Kiss1-dependent and independent release of luteinizing hormone and testosterone in perinatal male rats

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Abstract. Prenatal and postnatal biphasic increases in plasma testosterone levels derived from perinatal testes are considered critical for defeminizing/masculinizing the brain mechanism that regulates sexual behavior in male rats. Hypothalamic kisspeptin neurons are indispensable for stimulating GnRH and downstream gonadotropin, as well as the consequent testicular testosterone production/release in adult male rats. However, it is unclear whether kisspeptin is responsible for the increase in plasma testosterone levels in perinatal male rats. The present study aimed to investigate the role of Kiss1/kisspeptin in generating perinatal plasma LH and the consequent testosterone increase in male rats by comparing the plasma testosterone and LH profiles of wild-type (Kiss1+/+) and Kiss1−/− male rats. A biphasic pattern of plasma testosterone levels, with peaks in the prenatal and postnatal periods, was found in both Kiss1+/+ and Kiss1−/− male rats. Postnatal plasma testosterone and LH levels were significantly lower in Kiss1−/− male rats than in Kiss1+/+ male rats, whereas the levels in the prenatal embryonic period were comparable between the genotypes. Exogenous kisspeptin challenge significantly increased plasma testosterone and LH levels and the number of c-Fos-immunoreactive GnRH neurons in neonatal Kiss1−/− and Kiss1+/+ male rats. Kiss1 and Gpr54 (kisspeptin receptor gene) were found in the testes of neonatal rats, but kisspeptin treatment failed to stimulate testosterone release in the cultured testes of both genotypes. These findings suggest that postnatal, but not prenatal, testosterone increase in male rats is mainly induced by central kisspeptin-dependent stimulation of GnRH and consequent LH release.

Key words: Kisspeptin, Perinatal testosterone surges, Hypothalamic-pituitary-gonadal axis, Testes

A DRAMATIC INCREASE in plasma testosterone levels during the perinatal period has been observed in males of many mammalian species, including rodents, monkeys, and humans [1-4]. In rodents, perinatal testicular testosterone, which diffuses into the brain and is locally converted to estradiol by aromatase, is considered to be closely related to the development of the brain mechanism that regulates sexual behavior [5, 6]. Male rats castrated at birth display female-type sexual behavior (e.g., lordosis) in adulthood, but the effect of castration is reversed by immediate testosterone treatment after castration [7, 8]. Conversely, female-type lordosis behavior is suppressed in female rats treated with testosterone in early neonatal life, while neonatally estradiol-treated female rats show testosterone-induced male-type mounting behavior in adulthood [9, 10]. Thus, the neonatal testosterone surge and estradiol converted from testosterone are thought to defeminize/masculinize the brain to control sexual behavior in rodents. However, the mechanisms underlying the generation of perinatal testosterone surges in male rats are not fully understood.

In recent years, kisspeptin, encoded by the Kiss1 gene, has been accepted as a dominant stimulator of the hypothalamic-pituitary-gonadal (HPG) axis, controlling puberty onset and reproductive function in mammals [11-14]. Hypothalamic kisspeptin neurons, at the top of the HPG axis, directly stimulate GnRH secretion from GnRH neurons in the hypothalamus and consequent LH and follicle-stimulating hormone from the anterior...
pituitary to maintain gonadal activities and functions, including gametogenesis and steroidogenesis, in adult rodents [15-17]. Indeed, Kiss1 knockout (Kiss1−/−) male rats show a profound suppression of LH and testosterone secretion in adulthood, an absence of puberty onset, and atrophic testes [17, 18]. Furthermore, our previous study showed that castrated adult Kiss1−/− male rats implanted with testosterone showed no mounting behavior, while those implanted with a preovulatory level of estradiol displayed a high lordosis quotient score as found in female rats [18]. These findings suggest that kisspeptin is essential for defeminization/masculinization of the brain, controlling male-type sexual behavior in rodents.

Kisspeptin and GnRH neurons are detected as early as embryonic day 14.5 (E14.5) and E16.5, respectively, in the rat brain [19, 20], and neural circuits between kisspeptin and GnRH neurons in the mouse brain have already been established before birth [21]. Furthermore, plasma LH is detectable in male mice from E16 onwards, and LH can stimulate testosterone release from the cultured testes of E16.5 rats [21, 22]. These findings suggest that kisspeptin neurons may be involved in the generation of perinatal testosterone surges by stimulating the HPG axis.

To date, controversial results have been reported regarding the involvement of central kisspeptin and/or downstream GnRH/LH in testicular testosterone synthesis and/or release in newborn males. Our previous study showed that plasma testosterone levels in Kiss1−/− neonatal male rats within 2 h after birth were comparable to those in Kiss1+/+ male rats [18]. Similarly, plasma testosterone concentrations throughout the first 4 h after birth in male neonates of global Gpr54 KO (gene for GPR54, the kisspeptin receptor) knockout (Gpr54−/−) mice did not differ from those of wild-type mice [23]. Furthermore, plasma testosterone levels in hypogonadal (hpq) mice lacking GnRH and intratesticular testosterone levels in LH receptor knockout (Lhcgr−/−) mice on the day of birth (PND0) were comparable to those in wild-type mice [23, 24]. In contrast, Clarkson et al. showed that plasma testosterone concentration within 2 h after birth in GnRH neuron-specific Gpr54−/− male mice was significantly lower than in wild-type mice [25]. O’Shaughnessy et al. reported that testicular testosterone levels at PND0 in Lhcgr−/− mice were significantly lower than those in wild-type mice [26]. Expression of Kiss1 and Gpr54 has been reported in the testes of mice from PND7 onwards and in adult humans, goats, and rhesus monkeys [27-30]. Furthermore, previous studies have suggested that kisspeptin directly stimulates steroidogenesis in the testes of adult rhesus monkeys and goats [27, 28]. Hence, it is also possible that local kisspeptin in the testes directly stimulates testosterone synthesis in newborn male rats.

Thus, the present study aimed to determine whether the perinatal testosterone surge in male rats is triggered by central kisspeptin and downstream GnRH/LH or intratesticular kisspeptin-GPR54 signaling. We first examined the role of kisspeptin in the generation of perinatal testosterone surge by comparing plasma testosterone levels between Kiss1+/+ and Kiss1−/− perinatal male rats at several different time points during the prenatal embryonic and postnatal periods. We then explored the expression of c-Fos, a marker of neuronal activation, in GnRH neurons, and plasma LH and testosterone levels in neonatal Kiss1+/+ and Kiss1−/− male rats treated with peripheral vehicle or kisspeptin to examine whether neonatal GnRH neurons are capable of responding to the exogenous challenge of kisspeptin to induce downstream LH/testosterone release. Furthermore, we investigated the expression of 3β-hydroxysteroid dehydrogenase (3βHSD), a marker of fetal-type Leydig cells, in the neonatal testes of Kiss1+/+ and Kiss1−/− male rats to examine whether Kiss1 deletion affects the number of fetal-type Leydig cells in neonates. We also examined the effect of kisspeptin challenge on testosterone release from in vitro cultured neonatal testes obtained from Kiss1+/+ and Kiss1−/− male rats to determine whether kisspeptin directly acts on neonatal testes to stimulate testosterone release.

**Materials and Methods**

**Animals**

The Kiss1 knockout rat line (Kiss1−/−) was established as previously described [17] and maintained by crossing Kiss1−/− male and female rats or Kiss1+/− rats and Iar::Wistar-Imamichi rats (Institute of Animal Reproduction, Kasumigaura, Japan). Kiss1−/− and littermate Kiss1+/+ rats obtained by intercrossing Kiss1−/− rats were used for the experiments. The rats were housed in a controlled environment (14 h light: 10 h darkness with lights on at 0500 h, 23 ± 2°C) with free access to food (CE2; Clea Japan, Tokyo, Japan) and water. The day after mating and the day of birth were defined as the embryonic day 0.5 (E0.5) and postnatal day zero (PND0), respectively. Experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Agricultural and Life Sciences, The University of Tokyo, Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University, and Animal Care and Use Committee of the National Institute for Physiological Sciences, Okazaki, Japan.

**Blood and testes sampling from perinatal male rats**

Fetal blood samples were collected from the timed-pregnant female rats when they were sacrificed by
decapitation in the morning on E17.5, E18.5, and E19.5, and in the late afternoon on E21. Blood samples at birth (0 h after birth) were obtained within 20 min from the start of parturition from fetuses of pregnant rats decapitated when they started parturition. Blood samples of neonates were collected at three different time points, 0–1.5, 1.5–3 h, and 3–4.5 h after birth. Blood samples were collected using heparinized syringes and centrifuged at 15,000 × g for 20 min at 4°C to obtain plasma samples, which were stored at −30°C until testosterone and LH assays were performed. Testes collected within 2 h after birth were subjected to immunofluorescence staining for 3βHSD, in vitro culture, or reverse transcription-polymerase chain reaction (RT-PCR) analysis for Kiss1 and Gpr54 gene expression. Testes for 3βHSD immunofluorescence analysis were fixed in modified Davidson’s fluid (30% of a 37%–40% solution of formaldehyde, 15% ethanol, 5% glacial acetic acid, and 50% distilled H2O) overnight at room temperature and then transferred to 70% ethanol before paraffin embedding. Testes for RT-PCR analysis were snap-frozen in liquid nitrogen and stored at −80°C until ribonucleic acid (RNA) extraction.

**Postnatal treatment of kisspeptin and blood and brain sampling**

Within 2 h after birth, neonatal male rats were injected subcutaneously with rat kisspeptin (rKp-52, Peptide Institute, Osaka, Japan) at 1 nmol/50 μL under cryoanesthesia on ice. The dose and timing of injection were chosen according to our previous study, which rescued the defeminization of lordosis behavior in Kiss1−/− males [18]. Blood and brain samples were collected from the neonatal male rats 1 h after the kisspeptin injection. Plasma samples were obtained as described above and stored at −30°C until the testosterone and LH assays. The brain samples were subjected to immunofluorescence staining for GnRH and c-Fos. The brains were fixed with 4% paraformaldehyde in 0.05 M phosphate buffered (PB, pH 7.6) overnight at 4°C, transferred to 30% sucrose in 0.05 M PB at 4°C until they sank, and then immersed in optimal cutting temperature compound (Tissue-Tek, Sakura Finetek Japan, Tokyo, Japan) diluted with 0.05 M phosphate-buffered saline (PBS) overnight at 4°C. The brains were frozen and stored at −80°C until tissue sections were prepared.

**RT-PCR analysis for Kiss1 and Gpr54 expression in neonatal testes**

Total RNA was extracted from neonatal testes using Isogen reagent (Nippon Gene, Tokyo, Japan) per the manufacturer’s instructions. cDNA from each sample was synthesized with oligo deoxythymidine primers at 42°C for 60 min using a highly efficient reverse transcription kit (ReverTra Ace, Toyobo, Osaka, Japan). PCR was performed using ProFlex (Applied Biosystems, Foster City, CA) with Blend Taq polymerase (Toyobo, Osaka, Japan). The target genes were amplified using the following primers: Kiss1 (NM_181692, forward: 5′-AGCTGCTGTTCTCCTCCT GT-3′ and reverse: 5′-AGCGGTCCACACTCTGAC C-3′), Gpr54 (NM_023992, forward: 5′-TCCCTTCTGTG CTGCGTACCCT-3′ and reverse: 5′-AGCGGTCCACACT TCATGGCT-3′), and Actb (NM_031144, forward: 5′-TG TTACCAACTGGGACGACA-3′ and reverse: 5′-GGGG TGTTGAAAGGTCTCAAA-3′). The PCR conditions included 5 min of denaturation at 95°C, followed by 40 cycles (Kiss1 and Gpr54) or 22 cycles (Actb) of amplification (95°C for 30 s, 60°C for 20 s, and 72°C for 30 s), and 5 min elongation at 72°C. Five microliters of the amplified reaction mixture was electrophoresed on a 1.8% agarose gel, followed by staining with ethidium bromide.

**Immunofluorescence staining for GnRH and c-Fos in brains and 3βHSD in testes**

Every fourth serial coronal brain section (20 μm in thickness) of neonatal male rats was used for dual-immunofluorescence staining of GnRH and c-Fos. Antibody retrieval was performed at 95°C for 10 min in citrate buffer (pH 6.0). Sections were incubated for 90 min in blocking buffer (10% normal goat serum in 0.01 M PBS with 0.1% Triton-X). The sections were then incubated with mouse monoclonal anti-GnRH antibody (1:1,000; kindly provided by Dr. Min-Kyun Park, The University of Tokyo, Japan; Cat# LRH13, RRID: AB_2636958) [31] and rabbit polyclonal anti-c-Fos antibody (1:1,000, Millipore Cat# ABE457, RRID: AB_2631318) diluted with blocking buffer overnight at 4°C, followed by incubation with secondary antibody (Alexa Fluor 594 goat anti-mouse IgG, Cat# A11005, RRID: AB_2534073; Alexa Fluor 488 goat anti-rabbit IgG, Cat# A11034, RRID: AB_2576217; 1:800; Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature. The fluorescent signal was observed by fluorescence confocal microscopy (Carl Zeiss, Oberkochen, Germany), and the number of GnRH neurons and GnRH neurons co-expressing c-Fos in the preoptic area (POA, from 0.4 mm to −0.2 mm from the bregma) were counted according to the postnatal rat brain atlas [32].

Every tenth serial testis section (4 μm in thickness) was used for immunofluorescence staining of 3βHSD. Sections were incubated for 90 min in blocking buffer and then incubated with rabbit anti-3βHSD antibody (1:5,000; Cat# 3beta-HSD antibody, RRID: AB_2827771) diluted with blocking buffer overnight at 4°C, followed by incubation with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, 1:800; Invitrogen) for 2 h at room
Testis culture
The testes of neonatal male rats taken within 2 h after birth were cultured in a culture medium consisting of phenol red-free Dulbecco’s modified Eagle medium/ nutrient mixture F-12 (DMEM/F-12; Gibco, Grand Island, NY, USA) as previously described [34]. The neonatal testes were collected in cold PBS and cut into two pieces, which were cultured in 0.2 mL culture medium in 48-well culture plates for 4 h at 37°C, in a humidified atmosphere containing 95% air and 5% CO₂. The effects of in vitro kisspeptin or LH treatment on testosterone release from neonatal testes were evaluated by comparing one testis cultured in medium containing 100 ng/mL rat LH (NHPP, Baltimore, MD, USA) or rKp-52 (0.2 or 2 μM) with the other testis from the same neonate cultured in medium containing vehicle (distilled water). rKp-52, LH, or vehicle was added to the medium from the onset of testes culture, and the medium was collected 4 h after the start of incubation to measure the testosterone concentration.

Testosterone and LH assays
Plasma testosterone concentrations of perinatal male rats were determined by enzyme immunoassay (EIA) as previously described [18] after extraction with a mixture of diethyl ether and hexane (v/v = 2:3). Testosterone levels in the testis culture medium were measured using EIA without extraction. The lowest EIA quantification limit was 0.0625 ng/mL. The intra- and inter-assay coefficients of variation for plasma testosterone levels were 7.1% and 15.7% at 0.75 ng/mL, respectively. For the culture medium, the intra- and inter-assay coefficients of variation were 8.7% and 18.7% at 1.34 ng/mL, respectively. Plasma LH concentrations were determined by double-antibody radioimmunoassay (RIA) with a rat LH RIA kit provided by the National Hormone and Peptide Program (Baltimore, MD, USA), as previously described [17], and were expressed in terms of the NIDDK rat LH-RP-3. The lowest quantification limit of the LH RIA was 0.078 ng/mL and the intra- and inter-assay coefficients of variation were 4.3% and 5.6% at 0.45 ng/mL, respectively.

Statistical analysis
Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) (version 20.0; IBM Corp., Armonk, NY, USA). All data were log-transformed before analysis to normalize the distributions. Statistical differences in plasma testosterone and LH levels during the perinatal period between Kiss1+/+ and Kiss1−/− rats (genotypes and time points as main effects) were determined using a two-way analysis of variance (ANOVA) followed by the analysis of simple main effects with Bonferroni correction. Statistical differences in plasma LH and testosterone concentrations in neonatal male rats injected with vehicle or rKp-52, in the number of GnRH neurons and c-Fos-expressing GnRH neurons in neonatal male rats injected with vehicle or rKp-52, and in testosterone release from testes treated with LH or rKp-52 were determined using two-way ANOVA (genotypes and treatments as the main effects). Significant differences between Kiss1+/+ and Kiss1−/− male rats in the number of 3βHSD-positive cells were determined using a two-tailed Student’s t-test. All data are presented as mean ± SEM.

Results
Loss of Kiss1 diminished postnatal testosterone and LH surges
The profile of plasma testosterone levels during the perinatal period in Kiss1+/+ and Kiss1−/− male rats is shown in Fig. 1A. A biphasic pattern of plasma testosterone levels, with the lowest level at E21, was observed in Kiss1−/− male rats. Kiss1−/− rats also showed a biphasic pattern of plasma testosterone levels, with the lowest level at birth (0 h after birth). Two-way ANOVA revealed that there were significant main effects (genotypes [F1, 79] = 27.119, p < 0.001) and time points [F(7, 79) = 3.753, p = 0.011]) and a significant interaction between the main effects (genotypes and time points; F(7, 79) = 2.617, p = 0.018) on plasma testosterone levels. Specifically, testosterone levels during the prenatal period (from E17.5 to E19.5) were relatively high in Kiss1+/+ male rats and significantly higher in Kiss1−/− male rats than the levels at birth (0 h after birth). The testosterone levels in the postnatal periods (from 0–1.5 h to 1.5–3 h after birth) were relatively high compared to those at birth (0 h after birth), and then the levels at 3–4.5 h after birth tended to decrease to the comparable levels at birth (0 h after birth) in both Kiss1+/+ and Kiss1−/− male rats. Plasma testosterone levels in Kiss1−/− male rats were significantly lower than those in Kiss1+/+ male rats in the postnatal period (0–4.5 h after birth; ** p < 0.01), whereas no significant difference was found in testosterone levels between Kiss1+/+ and Kiss1−/− male rats at any time during the prenatal period (from E17.5 to E21).

To investigate whether the kisspeptin-dependent
increase in LH secretion is involved in the neonatal testosterone surge, plasma LH concentrations were determined in Kiss1+/+ and Kiss1−/− male rats at embryonic day 17.5 (E17.5), E18.5, E19.5, and E21 (one day before the expected day of birth) and after birth (0, 0–1.5, 1.5–3, and 3–4.5 h after birth). Asterisks indicate significant differences in the plasma testosterone levels between Kiss1+/+ and Kiss1−/− rats at each time point (** p < 0.01, the simple main effect of the two-way ANOVA). Different capital and lower-case letters indicate significant differences at different time points within Kiss1+/+ and Kiss1−/− rats, respectively (p < 0.05, the simple main effect of the two-way ANOVA). (B) Plasma LH concentrations in Kiss1+/+ and Kiss1−/− male rats at E21 and 0 h after birth. Asterisks indicate significant differences in the plasma LH levels (** p < 0.01, the simple main effect of the two-way ANOVA). Values are presented as the mean ± SEM. Numbers in columns indicate the number of animals used.

Fig. 1  Role of kisspeptin in the generation of neonatal testosterone and LH surge. (A) Plasma testosterone concentrations in Kiss1+/+ and Kiss1−/− male rats at embryonic day 17.5 (E17.5), E18.5, E19.5, and E21 (one day before the expected day of birth) and after birth (0, 0–1.5, 1.5–3, and 3–4.5 h after birth). Asterisks indicate significant differences in the plasma testosterone levels between Kiss1+/+ and Kiss1−/− rats at each time point (** p < 0.01, the simple main effect of the two-way ANOVA). Different capital and lower-case letters indicate significant differences at different time points within Kiss1+/+ and Kiss1−/− rats, respectively (p < 0.05, the simple main effect of the two-way ANOVA). (B) Plasma LH concentrations in Kiss1+/+ and Kiss1−/− male rats at E21 and 0 h after birth. Asterisks indicate significant differences in the plasma LH levels (** p < 0.01, the simple main effect of the two-way ANOVA).}

Two-way ANOVA revealed significant main effects (genotypes [F(1, 15) = 18.693, p = 0.001] and time points [F(1, 15) = 14.637, p = 0.002]) and a significant interaction between the main effects (genotypes and time points: F(1, 15) = 7.474, p = 0.015). Specifically, the plasma LH level at 0 h after birth was significantly higher than that at E21 in Kiss1+/+ male rats (** p = 0.001); however, no significant increase was found in the LH level at 0 h after birth compared with that at E21 in Kiss1−/− male rats (p = 0.415). Importantly, plasma LH levels at 0 h after birth in Kiss1+/+ male rats were significantly higher than in Kiss1−/− male rats (** p < 0.001).

Response of GnRH, LH, and testosterone to exogenous kisspeptin challenge in neonatal Kiss1+/+ and Kiss1−/− male rats

To further examine the effects of kisspeptin on the neonatal HPG axis, rKp-52 was injected within 2 h after birth into Kiss1+/+ and Kiss1−/− male neonates, as shown in Fig. 2A. GnRH-immunoreactive cells showing c-Fos signals were found 1 h after rKp-52 injection in both Kiss1+/+ and Kiss1−/− male rats, while they were rarely found in the vehicle-treated controls (Fig. 2B). Two-way ANOVA revealed that the number of GnRH-positive cells with c-Fos signal was significantly increased by rKp-52 treatment (main effect, F(1, 6) = 47.690, ## p < 0.001), while neither the loss of the Kiss1 gene nor rKp-52 treatment affected the total number of GnRH-positive cells in neonatal male rats (Fig. 2C). There was no significant interaction between the main effects (genotypes and treatments) on the number of GnRH neurons and the number of GnRH neurons with c-Fos (F(1, 8) = 0.289, p = 0.606 and F(1, 6) = 0.254, p = 0.632, respectively). The rKp-52 injection also increased plasma LH and testosterone levels in Kiss1+/+ and Kiss1−/− male rats. Two-way ANOVA revealed that plasma LH concentrations were significantly higher in the rKp-52-treated group than in the vehicle-treated group (main effect, F(1, 22) = 35.037, ## p < 0.001) and significantly lower in Kiss1−/− male rats than in Kiss1+/+ male rats (main effect, F(1, 22) = 9.720, ** p = 0.005; Fig. 2D). Plasma testosterone concentrations were also significantly higher in
Fig. 2 Effect of kisspeptin challenge on the stimulation of GnRH neurons and LH release. (A) Male neonates were subcutaneously injected with 1 nmol rat recombinant kisspeptin (rKp-52) or vehicle within 2 h after birth, and blood and brain samples were collected 1 h later. (B) Representative photomicrographs of GnRH (red) and c-Fos (green)-immunoreactive cells in the preoptic area (POA) of vehicle- or rKp-52-treated Kiss1+/+ (upper panel) and Kiss1−/− male neonates (lower panel). Scale bar = 100 μm, 10 μm (inset). (C) The number of GnRH cells and c-Fos-positive GnRH cells in the POA of each group. Open circles indicate the individual data points of the total number of GnRH cells, and solid circles indicate the individual data points of the c-Fos-positive GnRH cells. The rKp-52-treated group than in the vehicle-treated group (main effect, \( F(1, 24) = 6.028, \# p = 0.022 \), and significantly lower in Kiss1−/− male rats than in Kiss1+/+ male rats (main effect, \( F(1, 24) = 26.781, ** p < 0.001 \); Fig. 2E). There was no significant interaction between the main effects (genotypes and treatments) on plasma LH and testosterone levels (\( F(1, 22) = 0.260, p = 0.615 \) and \( F(1, 24) = 2.516, p = 0.126 \), respectively).

Loss of Kiss1 failed to affect the number of fetal-type Leydig cells

To examine whether fetal-type Leydig cells are affected by the loss of Kiss1, the number of fetal-type Leydig cells in testes obtained from Kiss1+/+ and Kiss1−/− male rats was measured (Fig. 3A). Immunoreactive cells of 3βHSD, a marker for fetal-type Leydig cells, were observed in the testes of Kiss1+/+ and Kiss1−/− rats (Fig. 3B). There was no significant difference in the number of 3βHSD-positive cells per unit area between the Kiss1+/+ and Kiss1−/− rats (\( p = 0.413, \) two-tailed Student’s t-test; Fig. 3C).
LH but not kisspeptin directly induced testosterone release from neonatal testes of Kiss1\(^{+/+}\) and Kiss1\(^{-/-}\) rats

To determine the direct effect of LH on testosterone release from neonatal testes, testes obtained from Kiss1\(^{+/+}\) and Kiss1\(^{-/-}\) neonatal male rats within 2 h after birth were cultured in vitro with LH or vehicle for 4 h (Fig. 4A). Two-way ANOVA revealed that testosterone release into the culture medium was significantly higher in LH-treated testes than in vehicle-treated testes (main effect, \(F(1, 8) = 42.938, \#\#p < 0.001\); Fig. 4B). Testosterone release was significantly lower in Kiss1\(^{-/-}\) testes than in Kiss1\(^{+/+}\) testes (main effect, \(F(1, 8) = 57.047, **p < 0.001\)). There was no significant interaction between the main effects (genotypes and treatments; \(F(1, 8) = 2.557, p = 0.148\)).

To evaluate the role of intratesticular kisspeptin-GPR54 signaling in testosterone release, the expression of Kiss1 and Gpr54 mRNA in neonatal testes of Kiss1\(^{+/+}\) and Kiss1\(^{-/-}\) rats. Lane M, DNA ladder marker. Lane N, negative control without a template. (D) Testosterone release from in vitro cultured Kiss1\(^{+/+}\) and Kiss1\(^{-/-}\) neonatal testes treated with rKp-52 (0.2 or 2 \(\mu\)M). Asterisks indicate a significant difference between the Kiss1\(^{+/+}\) and Kiss1\(^{-/-}\) groups (** \(p < 0.01\), the main effect of the two-way ANOVA). Values are represented as the mean ± SEM. Numbers in columns indicate the number of animals used.
the vehicle- and rKp-52-treated groups, while testosterone release was significantly lower in Kiss1<sup>−/−</sup> testes than in Kiss1<sup>+/+</sup> testes (main effect, F(1, 26) = 63.466, ** p < 0.001; Fig. 4D). There was no significant interaction between the main effects (genotypes and treatments; F(3, 26) = 0.899, p = 0.455).

**Discussion**

The present study demonstrated that central kisspeptin plays a role, at least partly, in inducing postnatal testosterone increase in male rats because postnatal plasma testosterone levels were significantly lower in Kiss1<sup>−/−</sup> male rats than in Kiss1<sup>+/+</sup> male rats. This notion is consistent with a previous study, which suggested that the postnatal testosterone surge depends on the kisspeptin-GnRH pathway by showing that the plasma testosterone concentration within 2 h after birth in GnRH neuron-specific Gpr54<sup>−/−</sup> male neonatal mice was significantly lower than that in wild-type mice [25]. Indeed, the current exogenous kisspeptin challenge could induce LH and testosterone release, as well as c-Fos (neural activation marker) expression in GnRH neurons in the postnatal period (2 h after birth) in Kiss1<sup>−/−</sup> male rats. Furthermore, the postnatal testosterone increase is likely due to central kisspeptin signaling but is unlikely due to intratesticular kisspeptin. *In vitro* kisspeptin treatment failed to increase testosterone release from postnatal testes, and 3βHSD expression in postnatal Kiss1<sup>−/−</sup> male rats was comparable to that in Kiss1<sup>+/+</sup> male rats. Our previous study showed that Kiss1<sup>−/−</sup> male rats displayed female-type sexual behavior, such as lordosis behavior, and neonatal kisspeptin administration caused defeminization of sexual behavior in Kiss1<sup>−/−</sup> male rats [18]. Together with the current study, these findings suggest that kisspeptin-induced activation of the HPG axis, at least partly, causes a postnatal testosterone surge and consequent defeminization of the brain mechanism responsible for lordosis behavior in male rats [35]. To the best of our knowledge, this is the first report to show a detailed analysis of both prenatal and postnatal testosterone surges in kisspeptin-deficient animals. Specifically, the current study revealed the importance of central kisspeptin and subsequent activation of the HPG axis in inducing postnatal testosterone surge by using Kiss1<sup>−/−</sup> male rats. Importantly, the present study suggests that the mechanism responsible for the perinatal biphasic testosterone surge is largely conserved among rodent species, such as rats and mice [25].

It has been reported that perinatal male rodents show biphasic (prenatal and postnatal) increases in plasma testosterone levels, and the prenatal testosterone surge occurring around E17–E19 in fetal male rats is critical for masculinizing external genitals [36-39]. The current study showed that plasma testosterone levels during the prenatal period were comparable in Kiss1<sup>+/+</sup> and Kiss1<sup>−/−</sup> male rats, suggesting that, unlike in the postnatal period, a prenatal increase in testosterone release is Kiss1 gene independent. This result is consistent with our previous study, which showed that testosterone levels in Kiss1<sup>−/−</sup> male rats were comparable to those in Kiss1<sup>+/+</sup> male rats at E18 of the prenatal period [18]. Fetal-type Leydig cells, whose origin differs from adult-type Leydig cells, appear shortly after gonadal differentiation and are responsible for prenatal and neonatal testosterone surges [40, 41]. In the present study, the number of fetal-type Leydig cells was comparable between Kiss1<sup>+/+</sup> and Kiss1<sup>−/−</sup> neonatal male rats. In addition, the anogenital distance was distinguishable from females at birth even in Kiss1<sup>−/−</sup> male rats that had a greater length between the anus and genitalia as in Kiss1<sup>+/+</sup> neonatal male rats. These results suggest that prenatal kisspeptin-independent testosterone synthesis is responsible for masculinizing the external genitals.

In the present study, a biphasic pattern of perinatal testosterone release was observed, showing lower testosterone levels at E21 and 0 h than at other pre- and postnatal time points in Kiss1<sup>+/+</sup> male rats. However, no statistical difference in plasma testosterone levels was detected between any time point in Kiss1<sup>+/+</sup> male rats. This is likely mainly due to the individual variation in the timing of the postnatal increase in testosterone level in Kiss1<sup>+/+</sup> male rats. Especially at birth, some neonates may have already started to increase testosterone release, since plasma testosterone levels at E21 were lowest in the current Kiss1<sup>+/+</sup> male rats and postnatal testosterone surge reportedly occurs in a short window, which starts to increase immediately after birth and peaks at around 2 h after birth [1, 5, 42]. Importantly, plasma testosterone levels at 0 h in Kiss1<sup>+/+</sup> male rats were significantly higher than those in Kiss1<sup>−/−</sup> male rats, suggesting that Kiss1-dependent testosterone release had already started at birth.

In the present study, Gpr54 mRNA was detected in the testes of both Kiss1<sup>+/+</sup> and Kiss1<sup>−/−</sup> male rats 2 h after birth, implying that kisspeptin-GPR54 signaling may have a physiological role in testicular function. However, it is unlikely that this signaling affects steroidogenesis in rats because the current exogenous kisspeptin challenge failed to induce testosterone release from both Kiss1<sup>+/+</sup> and Kiss1<sup>−/−</sup> testes. This notion is consistent with previous studies that reported that kisspeptin did not affect basal or gonadotropin-induced *in vitro* testosterone release from adult mouse testes [29, 43]. Taken together, these findings suggest that intra-testis kisspeptin-GPR54 signaling does not play a critical role in inducing
testosterone synthesis and release in both fetal- and adult-type Leydig cells in rodents. Conversely, the production of testosterone in Leydig cells isolated from the testes of adult goats was suppressed by a kisspeptin antagonist [44], and kisspeptin administration significantly increased human chorionic gonadotropin (hCG)-stimulated testosterone levels in adult male rhesus monkeys pretreated with acyline, a GnRH antagonist [28]. These findings raise the possibility of species differences in the role of intra-testis kisspeptin-GPR54 signaling in regulating testosterone synthesis/release from Leydig cells. Further studies are required to clarify the role of kisspeptin-GPR54 signaling in various mammalian species.

In the current study, in vitro testosterone release from postnatal (2 h after birth) Kiss1+/+testes was significantly higher than that from Kiss1−/−testes regardless of LH or rKp-52 treatment. It is speculated that the higher testosterone release from Kiss1+/+testes could be due to advanced exposure to endogenous LH induced by kisspeptin in vivo. Indeed, in vitro LH-induced testosterone release from Kiss1+/+neonatal testes was much higher than from Kiss1−/−testes. This result further supports the current in vivo result that a postnatal testosterone increase occurred after a plasma LH surge in Kiss1+/+ male rats but not in Kiss1−/− male rats, indicating the essential role of kisspeptin and the subsequent LH release in the induction of testosterone surge from postnatal rat testes.

In conclusion, the present study suggests that central kisspeptin-GPR54 signaling is indispensable for the induction of postnatal testosterone surge in male rats and that kisspeptin neurons activated after birth may stimulate GnRH and LH release, triggering the postnatal testosterone surge (Fig. 5). The present study also suggests that kisspeptin-GPR54 signaling is dispensable for generating the prenatal testosterone surge.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research to declare.

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