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

Attainment of reproductive capacity at puberty has been known to be initiated by an activation of the hypothalamic mechanism controlling tonic hypophysal 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 hyposcretion 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 hypophysal gonadotropin secretion [11–20]. Kisspeptin signaling in the hypothalamus is thought to be fundamental to initiate tonic hypophysal 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 hypothysyal 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
Animals

The mean ± SD of age, 28.2 ± 1.4 days (range, 26–31 days); the mean ± SD of age, 28.2 ± 1.4 days (range, 26–31 days) until the day their body weights reached 75 g. Animals, estrous cyclicity, were daily monitored in the morning. Animals pups were weaned on day 20 and housed individually. The body weight variation within and between litters. The female animals 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 ± 3°C 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 intake, 5.7 ± 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 4°C. 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 37°C. The sections were incubated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min and then hybridized overnight at 60°C with a digoxigenin (DIG)–labeled antisense 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 AF196983), 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°C twice. The sections were then treated with 20 μg/ml RNase A for 30 min at 37°C 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 37°C 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 37°C. Then, the sections were washed with DIG-1 buffer and DIG-3 buffer [100 mM Tris-Cl (pH 9.5), 100 mM NaCl and 50 mM MgCl₂]. 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-Cl (pH 7.6) and 1 mM EDTA (pH 8.0)]. The sections were mounted on gelatin-coated glass slides. The visualized cells throughout the A VPV (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.

Quantitative analysis of follicular development

The number of follicles were counted every fifth section under a light microscope (BX53, Olympus, Tokyo, Japan) with an alkaline phosphatase conjugated anti-DIG antibody (1:1,000; Roche Diagnostics, Indianapolis, IN, USA, RRID:AB_514497) for 2 h at 37°C. Then, the sections were washed with DIG-1 buffer and DIG-3 buffer [100 mM Tris-Cl (pH 9.5), 100 mM NaCl and 50 mM MgCl₂]. 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-Cl (pH 7.6) and 1 mM EDTA (pH 8.0)]. The sections were mounted on gelatin-coated glass slides. The visualized cells throughout the A VPV (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.

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 −20°C 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 NIH-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.
A significant difference was detected in these pulse parameters. Rats than growth-retarded controls in particular on day 50, but no

fed

ad libitum

and amplitude of LH pulses tended to be higher in growth-retarded controls on each day (P < 0.05, Fig. 3B). Frequency

of LH pulses were significantly higher in

secretion was strongly suppressed in age-matched food-restricted concentrations in all females on day 51. On the other hand, LH

feeding induced a surge-like increase in plasma LH

ad libitum

and 51 days of age. The female rats subjected to

feeding induced LH secretion in the growth-

et al.

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.

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).

(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 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 (Fig. 4C). No significant difference in the number of ARC Pdyn-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) compared to the age-matched food-restricted controls (P < 0.05, Fig. 4F).

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).

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 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).
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).
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 Graafian and non-Graafian tertiary follicles within 24 h after the resumption of ad libitum feeding in the chronic growth-retarded rat model, at 24 days later. The current feeding further exhibited that acute follicular development from the primordial to the secondary 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 pubertal 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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