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—Original Article—

Ad libitum feeding triggers puberty onset associated with increases in arcuate *Kiss1* and *Pdyn* expression in growth-retarded rats

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Abstract. Increasing evidence shows that puberty onset is largely dependent on body weight rather than chronological age. To investigate the mechanism involved in the energetic control of puberty onset, the present study examined effects of chronic food restriction during the prepubertal period and the resumption of *ad libitum* feeding for 24 and 48 h on estrous cyclicity, *Kiss1* (kisspeptin gene), *Tac3* (neurokinin B gene) and *Pdyn* (dynorphin A gene) expression in the hypothalamus, luteinizing hormone (LH) secretion and follicular development in female rats. When animals weighed 75 g, they were subjected to a restricted feeding to retard growth to 70–80 g by 49 days of age. Then, animals were subjected to *ad libitum* feeding or remained food-restricted. The growth-retarded rats did not show puberty onset associated with suppression of both *Kiss1* and *Pdyn* expression in the arcuate nucleus (ARC). 24-h *ad libitum* feeding increased tonic LH secretion and the number of Graafian and non-Graafian tertiary follicles with an increase in the numbers of ARC *Kiss1*- and *Pdyn*-expressing cells. 48-h *ad libitum* feeding induced the vaginal proestrus and a surge-like LH increase with an increase in *Kiss1*-expressing cells in the anteroventral periventricular nucleus (AVPV). These results suggest that the negative energy balance causes pubertal failure with suppression of ARC *Kiss1* and *Pdyn* expression and then subsequent gonadotropin secretion and ovarian function, while the positive energetic cues trigger puberty onset via an increase in ARC *Kiss1* and *Pdyn* expression and thus gonadotropin secretion and follicular development in female rats.

Key words: Dynorphin A, Follicular development, Kisspeptin, Luteinizing hormone, Puberty

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Attainment of reproductive capacity at puberty has been known to be initiated by an activation of the hypothalamic mechanism controlling tonic hypophyseal gonadotropin secretion in mammals [1–3]. It is well accepted that nutritional or energetic cues are important determinants of the initiation of tonic gonadotropin secretion during pubertal development, as evidenced by hyposecretion of gonadotropins when growth is retarded by food restriction in several mammalian species including cattle [4], sheep [5] and rats [6, 7]. Interestingly, such animals exhibited puberty onset when they reached "critical" body weights, at which normally grown animals exhibited puberty onset [5, 6]. The "critical" body weight hypothesis for determining puberty onset was initially proposed by Frisch and Revelle [8]. Their findings showed puberty onset in girls when they reached a body weight of around 47 kg at 17 years old in the 1840s, and at 13 years old in the 1960s along with nutritional improvements [9, 10]. Collectively, several lines of evidence suggested that the timing of puberty onset largely depends on body weight rather than chronological age in mammals [5–9].

Previous studies suggested that hypothalamic kisspeptin neurons, that govern mammalian reproduction, are involved in the mechanism regulating the pubertal increase in hypophyseal gonadotropin secretion [11–20]. Kisspeptin signaling in the hypothalamus is thought to be fundamental to initiate tonic hypophyseal gonadotropin secretion, because deficiency of kisspeptin or the kisspeptin receptor (also known as GPR54, deorphanized in 2001 [21, 22]) gene resulted in hypogonadotropic hypogonadism associated with pubertal failure in humans [11–13] and in animal models [14–20]. The hypothalamic arcuate nucleus (ARC) kisspeptin neurons, which co-express neurokinin B- and dynorphin A, thus referred to as KNDy neurons, are suggested to play a key role in generating pulsatile secretion of gonadotropin-releasing hormone (GnRH), which controls tonic hypophyseal gonadotropin secretion in mammals [3, 23, 24]. The currently working model suggests that each GnRH pulse is triggered by kisspeptin secreted from KNDy neurons, in which neurokinin B facilitates and dynorphin A inhibits the synchronized activity of KNDy neuronal cluster [23–26]. Indeed, Keen *et al*. [27] showed that pulsatile secretion of kisspeptin in the median eminence was well correlated with pulsatile secretion of GnRH detected in the median eminence in pubertal female monkeys. Further, central administration of neurokinin B or a dynorphin A antagonist increased the frequency of luteinizing hormone (LH) pulses and multiple unite activity volleys, which have been considered to reflect the rhythmic oscillations in the activity of KNDy neurons as an intrinsic source of the GnRH pulse generator, in sheep and goats [23–26]. On the other hand, anterior hypothalamic kisspeptin neuronal population in the anteroventral periventricular nucleus (AVPV) or preoptic area

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(POA) is now considered as a GnRH surge generator in response to the positive feedback action of estrogen derived from the preovulatory follicle(s) in mammals, because estrogen increases *Kiss1* (kisspeptin gene) expression or activates kisspeptin neurons in the AVPV or POA in all mammals examined to date [28–34]. Since energetic deficiency negatively regulates GnRH/LH pulse [35, 36] and surge [37], both ARC KNDy and AVPV/POA kisspeptin neurons would be major targets of negative energy balance in prepubertal period, during which energy is mainly consumed for body growth rather than reproductive function. Indeed, suppression of hypothalamic *Kiss1* and *Tac3* expression has been shown in peripubertal female rats subjected to short-time fasting [38, 39]. Suppression of hypothalamic *Kiss1* expression by short-time fasting has also been reported in adult rats and mice [40–42].

The present study aims to investigate whether energetic cues control puberty onset via regulating *Kiss1*, *Tac3* (neurokinin B gene) and *Pdyn* (dynorphin A gene) expression in the ARC KNDy neurons and AVPV/POA kisspeptin neurons. To this end, we used prepubertal chronic growth-retarded female rats [6, 43, 44]. This chronic growth-retarded rat model has an advantage to analyze the effect of energetic cues on KNDy gene expression during the pubertal transition, because previous studies demonstrated that the chronic growth-retarded rat model retained hypogonadotropic status during the food restriction and synchronously exhibited puberty onset few days after the resumption of *ad libitum* feeding [6, 43, 44]. The present study, therefore, examined the effects of chronic food restriction started at prepubertal period and the resumption of *ad libitum* feeding on pubertal sign, such as vaginal proestrus, *Kiss1, Tac3* and *Pdyn* expression in the ARC, LH secretion, uterine and ovarian weight and follicular development in the chronic growth-retarded rat model. AVPV *Kiss1* expression was also determined, because previous studies demonstrated that the growth-retarded rat model showed ovulation a few days after the resumption of *ad libitum* feeding [6, 43].

Materials and Methods

Animals

Wistar-Imamichi strain rats were purchased from the Institute for Animal Reproduction (Kasumigaura, Japan). The rats were kept in a room with a temperature of 23 ± 3ºC with a 14/10 h light/dark cycle (lights on at 0500 h) and, supplied with standard rodent chow (CE-2; CLEA Japan, Tokyo, Japan) and water *ad libitum* unless otherwise noted. Female rats (7–8 weeks of age) having at least two consecutive regular 4-day estrous cycles, which were determined by vaginal smears, were mated overnight with stud male rats (26–52 weeks of age) on the day of proestrus. Pregnant females, which were determined by the presence of vaginal plugs, were housed individually.

The day that a newborn litter was found at noon was designated postnatal day 0. The litter size was adjusted to eight on day 1 to minimize the growth variation within and between litters. The female pups were weaned on day 20 and housed individually. The body weight, food intake, vaginal opening and vaginal smear, to detect estrous cyclicity, were daily monitored in the morning. Animals were fed *ad libitum* until the day their body weights reached 75 g (the mean ± SD of age, 28.2 ± 1.4 days (range, 26–31 days); the mean ± SD of food intake at the last day of the *ad libitum* feeding, 12.8 ± 1.7 g (range, 8.8–15.0 g)), and then fed restricted quantities of chow, which was varied individually dependent on daily weight gain, once daily (0930 h) to keep their body weights at 70–80 g by day 49 (the initial food intake, 6.9 g; the mean ± SD daily food intake during food restriction, 6.1 ± 0.2 g (range, 4.8–6.9 g)). Then, half of the animals were subjected to *ad libitum* feeding thereafter (the mean ± SD daily food intake, 15.5 ± 1.5 g (range, 12.6–17.7 g)) and the other half remained food-restricted (the mean ± SD daily food intake, 5.7 ± 0.2 g (range, 5.2–6.0 g)). These *ad libitum*-fed animals and food-restricted controls were subjected to either brain, ovary and uterus sampling (n = 4 for each groups) or blood sampling (n = 4 for each groups) on days 50 or 51 (24 or 48 h after the resumption of *ad libitum* feeding, respectively), as previous studies showed the resumption of *ad libitum* feeding resulted in the first ovulation in the growth-retarded rat model at 2 or 3 days later [6, 43].

Care of the animals and all the experimental procedures used in these experiments were approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University, Japan.

Brain sampling

Brain samples were obtained from the animals subjected to *ad libitum* feeding for 24 or 48 h and the age-matched food-restricted controls on 50 and 51 days of age. The descending aorta was clamped and the upper body of rats were perfused with 0.05 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.05 M phosphate buffer between 1030 and 1330 h under deep anesthesia with an injection of sodium pentobarbital (40 mg/kg). The brains were immediately removed and post-fixed in the same fixative overnight at 4ºC. The brains were then immersed in 30% sucrose in 0.05 M phosphate buffer until the brain sank. Frozen frontal sections containing the AVPV and ARC (50-μm thickness) were made by a cryostat according to a rat brain atlas [45]. Every two AVPV section was used for *in situ* hybridization to visualize *Kiss1* expression as described previously [29]. Every fourth ARC section was used for *in situ* hybridization to visualize *Kiss1*, *Tac3* and *Pdyn* expression, respectively.

In situ hybridization for Kiss1, Tac3 and Pdyn

The sections were washed with PBS and treated with 1 μg/ml protease K for 15 min at 37ºC. The sections were incubated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min and then hybridized overnight at 60ºC with a digoxigenin (DIG)-labeled anti-sense cRNA probe for either *Kiss1*, *Tac3* or *Pdyn*. Each DIG-labeled anti-sense cRNA probe corresponded to nucleotides 33-349 of *Kiss1* (GenBank accession no AY196983), 180-304 of *Tac3* (GenBank accession no NM_019162) and 315-731 of *Pdyn* (GenBank accession no NM_019374), respectively. After hybridization, the sections were washed with 2 × SSC containing 50% formamide for 15 min at 60 ºC twice. The sections were then treated with 20 μg/ml RNase A for 30 min at 37ºC and then immersed sequentially in 2 × SSC, 0.5 × SSC and DIG- 1 buffer [100 mM Tris-HCl (pH7.5), 150 mM NaCl and 0.01% Tween 20] for 15 min, twice each. Following this, the sections were immersed in 1.5% blocking reagent (Boehringer Mannheim, Mannheim, Germany) in DIG-1 buffer for 1 h at 37ºC and incubated

with an alkaline phosphatase conjugated anti-DIG antibody (1:1,000; Roche Diagnostics, Indianapolis, IN, USA, RRID:AB_514497) for 2 h at 37ºC. Then, the sections were washed with DIG-1 buffer and DIG-3 buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 50 mM $MgCl_2$]. Following this, the sections were treated with a chromogen solution (337 µg/ml 4-nitro blue tetrazolium chloride and 175 µg/ml 5-bromo-4-chloro-3-indolyl-phosphate in DIG-3 buffer, Roche Diagnostics) for 1 h. The reaction was stopped by adding a reaction stop solution [10 mM Tris-HCl (pH 7.6) and 1 mM EDTA (pH 8.0)]. The sections were mounted on gelatin-coated glass slides. The visualized cells throughout the AVPV (from 0.12 mm anterior to 0.60 mm posterior to the bregma) and ARC (from 1.72 to 4.36 mm posterior to the bregma) were unilaterally counted under a light microscope (BX53, Olympus, Tokyo, Japan): two of the authors independently counted the visualized cells, which detected subjectively, in all the sections, and the averages were calculated in each individual. Specificity of antisense cRNA probes for *Kiss1*, *Tac3* and *Pdyn* were verified by control experiments using sense cRNA probes. No signals were found in the sections incubated with sense cRNA probes for *Kiss1*, *Tac3* or *Pdyn.*

Ovary and uterus sampling

Ovarian and uteri samples were obtained from the animals subjected to *ad libitum* feeding for 24 or 48 h and the age-matched food-restricted controls on 50 and 51 days of age. Ovaries and uteri were collected and weighed immediately after the perfusion of the upper body with the fixative solution. The ovaries were then fixed in 10% neutral buffered formalin for 24 h, dehydrated through increasing concentrations of ethanol, and were embedded with paraffin. Serial 8-µm sections of ovaries were made by using a microtome (RM2235, Leica, Wetzlar, Germany) and mounted on gelatin-coated glass slides. Ovarian sections on the slides were deparaffinized and stained with hematoxylin and eosin and then subjected to the quantitative analysis of follicular development.

Quantitative analysis of follicular development

The number of follicles were counted every fifth section under a light microscope (BX53) according to Yamada *et al*. [46]. To avoid doubly counting the same follicle, only follicles with nucleoli of oocytes were counted. The stages of follicles were classified according to Pedersen and Peters [47]. Briefly, the primordial follicle is defined as a small oocyte surrounded with epithelial cells; the primary follicle is defined as a growing oocyte surrounded with complete single layer of cuboidal epithelial cells; the secondary follicle is defined as a growing oocyte surrounded with multiple layers of granulosa cells without a visible cavity; the tertiary follicle is defined as a large oocyte surrounded with multiple layers of granulosa cells with one or more cavities containing the follicle fluid. The atretic follicles, defined by any one of the following features —loose granulosa layers, dead granulosa cells scattered into antrum, pyknotic granulosa cells, or degenerated oocyte— were not included.

The tertiary follicle was further classified in terms of the mean diameter that was calculated using the maximal diameter and the diameter at a right angle to it by using Image J software (https://imagej.nih.gov/ij/). According to Hirshfield and Midgrey [48], the tertiary follicle with a mean diameter greater than 390 µm was termed as Graafian.

Blood sampling

Blood samples were obtained from the animals subjected to *ad libitum* feeding for 24 or 48 h and the age-matched food-restricted controls on 50 and 51 days of age. Briefly, blood samples (50 µl) were collected from freely moving conscious rats for 3 h, at 6-min intervals, starting at 1300 h to detect either tonic or surge-mode of LH secretion, through a silicon cannula (0.5 mm inner diameter; 1.0 mm outer diameter; Shin-Etsu polymer, Tokyo, Japan) inserted into the right atrium through the jugular vein on the day before blood sampling under anesthesia with an injection of ketamine (26.7 mg/kg)-xylazine (5.3 mg/kg) mixture and isoflurane inhalation (1–3% in air). An equivalent volume of rat blood cells, taken from donor rats and diluted with heparinized saline, was replaced through the cannula after each blood collection to keep the hematocrit constant. Plasma were separated by centrifugation, collected and stored at –20ºC until assayed for LH.

Radioimmunoassay and LH pulse parameter analysis

Plasma LH concentrations were determined by a double-antibody radioimmunoassay (RIA) using a rat LH RIA kit provided by the National Hormone and Peptide Program (Torrance, CA, USA) and were expressed in terms of the NIDDK rat LH-RP-3. The detectable levels ranged from 3.9 to 2500 pg/tube for 25 µl plasma samples. Intra- and inter-assay coefficients of variation were 5.8% and 7.9% at the level of 1.8 ng/ml, respectively.

LH pulse parameters were determined by the PULSAR computer program [49]. Mean LH concentrations as well as the baseline level, frequency and amplitude of LH pulses were calculated for the 3-h sampling period in each individual and then groups.

Statistical analysis

Statistical differences in the number of *Kiss1*-, *Tac3*- or *Pdyn*-expressing cells, LH pulse parameters, body weights, ovarian weights, uterine weights as well as the number of primordial, primary, secondary and tertiary follicles between the *ad libitum*-fed rats and age-matched food-restricted controls were determined by Student's t-test using the SAS University Edition (https://www.sas.com/).

Results

Ad libitum feeding induced catch-up growth and puberty onset in the growth-retarded female rats

The growth-retarded female rats that received restricted food showed hypogonadotropic status: they showed vaginal opening (the mean ± SD of age, 30 ± 1 days) and then persistent vaginal diestrus during the experimental period (until 51 days of age). On the other hand, all animals subjected to *ad libitum* feeding for 48 h from 49 days of age showed the first vaginal proestrus on day 51. Body weights were significantly heavier in the *ad libitum*-fed rats on both 50 and 51 days of age (24 and 48 h after the resumption of *ad libitum* feeding) compared to the age-matched food-restricted controls ($P < 0.05$, Fig. 1). Ovarian and uterine weights were also significantly higher in the *ad libitum*-fed rats on both 50 and 51 days of age compared to the age-matched food-restricted controls

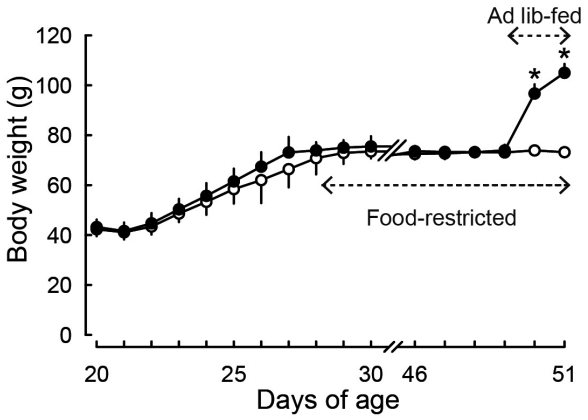

Fig. 1. *Ad libitum* feeding induced catch-up growth in growth-retarded female rats. The rats weaned on day 20 were fed *ad libitum* until the day when their body weights reached 75 g and then subjected to a restricted feeding to keep their body weights at 70–80 g by day 49. Then, half of the animals were subjected to *ad libitum* feeding thereafter and the other half were remained food-restricted. Values are expressed as the mean ± SD (n = 8 each). Asterisks indicate significant difference in body weights between the *ad libitum*-fed rats and the age-matched food-restricted controls (* P < 0.05, Student's t-test).

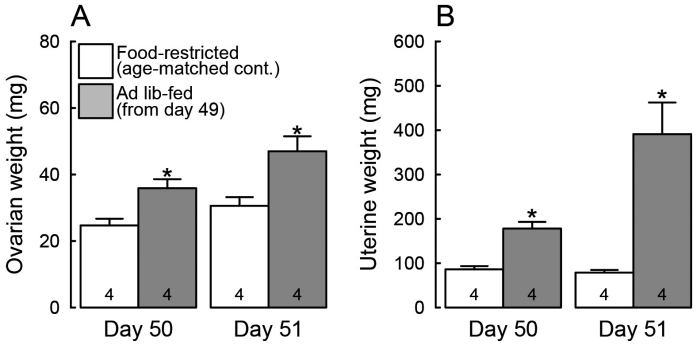

Fig. 2. *Ad libitum* feeding increased ovarian and uterine weights in the growth-retarded female rats. Ovarian (A) and uterine (B) weights of animals subjected to *ad libitum* feeding from 49 days of age and the age-matched food-restricted controls on 50 and 51 days of age. Values are expressed as the mean ± SEM. Numbers in each column indicate the numbers of animals used. Asterisks indicate significant difference in ovarian or uterine weight between the *ad libitum*-fed rats and the age-matched food-restricted controls (* P < 0.05, Student's t-test).

(P < 0.05, Fig. 2A, B).

Ad libitum feeding induced LH secretion in the growth-retarded female rats

Figure 3A shows representative plasma LH profiles of *ad libitum*-fed rats and the age-matched food-restricted controls on both 50 and 51 days of age. The female rats subjected to *ad libitum* feeding for 24 h showed an increase in tonic LH secretion on day 50. 48-h *ad libitum* feeding induced a surge-like increase in plasma LH concentrations in all females on day 51. On the other hand, LH secretion was strongly suppressed in age-matched food-restricted controls. Specifically, mean LH concentrations and the baseline levels of LH pulses were significantly higher in *ad libitum*-fed rats than growth-retarded controls on each day (P < 0.05, Fig. 3B). Frequency and amplitude of LH pulses tended to be higher in *ad libitum*-fed rats than growth-retarded controls in particular on day 50, but no significant difference was detected in these pulse parameters.

Ad libitum feeding increased the number of Kiss1- and Pdyn-expressing cells in the ARC of the growth- retarded female rats

Figure 4A shows *Kiss1*-expressing cells in the ARC of representative female rats subjected to *ad libitum* feeding and the age-matched food-restricted controls. In the *ad libitum*-fed rats, a large number of *Kiss1*-expressing cells were found throughout the ARC on day 50 (24 h after the resumption of *ad libitum* feeding) and a moderate number of *Kiss1*-expressing cells were found scattered in the ARC on day 51 (48 h after the resumption). On the other hand, a small number of *Kiss1*-expressing cells were found in the ARC of age-matched food-restricted controls on days 50 and 51. The number of ARC *Kiss1*-expressing cells was significantly higher in *ad libitum*-fed rats on day 50 compared to the age-matched food-restricted controls (P < 0.05, Fig. 4B). On the other hand, a number of *Tac3*-expressing cells were found in the ARC of both *ad libitum*-fed rats and age-matched growth-retarded controls on both 50 and 51 days of age (Fig. 4C). No significant difference in the number of ARC *Tac3*-expressing cells between groups (Fig. 4D). Figure 4E shows *Pdyn*-expressing cells in the ARC of female rats subjected to *ad libitum* feeding and the age-matched food-restricted controls. In the *ad libitum*-fed rats, a large number of *Pdyn*-expressing cells were found throughout the ARC on both 50 and 51 days of age (24 and 48 h after the resumption of *ad libitum* feeding, respectively). On the other hand, a small number of *Pdyn*-expressing cells were found in the ARC of age-matched food-restricted controls on both 50 and 51 days of age. The number of ARC *Pdyn*-expressing cells were significantly higher in *ad libitum*-fed rats on both 50 and 51 days of age (24 and 48 h after the resumption of *ad libitum* feeding) compared to the age-matched food-restricted controls (P < 0.05, Fig. 4F).

Ad libitum feeding increased the number of Kiss1-expressing cells in the AVPV of the growth-retarded female rats

Figure 5A shows *Kiss1*-expressing cells in the AVPV of representative female rats subjected to *ad libitum* feeding and the age-matched food-restricted controls. In the *ad libitum*-fed rats, a large number of *Kiss1*-expressing cells were found throughout the AVPV on both 50 and 51 days of age (24 and 48 h after the resumption of *ad libitum* feeding, respectively). On the other hand, a small number of *Kiss1*-expressing cells were found in the AVPV of age-matched food-restricted controls on both 50 and 51 days of age. The number of AVPV *Kiss1*-expressing cells was significantly higher in *ad libitum*-fed rats on both 50 and 51 days of age compared to the age-matched food-restricted controls (P < 0.05; Fig. 5B).

Ad libitum feeding increased the number of Graafian and non-Graafian tertiary follicles in the growth- retarded female rats

Figure 6A shows Graafian and non-Graafian tertiary follicles in the

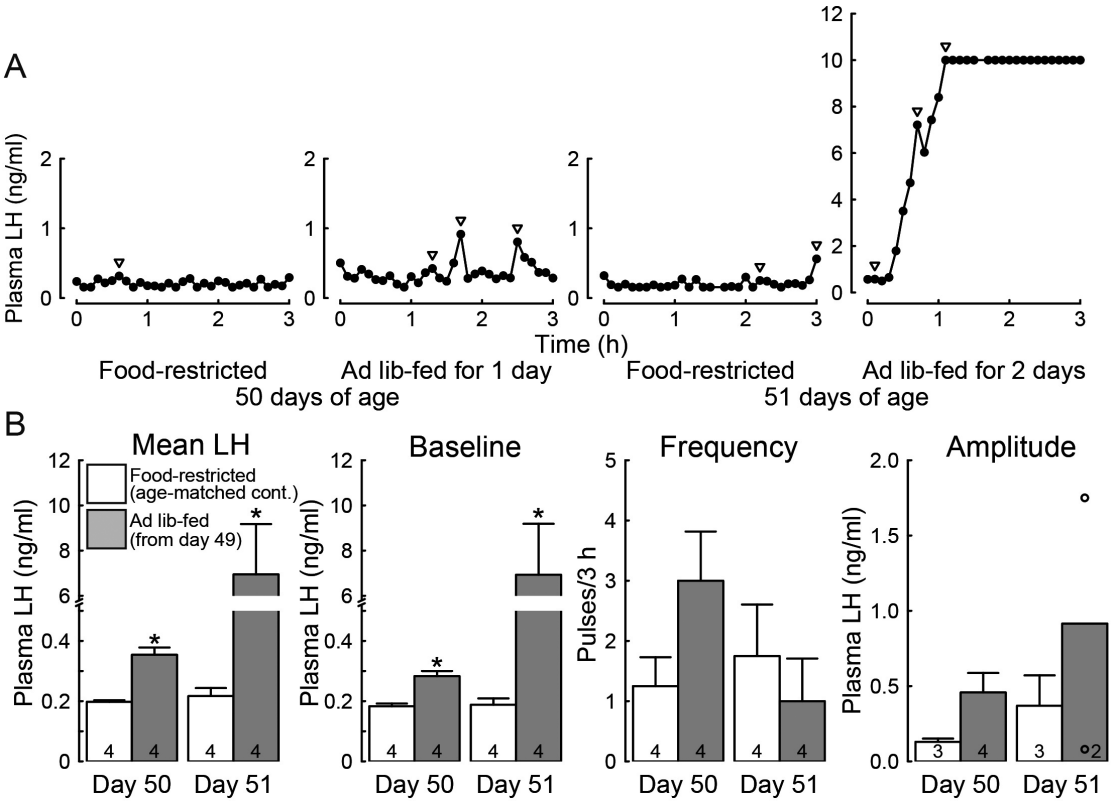

Fig. 3. *Ad libitum* feeding induced tonic and surge-like increase in luteinizing hormone (LH) secretion in the growth-retarded female rats. (A) Representative plasma LH profiles in the *ad libitum*-fed rats on 50 and 51 days of age (24 and 48 h after the resumption of *ad libitum* feeding) and the age-matched food-restricted controls. Arrowheads indicate LH pulses identified with the PULSAR computer program. Blood sampling was started from 1300 h. Note that the *ad libitum*-fed animals on 51 days of age showed a surge-like increase in plasma LH concentration and the concentrations were often higher than the upper detectable level. (B) Mean LH concentrations, baseline, frequency and amplitude of LH pulses in the *ad libitum*-fed rats on 50 and 51 days of age (24 and 48 h after the resumption of *ad libitum* feeding) and age-matched food-restricted controls. Values are expressed as the mean ± SEM. Numbers in each column in the graphs for mean LH concentration, and baseline and frequency of LH pulses, indicate the numbers of animals used. Numbers in each column in the graph for the amplitude of LH pulse indicate the numbers of animals showing detectable LH pulses during the 3-h sampling period. Note that LH pulses were detected in two out of four *ad libitum*-fed animals that showed a surge-like LH increase. Open circles indicate the mean values of pulse amplitude in those individuals. Asterisks indicate significant difference in mean LH concentration or baseline of LH pulses between the *ad libitum*-fed rats and the age-matched food-restricted controls (* $P < 0.05$, Student's t-test).

ovary of representative female rats subjected *to ad libitum* feeding and the age-matched food-restricted controls. The *ad libitum*-fed rats showed Graafian follicles with a diameter greater than 390 µm on both 50 and 51 days of age (24 and 48 h after the resumption of *ad libitum* feeding), while the age-matched food-restricted controls showed only non-Graafian tertiary follicles with a diameter smaller than 390 µm (Fig. 6B). The number of Graafian and non-Graafian tertiary follicles was significantly higher in *ad libitum*-fed rats on both 50 and 51 days of age compared to the age-matched food-restricted controls ($P < 0.05$; Fig. 6C and 6D). The number of primordial, primary and secondary follicles were not significantly different between *ad libitum*-fed rats and the age-matched food-restricted controls (Fig. 6E, 6F and 6G).

Discussion

The present study demonstrated that positive energy balance triggered completion of gene expression in ARC KNDy neurons, which may in turn result in puberty onset, because our chronic food-restricted female rats showed hypogonadotropic status along with suppression of ARC *Kiss1* and *Pdyn* expression. The resumption of *ad libitum* feeding induced puberty onset along with increases in ARC *Kiss1* and *Pdyn* expression in the chronic growth-retarded female rat model within 24 h. To our knowledge, this is the first report to show the energetic down- and up-regulations of ARC *Kiss1* and *Pdyn* expression associated with pubertal transition in the chronic growth-retarded female rats. On the other hand, it is unlikely that ARC *Tac3* expression is affected by chronic undernutrition —albeit ARC *Tac3* expression was reportedly suppressed by short-time fasting in the peripubertal period [39]—, because the number of *Tac3*-expressing cells were comparable between the *ad libitum*-fed rats and food-restricted controls. Interestingly, ARC *Kiss1*, *Tac3* and *Pdyn* gene expression were also separately controlled during the lactational anestrus: our recent study [50] showed that *Kiss1* expression was solely suppressed in ARC of lactating mother rats. The present results suggest that the chronic negative energy balance impairs

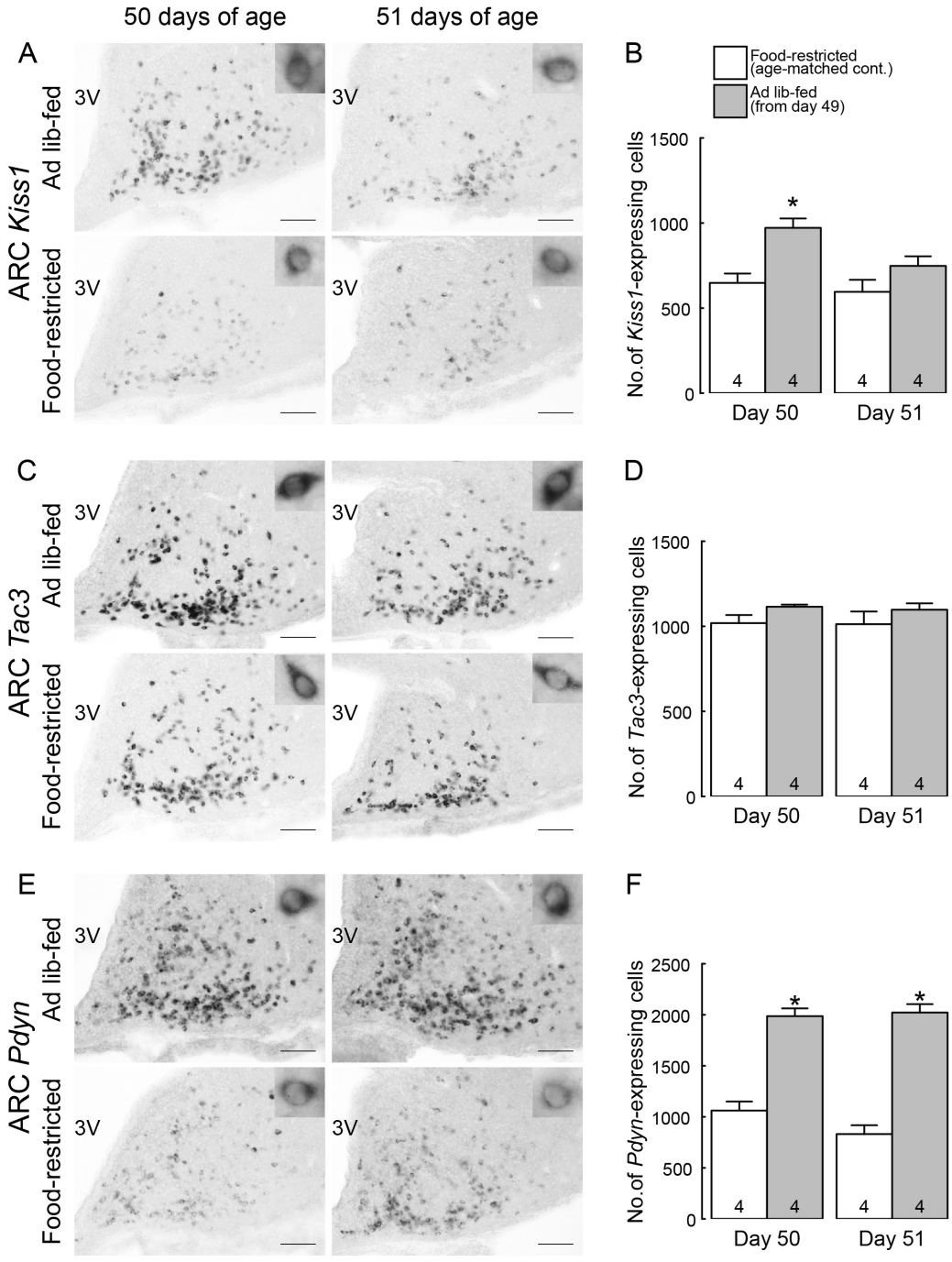

Fig. 4. *Ad libitum* feeding increased the number of *Kiss1*- and *Pdyn*-expressing cells in the arcuate nucleus (ARC) of the growth-retarded female rats. (A) *Kiss1*-expressing cells in the ARC of representative female rats subjected to *ad libitum* feeding on 50 and 51 days of age (24 and 48 h after the resumption of *ad libitum* feeding) and age-matched food-restricted controls. (B) The number of *Kiss1*-expressing cells throughout the ARC of *ad libitum*-fed rats on 50 and 51 days of age and age-matched food-restricted controls. (C) *Tac3*-expressing cells in the ARC of representative female rats subjected to *ad libitum* feeding on 50 and 51 days of age and age-matched food-restricted controls. (D) The number of *Tac3*-expressing cells throughout the ARC of *ad libitum*-fed rats on 50 and 51 days of age and age-matched food-restricted controls. (E) *Pdyn*-expressing cells in the ARC of representative female rats subjected to *ad libitum* feeding on 50 and 51 days of age and age-matched food-restricted controls. (F) The number of *Pdyn*-expressing cells throughout the ARC of *ad libitum*-fed rats on 50 and 51 days of age and age-matched food-restricted controls. *Insets* show representative cells showing *Kiss1* (A), *Tac3* (C) or *Pdyn* (E) expression at higher magnification. Scale bars, 100 μm; 3V, third ventricle. Values are expressed as the mean ± SEM. Numbers in each column indicate the numbers of animals used. Asterisks indicate significant difference in the number of ARC *Kiss1*- or *Pdyn*-expressing cells between the *ad libitum*-fed rats and the age-matched food-restricted controls (* $P < 0.05$, Student's t-test).

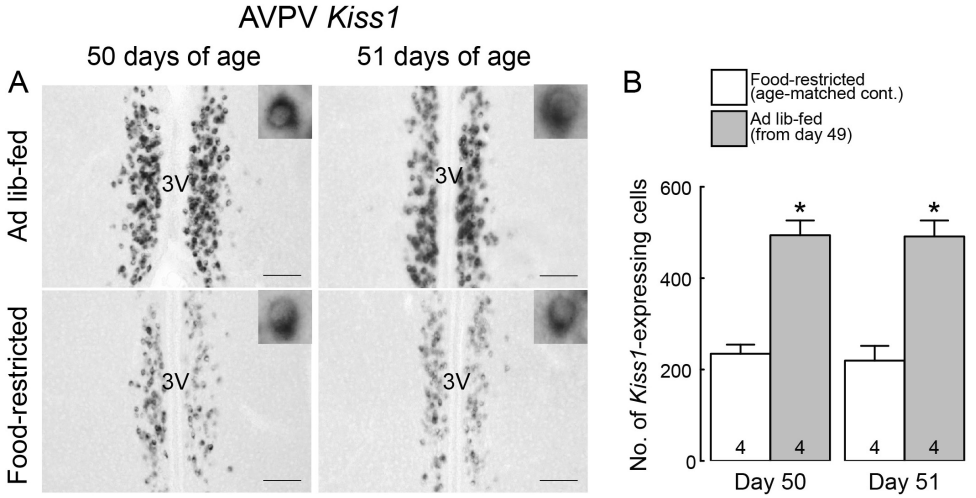

Fig. 5. *Ad libitum* feeding increased the number of *Kiss1*-expressing cells in the anteroventral periventricular nucleus (AVPV) of the growth-retarded female rats. (A) *Kiss1*-expressing cells in the AVPV of representative female rats subjected to *ad libitum* feeding on 50 and 51 days of age (24 and 48 h after the resumption of *ad libitum* feeding) and age-matched food-restricted controls. *Insets* show *Kiss1*-expressing cells at higher magnification. Scale bars, 100 μm; 3V, third ventricle. (B) The number of *Kiss1*-expressing cells throughout the AVPV of *ad libitum*-fed rats on 50 and 51 days of age and age-matched food-restricted controls. Values are expressed as the mean ± SEM. Numbers in each column indicate the numbers of animals used. Asterisks indicate significant difference in the number of AVPV *Kiss1*-expressing cells between the *ad libitum*-fed rats and the age-matched food-restricted controls (* $P < 0.05$, Student's t-test).

KNDy neurons, namely the GnRH pulse generator, via suppression of *Kiss1* and *Pdyn* expression, and the positive energetic cues trigger the completion of the functional GnRH pulse generator via increases in ARC *Kiss1* and *Pdyn* expression. Indeed, the chronic food-restricted rats showed suppression of LH secretion, whereas *ad libitum*-fed rats showed an increase in tonic LH secretion. Importantly, ARC *Pdyn* expression and LH secretion was suppressed by chronic food restriction and increased by the resumption of *ad libitum* feeding, despite of that dynorphin has been consider to play an inhibitory role in tonic LH secretion via inhibition of the synchronized activity of KNDy neuronal cluster [23, 24]. Taken together, the present results suggest that the negative energy balance inhibits pubertal onset of gonadotropin secretion by the suppression of ARC *Kiss1* and *Pdyn* expression, and the positive energetic cues induce pubertal onset of gonadotropin secretion by the completion of gene expression in ARC KNDy neurons in female rats.

Furthermore, it was demonstrated that chronic food restriction inhibited follicular development especially from the secondary to the tertiary stages and the resumption of *ad libitum* feeding induced follicular development with an increase in the number of Graafian and non-Graafian tertiary follicles within 24 h after feeding. Previously, Lintern-Moore *et al*. [51, 52] showed recovery of follicular development by the resumption of *ad libitum* feeding in the chronic growth-retarded rats, at 24 days later. The current study further exhibited that acute follicular development from the secondary follicles to the Graafian follicles occurs within 24 h by the resumption of *ad libitum* feeding in the chronic growth-retarded rats. Moreover, it showed that the numbers of primordial, primary, and secondary follicles were comparable between *ad libitum*-fed rats and food-restricted controls. These results are consistent with our understanding of the gonadotropin-dependency for the follicular development in mammals: gonadotropins are indispensable for the promotion of follicular development from the secondary to the tertiary stages, whereas they are dispensable for the promotion of follicular development from the primordial to the secondary stages [53–55]. Taken together with the present results of an increase in tonic LH secretion induced by the resumption of *ad libitum* feeding, an excess amount of energy is required to increase tonic gonadotropin secretion and hence promotes the further follicular development in the ovary.

The present study also indicated an increase in *Kiss1* expression in the AVPV of growth-retarded rat model 24 and 48 h after the resumption of *ad libitum* feeding. The increase in AVPV *Kiss1* expression is likely caused by an increase in circulating estrogen that is synthesized by tertiary follicles stimulated by tonic gonadotropin secretion. Indeed, the animals subjected to *ad libitum* feeding for 24 and 48 h showed an increase in uterine weights and the animals subjected to 48-h *ad libitum* feeding showed vaginal proestrus, both of which are reliable biomarkers to show an increase in circulating levels of estrogen [56, 57]. It is well known that AVPV *Kiss1* expression is positively controlled by estrogen in rodents [28–30] and thus kisspeptin neurons are considered to be responsible for GnRH/LH surge generation. Indeed, the current 48-h *ad libitum* feeding caused a surge-like increase in LH secretion. This result is well consistent with previous studies showing the resumption of *ad libitum* feeding resulted in the first ovulation in growth-retarded rat model on 2 or 3 days later [6, 43]. It is likely that the circulating estrogen also exert a negative influence on ARC *Kiss1* expression 48 h after the resumption of *ad libitum* feeding. Our previous studies showed that both diestrous and proestrous levels of estrogen increase AVPV *Kiss1* expression [29], whereas only proestrous levels of estrogen suppressed ARC *Kiss1* expression in female rats [29, 58]. This could be a reason why the number of AVPV *Kiss1*-expressing cells were

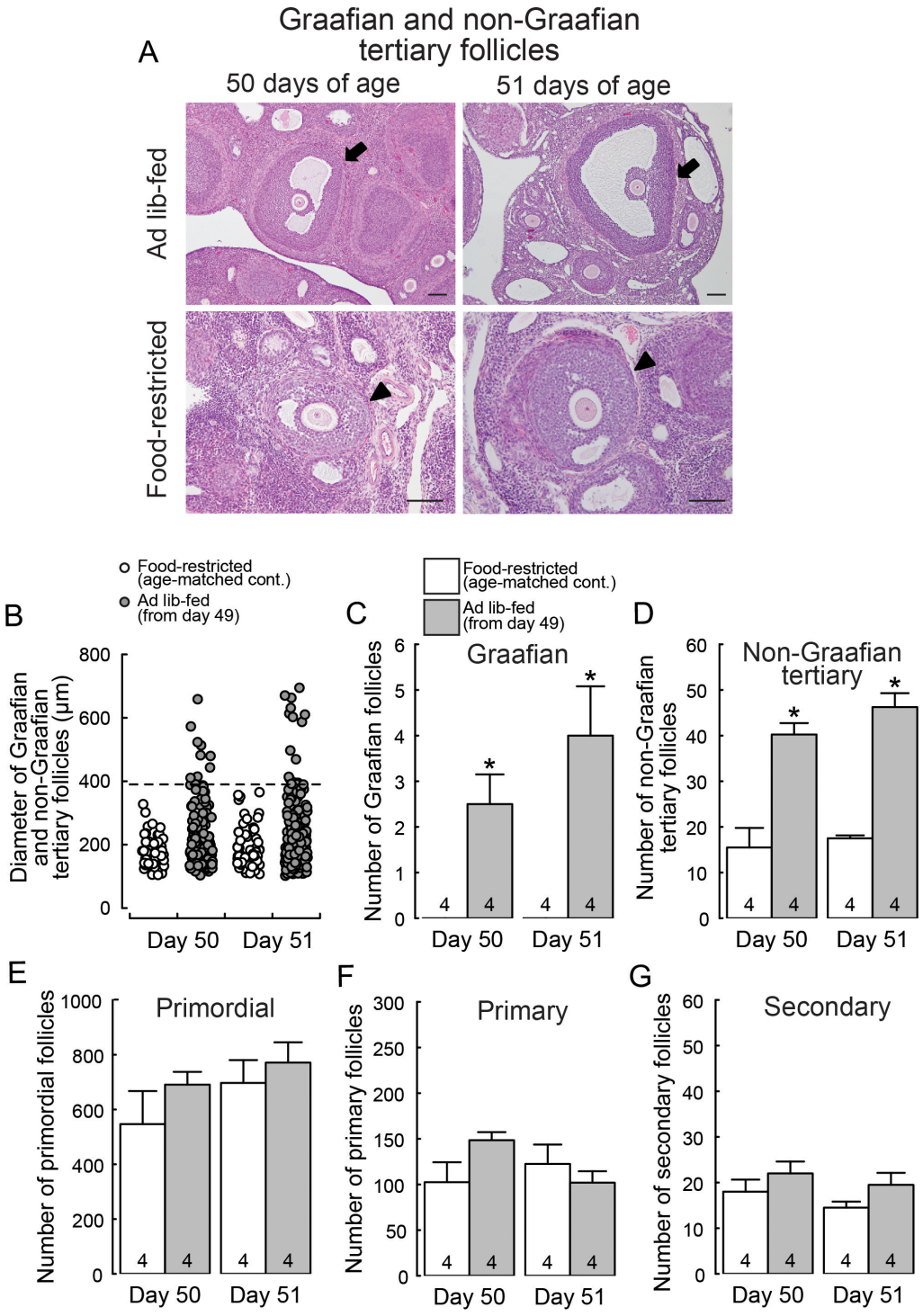

Fig. 6. *Ad libitum* feeding increased the number of Graafian and non-Graafian tertiary follicles in the growth-retarded female rats. (A) Representative photomicrographs of ovarian sections showing Graafian follicles with a diameter greater than 390 μm (arrows) in the *ad libitum*-fed rats on 50 and 51 days of age (24 and 48 h after the resumption of *ad libitum* feeding) and ovarian sections showing only non-Graafian tertiary follicles with a diameter smaller than 390 μm (arrowheads) in the age-matched food-restricted controls. Note that the magnification of the upper two images is different from that of the lower two images. Scale bar, 100 μm. (B) The mean diameter in each Graafian and non-Graafian tertiary follicle. The tertiary follicle with a diameter greater than 390 μm was termed as Graafian. The numbers of Graafian (C) and non-Graafian tertiary (D) follicles in the *ad libitum*-fed rats on 50 and 51 days of age and age-matched food-restricted controls. Values are expressed as the mean ± SEM. Numbers in each column in the graphs indicate the numbers of animals used. Asterisks indicate significant difference in the numbers of Graafian and non-Graafian tertiary follicles between the *ad libitum*-fed rats and the age-matched food-restricted controls (* $P < 0.05$, Student's t-test). The number of primordial (E), primary (F) and secondary (G) follicles in the *ad libitum*-fed rats on 50 and 51 days of age and the age-matched food-restricted controls.

comparable between the 24- and 48-h *ad libitum*-fed rats and why the number of ARC *Kiss1*-expressing cells tended to decrease in the 48-h *ad libitum*-fed rats.

Under a positive energy balance, energy storage in body fat has been considered to be a possible determinant for pubertal onset [59]. Body fat is the most labile component of body weight and therefore would reflect environmental changes in food supplies more rapidly than other tissues [59]. Thus, researchers paid much attention to leptin, the first hormone discovered from fat tissue [60]. Leptin is now considered one of the permissive factors that allow pubertal development to proceed [61]. On the other hand, many studies suggest that glucose, fatty acids, and ketone bodies are signals in the energetic control of LH secretion in rats [36, 62, 63]. Further studies are needed to clarify a key signal or a neural pathway, that relays the attainment of energy storage from the peripheral to KNDy neurons at the pubertal onset in mammals.

In conclusion, the present study suggests that energetic cues rather than chronological ages to define the puberty onset via affecting the ARC *Kiss1* and *Pdyn* expression: the negative energy balance causes pubertal failure probably because of the suppression of ARC *Kiss1* and *Pdyn* expression causing deficiency of gonadotropin secretion, while the positive energetic cues induce puberty onset by completion of functional GnRH pulse generator via increases in ARC *Kiss1* and *Pdyn* expression and hence an increase in tonic GnRH/gonadotropin secretion and follicular development, which in turn induces GnRH/LH surge leading to ovulation in female rats.

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