**Abstract.** Puberty is associated with an increase in gonadotropin secretion as a result of an increase in gonadotropin-releasing hormone (GnRH) secretion. Kisspeptin is considered to play a key role in puberty onset in many mammalian species, including rodents, ruminants and primates. The present study aimed to determine if changes in hypothalamic expression of the KISS1 gene, encoding kisspeptin, are associated with the onset of puberty in pigs. The animals (n=4 in each group) were perfused with 4% paraformaldehyde at 0, 1, 2, 3 and 4 months old, as prepubertal stages, and at 5 months old, as the peripubertal stage, following each blood sampling. KISS1 gene expressions in coronal sections of brains were visualized by in situ hybridization. Plasma luteinizing hormone (LH) was measured by radioimmunoassay. KISS1 mRNA signals were observed in the arcuate nucleus (ARC) at all ages examined without any significant difference in the number of KISS1 mRNA signals. The hormone (GnRH) secretion is associated with an increase in gonadotropin-releasing hormone (GnRH) secretion as a result of an increase in gonadotropin-releasing hormone (GnRH) secretion [1, 2]. Pulsatile GnRH/luteinizing hormone (LH) secretion is accelerated towards completion of puberty [3], leading to the onset of reproductive cyclicity in female mammals.

To date, kisspeptin, a neuropeptide encoded by the Kiss1 gene, and its receptor are considered to be involved in puberty onset via direct stimulation of GnRH secretion [4–7]. The loss-of-function mutations for Gpr54, encoding a kisspeptin receptor, cause sexual immaturity in humans [8, 9] and mice [9]. There are two major populations of kisspeptin neurons: one is the anterior hypothalamus, such as the anteroventral periventricular (AVPV)/preoptic area (POA), and the other is the hypothalamic arcuate nucleus (ARC) [10]. Initial studies in mice [11] and monkeys [12] revealed that Kiss1 expression in the whole hypothalamus increases when animals undergo pubertal maturation. More specifically, a pubertal increase in gene expression was also reported in the AVPV/POA and ARC in rats [13–15] and in sheep [16]. An increased number of kisspeptin immuno-positive fibers were found on GnRH neuronal cell bodies during pubertal development in mice, indicating that morphological proximity of the kisspeptin and GnRH neurons is involved in puberty onset [17]. These studies provide circumstantial evidence indicating that acceleration of kisspeptin-GPR54 signaling triggers the onset of puberty by stimulating the hypothalamic-pituitary-gonadal (HPG) axis in mammals.

The central mechanism underlying the hormonal change during pubertal development in pigs has yet to be revealed. Our previous study showed that KISS1-expressing cells are located in the paraventricular nucleus (PeN) and ARC in the adult female pig, as shown in other mammals [18]. Exogenous kisspeptin administration induces LH secretion in prepubertal gilts [19], which suggests that the HPG axis is responsive to kisspeptin during pubertal maturation in pigs. These studies imply a possible involvement of kisspeptin in maturation of the HPG axis in pigs.

The present study aimed to determine whether or not changes in hypothalamic expression of the KISS1 gene is associated with the onset of puberty in gilts to gain better comprehension of the neuropeptide involvement in reproductive maturation in the pig.
Materials and Methods

Animals
Female Landrace (L) and Large White (W) pigs, an F1 hybrid of Landrace and Large White pigs (LW) and a three-way cross of LW and Duroc pigs were used at 0, 1, 2, 3, 4 and 5 months of age. The gilts were kept at the Aichi Agricultural Research Center (Nagakute, Aichi, Japan) and fed a standard diet with free access to water. The body weights of the animals at each age are shown in Figure 1. Estrus behaviors of the 5-month-old females were assessed by the back pressure test in the presence of a mature boar. Those that showed a standing estrus reflex within a week before the day of sampling were used as peripubertal animals. All animal treatment procedures were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals [20].

Tissue sampling
For perfusion, all the animals were intravenously injected with heparin (4000 units/10 kg body weight) followed by administration of sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan) for deep anesthesia. Blood samples were collected before anesthesia. After collecting ovaries, the brains of the animals were perfused through the carotid arteries with 0.3–5 l of 0.05 M PBS (according to the body size) containing 13000 U/l heparin, followed by perfusion of the same volume of 4% paraformaldehyde in 0.05 M PB. Plasma samples were kept at −20 °C until radioimmunoassay (RIA) for LH, follicle-stimulating hormone (FSH), estradiol (E$_2$) and progesterone. The hypothalamic tissue was dissected out, postfixed overnight in the same fixative at 4 °C, immersed in 30% sucrose in 0.05 M PB at 4 °C and kept at −80 °C in OCT compound (Sakura Finetek Japan, Tokyo, Japan) until analysis of KISS1 mRNA by in situ hybridization. Tissue sampling was undertaken between 1000 and 1500 h on each day from April to August 2009 (3 gilts of each age group at 1 to 5 months of age), on each day in September 2010 (3 gilts of 0 months of age) and on each day from September to November 2011 (1 gilt for each age group).

KISS1 in situ hybridization
KISS1 expression in the pig hypothalamus was determined by in situ hybridization as described previously [18]. Briefly, the hypothalamic coronal sections (50 μm thick) including the PeN or ARC were made on a cryostat, and every fourth section was used for the analysis. Digoxigenin (DIG)-labeled antisense cRNA probes for porcine KISS1 mRNA were hybridized at 60 °C overnight. Hybridized probes were detected using an alkaline phosphatase-conjugated anti-DIG Fab fragment (Roche Diagnostics, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium chloride (Roche Diagnostics). The numbers of KISS1-expressing cells in the ARC, covered by 17–23 sections, were counted unilaterally under a light microscope in every individual animal.

Radioimmunoassay
Plasma LH and FSH concentrations were measured by double antibody RIA using kits provided by the National Hormone and Peptide Program (Baltimore, MD, USA) and were expressed in terms of NIDDK-pLH (AFP11043B) and NIDDK-pFSH (AFP10640B), respectively. AFP11046B and AFP10640B were also used for $^{125}$I labeling. The least detectable levels for 50 μl plasma sample were 0.02 ng/ml for LH and 0.078 ng/ml for FSH. The intra-assay coefficients of variation were 7.4% at 0.71 ng/ml for LH and 4.8% at 0.63 ng/ml for FSH. The plasma E$_2$ and progesterone concentrations were measured by radioimmunoassay. Values are means ± SEM (n=3 for E$_2$, n=4 for body weight, P$_4$, LH and FSH). Values with different letters are significantly different (P<0.05, one-way ANOVA followed by Bonferroni test).

![Fig. 1. Changes in body weight, plasma LH, FSH, estradiol (E$_2$) and progesterone (P$_4$) levels in pre- and peri-pubertal pigs. Blood samples were taken from pre- (0–4 months of age) and peri- (5 months of age) pubertal pigs immediately before brain collection. Plasma LH, FSH, E$_2$ and P$_4$ concentrations were measured by radioimmunoassay. Values are means ± SEM (n=3 for E$_2$, n=4 for body weight, P$_4$, LH and FSH). Values with different letters are significantly different (P<0.05, one-way ANOVA followed by Bonferroni test).](image-url)
Statistical analysis

One-way ANOVA was performed followed by the Bonferroni test to determine the statistical difference in the numbers of KISS1-expressing cells in the ARC and the plasma LH, FSH, E2, and progesterone concentrations between the groups at different developmental stages.

Results

Plasma LH, FSH, E2 and progesterone concentrations at pre- and peripubertal stages in the pig

The plasma LH concentration of the 0-month-old piglets was significantly higher than those of 1-, 2-, or 5-month-old groups (P<0.05, one-way ANOVA followed by the Bonferroni test, Fig. 1). The plasma LH levels showed a transient decrease at 1 month of age and then increased gradually from 2 to 4 months of age. The plasma FSH concentrations increased slightly as the animal age underwent development, reaching their peak at 2 months of age, and decreased until 5 months of age. There was a significant difference in the plasma FSH level between 2 and 5 months of age (P<0.05). No significant difference was found in plasma E2 levels among any groups. The plasma progesterone concentrations were high in one animal at 5 months of age, but no significant difference was found among any groups (note that the animal that showed the high progesterone level had corpora lutea).

Development of ovaries at pre- and peripubertal stages of the pig

Photographs of ovaries of representative animals at 0, 1, 2, 3, 4 and 5 month of age are shown in Figure 2A–H. In two out of four animals at 3 months of age, the ovary size dramatically increased (Fig. 2E), while in the other two (Fig. 2D), the ovary size was comparable to those at 0, 1 and 2 months of age (Fig. 2A–C). Many apparent antral follicles were observed in all animals at 4 and 5 months of age (Fig. 2F–G). In an ovary of one 5-month-old animal, corpora lutea were observed as shown in Fig. 2H.

KISS1-expressing cells in the pig hypothalamus at pre- and peri-pubertal stages

Figure 3 shows a schematic illustration indicating the PeN (left panel) and ARC (right panel) in the pig hypothalamus and representative photomicrographs of brain sections including the PeN or ARC at 5 and 3 months of age, respectively. Many KISS1-positive cells were found in the ARC, while few positive cells were found in the PeN at any stages examined. Figure 4A shows representative photomicrographs indicating the magnified ARC region at each age. KISS1 mRNA signals were found in the ARC of all the individuals at 0, 1, 2, 3, 4 and 5 months of age. The distribution and strength of the KISS1 mRNA signals in the prepubertal period (0–4 months of age) were similar to those in peripubertal animals at 5 months of age. The total numbers of KISS1-expressing cells in the ARC showed no significant difference between ages (Fig. 4B).

Discussion

The present study demonstrated that many KISS1-expressing cells were observed in the ARC of the pig hypothalamus throughout the developing period, and the numbers of KISS1-expressing cells in the ARC in peripubertal gilts were comparable to those in 5-month-old peripubertal pigs showing behavioral estrus. Kisspeptin-GPR54 signaling is considered to play an important role in pubertal onset in other mammalian species, such as mice and humans [8, 9], because loss-of-function mutations for Gpr54 cause the absence of puberty in these species. A previous study showed that exogenous kisspeptin dramatically stimulates LH secretion in prepubertal gilts [19], suggesting that downstream effectors of kisspeptin neurons such as GnRH neurons and gonadotrophs in the anterior pituitary are responsive to kisspeptin stimulus. Therefore, kisspeptin secretion rather than kisspeptin synthesis may be inhibited in the prepubertal period in the pig. In this context, a pubertal increase in expression of other neurotransmitters such as neurokinin B, a stimulator for kisspeptin neuronal activity [22], could be involved in pubertal onset in pigs rather than kisspeptin synthesis as suggested in mice [23].

The current study suggests that kisspeptin neurons in the ARC rather than in the PeN are responsible for the initial step of pubertal maturation of the HPG axis in pigs, as we found a consistent number of KISS1-expressing cells in the ARC and few of them in the PeN during the developmental period. Rats and monkeys showed a dramatic increase in KISS1/kisspeptin expression in the ARC [12, 14], while pigs showed constant ARC KISS1 expression in both pre- and peripubertal stages in pigs. This fact again suggests that establishment of a kisspeptin-releasing system from the ARC kisspeptin neurons could be responsible for pubertal onset in the pig. Analysis of kisspeptin expression at the peptidergic level by immunohistochemistry may provide another aspect to understand the synthesis and secretion of kisspeptin. Regarding KISS1/kisspeptin expressions in the anterior hypothalamus, such as AVPV and POA, a pubertal increase in KISS1/kisspeptin expression in the AVPV/POA was evident in rodents [14, 15, 17, 24] and sheep [16], while we found few KISS1-expressing cells in the PeN, even in the peripubertal pigs at 5 months of age. PeN kisspeptin neurons are considered a GnRH/LH surge center in pigs, as KISS1 expression is stimulated by preovulatory level of estrogen in adult ovarietomized pigs [18]. In this context, the low level of KISS1 expression in the PeN in pubertal gilts in the present study could be due to immature stages of developing follicles, even in the 5-month-old peripubertal pigs.

In the current study, the plasma LH concentration was highest in the neonatal period (0 month) in the gilts and showed a subsequent decrease in the peripubertal period, which is consistent with the concentrations in ruminants [25]. This decrease could have resulted from the developing negative feedback mechanism in the HPG axis during the prepubertal period. The plasma FSH concentration was decreased when ovaries showed drastic development at 3 months of age, suggesting that inhibin secreted from developing follicles may be involved in FSH suppression. In rats, prepubertal Kiss1/kisspeptin expressions in the ARC are strongly inhibited by estrogen negative feedback [14]. On the other hand, ARC KISS1 expression was constantly detected during the developmental period in pigs. This suggests that unlike rats, prepubertal estrogen negative feedback on ARC KISS1 expression is not evident in pigs. This notion is consistent with the fact that the preovulatory level of estrogen has little inhibitory effect on ARC KISS1 expression in the adult female pig [18]. Therefore, the sensitivity to estrogen with respect to regulation of ARC KISS1 expression in pigs is likely different from those in...
Fig. 2. Ovaries in representative pigs at 0, 1, 2, 3, 4 and 5 months old. Numbers above the photographs indicate the ages of animals. At 3 months of age, half of the pigs showed a dramatic increase in ovary size, while the other half showed comparable sizes of ovaries at 0, 1 and 2 months of age. Apparent antral follicles were observed in all animals at 4 and 5 months of age. There were corpora lutea in an ovary from one animal at 5 months of age. CL, corpora lutea.

Fig. 3. Schematic illustration of the pig hypothalamus including the PeN (left) and ARC (right) and representative photographs showing the PeN and ARC of gilts at 5 months and 3 months of age, respectively. Few KISS1-expressing cells were found in the PeN in 5-month-old pigs. 3V, third ventricle; AC, anterior commissure; PeN, periventricular nucleus; VMH, ventromedial hypothalamic nuclei; ARC, arcuate nucleus; PVH, hypothalamic paraventricular nucleus.

Fig. 4. KISS1 mRNA signals in the hypothalamic arcuate nucleus (ARC) at pre- (0, 1, 2, 3 and 4 months old) and peri-pubertal (5 months old) stages in pigs detected by in situ hybridization with KISS1-specific cRNA probes. A, KISS1 mRNA signals in the ARC of representative animals at 0, 1, 2, 3, 4 and 5 months old. B, Numbers of KISS1-expressing cells in the ARC at each age in months (n=4). No significant difference was found in the numbers among ages by statistical analysis (one-way ANOVA). Values are means ± SEM (n=4).
rodents at pre- and post-pubertal stages. A previous study implied that the downstream effectors, such as hypothalamic GnRH neurones and pituitary gonadotrophs, function properly in prepubertal pigs, as intravenous injection of GnRH induces LH secretion in prepubertal pigs at 40, 80 and 120 days old [26]. GnRH immunoactivity was observed in the hypothalamus of fetal and postnatal pigs [27]. These studies indicate that GnRH and its receptor signaling are ready to function at the onset of puberty in pigs. During pubertal development, Gpr54 expression increases in rats [14] and monkeys [12]. In mice, a pubertal increase in Gpr54 expression in GnRH neurons was found in the neonatal stage [28]. Future study is required to clarify if an increase in Gpr54 expression is involved in the pubertal acceleration of GnRH/LH release in the pig. The present study indicates that hypothalamic KISS1 is consistently expressed in the developing period and shows no association between the gene expression level and the timing of puberty onset in pigs. Therefore, the increase of KISS1 gene expression is not a prior indicator of puberty in pigs, as previously reported in mice [23]. Neuronal factors that trigger an increase in kisspeptin and then GnRH secretion in developing pigs require further investigation.

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References

1. Loose MD, Terasawa E. Pulsatile infusion of luteinizing hormone-releasing hormone induces precocious puberty (vaginal opening and first ovulation) in the immature female guinea pig. Biol Reprod 1985; 33: 1084–1093. [Medline] [CrossRef]
2. Watanabe G, Terasawa E. In vivo release of luteinizing hormone releasing hormone increases with puberty in the female theus monkey. Endocrinology 1989; 125: 92–99. [Medline] [CrossRef]
3. Harris GC, Levine JE. Pulsatile activation of pulsatile gonadotropin-releasing hormone release in male rats as revealed by microdialysis. Endocrinology 2003; 144: 163–171. [Medline] [CrossRef]
4. Messager S, Chatzlidaki KE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinger E, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proc Natl Acad Sci USA 2005; 102: 1761–1766. [Medline] [CrossRef]
5. Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acshido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA. A role for kisspeptin in the regulation of gonadotropin secretion in the mouse. Endocrinology 2004; 145: 4073–4077. [Medline] [CrossRef]
6. Matsui H, Takatsu Y, Kumanos O, Matsumoto H, Ohtaki T. Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. Biochem Biophys Res Comm 2004; 329: 383–388. [Medline] [CrossRef]
7. d’Angleym de Tassagne X, Fagg LA, Carlton MB, Colledge WH. Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. Endocrinology 2008; 149: 3926–3932. [Medline] [CrossRef]
8. de Houx N, Genin E, Carel JC, Matsuda F, Chaussee JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KISS1-derived peptide receptor GPR54. Am J Hum Genet 2003; 73: 10972–10976. [Medline] [CrossRef]
9. Seminara SB, Messager S, Chatzlidaki EE, Thresher RR, Aparicio JS, Shagury JK, Bo-Abbas Y, Kusuhong W, Schinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O’Rahilly S, Carlton MB, Crowley WF Jr, Aparicio SA, Colledge WH. The GPR54 gene as a regulator of puberty. N Engl J Med 2003; 349: 1614–1627. [Medline] [CrossRef]
10. Asachi S, Yamada S, Takahashi Y, Matui H, Kinosita M, Takase K, Sugihara H, Ohtaki T, Matsumoto H, Uenoyma Y, Tsukamura H, Inoue K, Maeda K. Involvement of anterograde periventricular metastin/kisspeptin neurons in estrogen positive feedback action on lutetinizing hormone release in female rats. J Reprod Dev 2007; 53: 367–378. [Medline] [CrossRef]
11. Navarro VM, Castellano JM, Fernández-Fernández R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pittlla L, Tena-Sepere M, Developmental and hormonally regulated messenger ribonucleic acid expression of KISS-1 and its putative receptor, GPR54, in rat hypothalamus and potential lutetinizing hormone-releasing-activity of KISS-1 peptide. Endocrinology 2004; 145: 4565–4574. [Medline] [CrossRef]
12. Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM. Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proc Natl Acad Sci USA 2005; 102: 2129–2134. [Medline] [CrossRef]
13. Beatson AH, Anel L, Simonneaux V, Tena-Sempere M, Juul A, Mikkelsen JD. Maturation of kisspeptinergic neurons coincides with puberty onset in male rats. Peptides 2010; 31: 275–283. [Medline] [CrossRef]
14. Takase K, Uenoyma Y, Inoue N, Matsui H, Yamada S, Shimizu M, Homma T, Tomikawa J, Kanda S, Matsumoto H, Oka Y, Tsukamura H, Maeda K. Possible role of estrogen in pubertal increase of Kiss1/kisspeptin expression in discrete hypothalamic areas of female rats. J Neuroendocrinol 2009; 21: 527–537. [Medline] [CrossRef]
15. Takumi K, Ibujma N, Ozawa H. Developmental changes in the expression of kisspeptin mRNA in rat hypothalamus. J Mol Neurosci 2011; 45: 138–145. [Medline] [CrossRef]
16. Redmond JS, Baetz-Sandoval GM, Spell KM, Spencer TE, Lents CA, Williams GL, Amstalden M. Developmental changes in hypothalamic KISS1 expression during activation of the pulsatile release of luteinising hormone in mating ewe lambs. J Neuroendocrinol 2011; 23: 815–822. [Medline] [CrossRef]
17. Clarkson J, Herbsom AE. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology 2006; 147: 5817–5825. [Medline] [CrossRef]
18. Tomikawa J, Homma T, Tajima S, Shibata T, Inamoto Y, Takase K, Inoue N, Ohkura S, Uenoyma Y, Maeda K, Takamura H. Molecular characterization and estrogen regulation of hypothalamic KISS1 gene in the pig. Biol Reprod 2010; 82: 313–319. [Medline] [CrossRef]
19. Tamaki Y, Harada K, Tsukamura H, Inoue T, Ohkura S, Ono K. Involvement of ovarian steroids and endogenous opioids in the fasting-induced suppression of pulsatile LH release in ovariectomized rats. J Endocrinol 1991; 129: 321–328. [Medline] [CrossRef]
20. de Croft S, Booth E, Herbsom AE. Neurokinin B activates arcuate kisspeptin neurons through multiple tachykinin receptors in the male mouse. Endocrinology 2013; 154: 2750–2760. [Medline] [CrossRef]
21. Gill JC, Navarro VM, Kwong C, Noel SD, Martin C, Xu S, Clifton DK, Carroll RS, Steiner RA, Kaiser UB. Increased neurokinin B (Taq2) expression in the mouse arcuate nucleus is an early marker of pubertal onset with differential sensitivity to sex steroid-negative feedback than Kiss1. Endocrinology 2012; 153: 4883–4893. [Medline] [CrossRef]
22. Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakowich SK, Clifton DK, Steiner RA, Herbsom AE. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci 2005; 25: 11349–11356. [Medline] [CrossRef]
23. Rawlings NC, Evans AC, Honaramouz A, Bartlewski PM. Antral follicle growth and endocrine changes in prepubertal cattle, sheep and goats. Anim Reprod Sci 2003; 78: 259–270. [Medline] [CrossRef]
24. Fleming MS, Dailey RA. Longitudinal study of the surge of gonadotropins induced by exogenous hormones in prepuberal gilts. Endocrinology 1985; 116: 1893–1896. [Medline] [CrossRef]
25. Pearson PL, Anderson LL, Jacobson CD. The pubertal ontogeny of lutetinizing hormone-releasing hormone-like immunoreactivity in the dienochephalon and telencephalon of the male Meishan pig brain. Brain Res Dev Brain Res 1996; 95: 157–168. [Medline] [CrossRef]
26. Herbsom AE, de Tassagne X, Doran J, Colledge WH. Distribution and postnatal development of Gpr54 gene expression in mouse brain and gonadotropin-releasing hormone neurons. Endocrinology 2010; 151: 312–321. [Medline] [CrossRef]