) for 30 min according to the manufacturer's instructions. After the slides were rinsed again, they were incubated for 10–20 min at room temperature with diaminobenzidine to visualize immunostaining.

Finally, the slides were rinsed with distilled water twice for 10 min, counterstained with hematoxylin for 30 s, and hydrated with ethanol and xylene before adding a mounting medium (Hecht-Assistant, Sondheim, Germany). Sections were observed under an optical microscope (Axioskop 40, Carl Zeiss, Göttingen, Germany), and images were collected using the AxioCam ERc 5s (Carl Zeiss) digital camera.

Western blot

- The tissues or cells were rinsed once with cold PBS and collected. They were ground with a mechanical homogenizer in a cold lysis buffer [150 nM NaCl, 0.1% Triton X-100, 50 mM Tris-HCl (pH 8.0), protease inhibitor, and phosphatase inhibitor]. Protein concentrations were determined using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions. Samples containing 30–60 μg of protein were separated by 15% SDS-PAGE. The separated proteins were transferred to a polyvinylidene fluoride membrane. The membrane was blocked by immersing it in PBS containing 0.01% Tween 20 (PBST) and 2.5% BSA for 8 h at room temperature, followed by incubation with our developed chicken anti-mouse *KISS1R* antibody (1:200,000 dilution or serial diluted concentrations) in PBST with 0.5% BSA for 18 h at room temperature. Then, the membrane was washed three times with PBST and incubated for 2 h with peroxidase-conjugated goat antirabbit IgG (1:25,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or goat anti-chicken IgY. The membrane was washed with PBST, and bound antibodies were visualized using the enhanced chemiluminescence system (Merck Millipore, Burlington, MA, USA). The images were presented on Kodak X-OMAT film (Eastman Kodak Company, Rochester, NY, USA).

RNA extraction and cDNA synthesis

- Total RNA was extracted from tissues or cells by using the TRIsure reagent (Bioline Inc., Taunton, MA, USA). We used the PrimeScript RT Reagent Kit (Takara Bio Inc., Shiga, Japan) to synthesize cDNA. Total RNA (500 ng) was mixed with 25 pM oligo(dT) primer, 50 pM random hexamers, an enzyme mix, and a reaction buffer and then incubated at 37°C for 15 min. Reverse transcriptase was inactivated by heating to 85°C for 5 s, and the cDNA products were stored at 4°C for analysis.

Quantitative real-time PCR

- Transcripts were quantified using the Fast SYBR Green Master Mix (Applied Biosystems) in a total volume of 10 μL. Samples were heated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Then, we conducted melting curve analysis to observe the appearance of nontarget products that could affect the final data.

Statistical analysis

- SigmaPlot (Systat Software Inc., San Jose, CA, USA)