Effect of diet-induced obesity on kisspeptin-neurokinin B-dynorphin A neurons in the arcuate nucleus and luteinizing hormone secretion in sex hormone-primed male and female rats

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Metabolic stress resulting from either lack or excess of nutrients often causes infertility in both sexes. Kisspeptin-neurokinin B-dynorphin A (KNDy) neurons in the arcuate nucleus (ARC) has been suggested to be a key players in reproduction via direct stimulation of the pulsatile gonadotropin-releasing hormone (GnRH) and subsequent gonadotropin release in mammalian species. In this study, we investigated the effect of high-fat diet (HFD) on hypothalamic KNDy gene expression to examine the pathogenic mechanism underlying obesity-induced infertility in male and female rats. Male and female rats at 7 weeks of age were fed with either a standard or HFD for 4 months. In the male rats, the HFD caused a significant suppression of ARC Kiss1 and Pdyn gene expressions, but did not affect the plasma luteinizing hormone (LH) levels and sizes of the morphology of the testis and epididymis. In the female rats, 56% of the HFD-fed female rats exhibited irregular estrous cycles, whereas the remaining rats showed regular cycles. Two of the 10 rats that showed HFD-induced irregular estrous cycles showed profound suppression of LH pulse frequency and the number of ARC Kiss1-expressing cells, whereas the other females showed normal LH pulses and ARC Kiss1 expression. Our finding shows that suppression of ARC Kiss1 expression might be the initial pathological change of hypogonadotropic hypogonadism in HFD-fed male rats, while the obese-related infertility in the female rats may be mainly induced by KNDy-independent pathways. Taken together, ARC kisspeptin neurons in male rats may be susceptible to HFD-induced obesity compared with those in female rats.

1. Introduction

Metabolic stress resulting from the lack or excess of nutrients is a predominant factor causing infertility in mammals. Availability of metabolic fuels is considered detected by central and peripheral energy sensors to control the gonadal activity in mammals [1,2]. Indeed, severe malnutrition such as dietary restrictions and eating disorders showed menstrual disturbance [3]. On the other hand, conversely, over-nutrition, including obesity and metabolic disorders, also caused sub-fertility such as oligomenorrhea [4], anovulation [5], lower quality of embryos [6], and decreased implantation rate [7]. Male subfertility has been associated with overweight and obesity in American [8], Danish [9], Norwegian [10] and Chinese [11] epidemiological studies. [5]. In rodents, high-fat diet (HFD)-induced obesity showed deteriorated gonadal functions in both sexes and decreased litter size [12,13]. Thus, the detrimental effects of nutritional excess on fertility are a common phenomenon regardless of sex or genetic factors, and HFD-fed rodents are considered to provide an appropriate model for analyzing the pathogenesis of infertility in obese men and women.

The hypothalamo-pituitary-gonadal (HPG) axis has been well established to play a key role in controlling gonadal functions such as follicular development, ovulation, spermatogenesis, and steroidogenesis in mammals. The gonadotropin-releasing hormone (GnRH) stimulates the production and release of gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), and the release of GnRH is fine-tuned by the negative feedback action of gonadal steroids. Studies on the relationship of obesity and fertility in women are largely related to polycystic ovary syndrome (PCOS). Most women with PCOS show hyperandrogenism, hypergonadism and increased plasma LH levels [5,14,15]. On the other hand, Conversely, obese women without hyperandrogenism are considered to provide an appropriate model for analyzing the pathogenesis of infertility in obese men and women.
show a decrease in gonadotropin and estrogen levels [16,17], which suggests that obesity may attenuate the hypothalamic function in women. Regarding men, decreased plasma testosterone and gonadotropin levels were associated with severe obesity [18,19]. Male obesity-related hypothalamic hypogonadism was reversed by weight loss, resulting in the recovery of androgen levels [20]. Furthermore, LH secretory responses to GnRH administration in obese hypogonadotropic men were spared at a similar level to that in healthy control men [21], which suggests that the hypothalamic reproductive centers but not the pituitary in men would be affected by severe obesity.

Accumulating evidence suggests that hypothalamic kisspeptin neurons are a key regulator of reproduction via direct stimulation of GnRH and subsequent gonadotropin release in mammalian species such as nonhuman primates, ruminants, and rodents [13]. A population of kisspeptin neurons in the arcuate nucleus (ARC) responsible for pulsatile GnRH/gonadotropin release [27,28] are considered to be direct targets for the negative feedback actions of sex steroid hormones in both males and females [16,17]. ARC kisspeptin neurons coexpress two other neuropeptides, neurokinin B and dynorphin A; therefore, they are referred to as KNDy (kisspeptin, neurokinin B, and dynorphin A) neurons [18]. KNDy gene expressions reflect dynamic changes in gonadotropin secretion under both acute and chronic stress conditions such as restraint, insulin-induced hypoglycemia, or lipopolysaccharide stress and developmental exposure to estrogenic chemicals [19–22]. These findings suggest that KNDy neurons function as hub neurons to integrate information of various environmental stress factors and mediate negative regulation of gonadotropin secretion and then gonadal functions in both sexes of mammals. Anderson and coworkers firstly showed that HFD from 3 to 24 weeks of age reduced the ARC Kiss1 gene expression level in female mice [23]. The result suggests that diet-induced obesity (DIO) can lead to suppression of KNDy gene expression in ARC, resulting in dysfunction of GnRH/LH pulse generation and hypogonadotropic subfertility in mammals. Some reports, however, showed controversial results that HFD had no effects on ARC kisspeptin immunoreactivity in intact male and female rats [24,25]. Thus, the mechanism mediating obesity-induced hypogonadotropism is still remains controversial. Elucidating the role of KNDy neurons would be of benefit to understand the central mechanism involved in obesity-induced subfertility of men and women.

The present study aimed to investigate the involvement of ARC KNDy neurons in obesity-induced hypogonadism followed by subfertility in male and female rats. We examined an animal model of HFD-KNDy neurons in obesity-induced hypogonadism followed by subfertility in both sexes of mammals. Anderson and coworkers firstly showed that HFD from 3 to 24 weeks of age reduced the ARC Kiss1 gene expression level in female mice [23]. The result suggests that diet-induced obesity (DIO) can lead to suppression of KNDy gene expression in ARC, resulting in dysfunction of GnRH/LH pulse generation and hypogonadotropic subfertility in mammals. Some reports, however, showed controversial results that HFD had no effects on ARC kisspeptin immunoreactivity in intact male and female rats [24,25]. Thus, the mechanism mediating obesity-induced hypogonadotropism is still remains controversial. Elucidating the role of KNDy neurons would be of benefit to understand the central mechanism involved in obesity-induced subfertility of men and women.

2. Materials and Methods

2.1. Animals and induction of obesity

Wistar-Imamichi strain rats (7-weeks old) were purchased from the Institute for Animal Reproduction (Kasumigaura, Japan). Rats were housed in facilities at Nippon Medical School in a controlled environment (14 h: 10 h light-dark cycle, lights on at 06:00 h, at 22 °C ± 2 °C) with free access to food and water. Rats were randomly placed on a normal diet with 10% of calories from fat (catalog D12450 H; Research Diets, New Brunswick, NJ) or a HFD with 45% calories from fat (catalog D12451; Research Diets) for the duration of the study (Table 1). These diets have been formulated specifically for scientific research and have been used widely in research with rats and has been reported to induce heavier body weight at >4 weeks after starting feeding compared with the normal-fed counterparts [26,27]. The animals were fed the diets for 4 months (17–18 weeks) and expression, while some females regularly weighed. The reason feeding HFD for 17–18 weeks is that HFD-fed female rats showed irregular estrous cycles weighed regularly. when fed an HFD for this duration. Additionally, HFD rats exhibited regular estrous cycles until 12 weeks (data not shown). Surgical procedures for the animals were performed under isoflurane anesthesia. The animals were euthanized under deep anesthesia with sodium pentobarbital (100 mg/kg, intraperitoneally [i.p.]) and medetomidine hydrochloride (0.5 mg/kg, i.p.) to collect brain tissues. The Nippon Medical School’s committee on animal research approved all the procedures and housing conditions used in the study.

2.2. Effects of HFD on metabolic parameters, plasma testosterone and LH levels, and hypothalamic KNDy gene expression level in male rats

After 16 weeks of receiving the diets, all the male rats were bilaterally castrated (CAST) to remove the influences of endogenous gonadal steroids, and received a subcutaneous (s.c.) Silastic tubing (1.5-mm inner diameter, 3.0-mm outer diameter, and 10-mm length; Dow Corning) containing crystalline testosterone (Sigma, St. Louis, MO, USA) 1 week before the brain collection. We referred to a previous study to determine the length of the tube because the plasma testosterone concentrations in the animals implanted with the tube were sufficient for LH suppression in nutritional stress, which is a steroid-dependent change [28]. In this study, plasma testosterone and LH levels were measured in rats implanted with the testosterone tube to confirm the concentrations. The plasma testosterone concentrations were 0.05 ± 0.004, 0.52 ± 0.10, and 2.66 ± 0.61 ng/μL (mean ± SEM, n = 3) in the CAST, CAST + T, and intact male rats, respectively. The plasma LH concentrations were 1.18 ± 0.09, 0.92 ± 0.04, and 0.30 ± 0.15 ng/μL (n = 3) in the CAST, CAST + T, and intact male rats, respectively.

Blood samples (500 μL) were obtained under anesthesia immediately before castration to analyze plasma testosterone and estradiol concentrations in the male rats. A testes and epididymis were weighed, fixed in Bouin’s fluid, and their paraffin sections (5 μm) were histologically examined under a microscope after general staining with hematoxylin-eosin (HE). A week after castration, visceral white adipose tissue

### Table 1

| Diet constituents (from Research Diets, New Brunswick, NJ) | Control diet (D12450H) | High-fat diet (D12451) |
|----------------------------------------------------------|------------------------|------------------------|
| **Composition**                                           |                        |                        |
| Protein                                                  | 19.2 ± 0.9             | 19.2 ± 0.9             |
| Carbohydrate                                             | 67 ± 2.0               | 67 ± 2.0               |
| Fat                                                      | 67.3 ± 0.1             | 24 ± 4.5               |
| **Total**                                                | 100 ± 0.3              | 100 ± 0.3              |
| kcal/g                                                   | 3.85 ± 0.10            | 4.73 ± 0.10            |
| **Ingredients**                                          |                        |                        |
| Casein                                                   | 200 ± 8.0              | 200 ± 8.0              |
| L-Cysteine                                               | 3 ± 0.12               | 3 ± 0.12               |
| Corn starch                                              | 452.2 ± 18.08          | 72.3 ± 2.91            |
| Maltodextrin                                             | 75 ± 3.0               | 100 ± 4.00             |
| Sucrose                                                  | 172.8 ± 6.91           | 172.8 ± 6.91           |
| Cellulose                                                | 50 ± 0.0               | 50 ± 0.0               |
| Soybean Oil                                              | 25 ± 2.25              | 25 ± 2.25              |
| Lard                                                     | 20 ± 1.80              | 177.5 ± 15.98          |
2.3. Effects of HFD on metabolic parameters, estrous cycles, plasma estradiol levels, pulsatile LH secretions, and hypothalamic KNDy gene expressions in female rats

The vaginal smears in all the female rats were daily examined 14 weeks after the initial exposure to HFD or normal diet to determine estrous cyclicity. After analysis of the vaginal smears for 2 weeks, some female animals were bilaterally ovariectomized and received a sc Silastic tubing (1.5-mm inner diameter, 3.0-mm outer diameter, and 25-mm length; Dow Corning) containing estradiol-17β (E2) (Sigma) dissolved in sesame oil at 20 μg/mL. A previous report indicated that rats subjected to this E2 treatment had diestrous plasma E2 levels [29]. A week after ovariectomy, blood samples were collected for 3 h at 6-min intervals from freely-moving conscious rats from 1300 h to detect pulsatile LH release. Blood samples (100 μL) were taken from the right atrial cannula (0.5-mm inner diameter and 1.0-mm outer diameter, Shin-Etsu Polymer, Tokyo, Japan) that had been inserted through the jugular vein on a day before blood sampling. Each blood sample was replaced with an equivalent volume of washed red blood cells obtained from other Wistar-Imamichi strain rats to keep the hematocrit constant. Plasma samples were obtained by an immediate centrifugation and stored at −25 °C until assayed for LH. Brain tissues and additional blood samples (500 μL) for leptin and triglyceride assays were collected on the next day of the frequent blood sampling.

Other intact female animals were used to examine plasma estradiol levels and a uterine weight. Blood samples (500 μL) were obtained from the internal jugular veins under anesthesia immediately on the proestrous day for E2 assay and diestrous day for E2, leptin, and triglyceride assays. In addition, the visceral white adipose tissue, uterine, and ovary were weighed and brain tissues were collected in the diestrous rats. Plasma samples were obtained by an immediate centrifugation and stored at −25 °C until the assays.

2.4. In situ hybridization (ISH)

Animals were deeply anesthetized with sodium pentobarbital and perfused with 0.05 M phosphate-buffered (PB) saline followed by 4% PFA. Brains were immediately removed, postfixed in the same fixative overnight at 4 °C, and then immersed in 20% sucrose in 0.05 M PB at 4 °C. The serial coronal sections (50 μm in thickness) were obtained and used a cryostat for histological analysis. Every second section of the anterodorsal periventricular nucleus (AVPV from 0.6 mm anterior to 0.6 mm posterior to the bregma, containing the POA) for Kiss1 (only female) and Gnrh1 ISH and every fourth section through the ARC from 1.72 mm to 4.36 mm posterior to the bregma) for Kiss1, Tac3, and Pdyn ISH were obtained from each CAST + T male and OXV + E2 female rat according to the brain atlas [30]. In intact female rats, only ARC Kiss1 ISH were performed.

mRNA expression was detected by free-floating ISH using specific digoxigenin (DIG)-labeled probes, as described elsewhere [31]. DIG-labeled antisense RNA probes for rat Kiss1 (position 379–867, GenBank accession no. XM_008769443), Tac3 (position 180–483, GenBank accession no. NM_019162), Pdyn (position 315–731, GenBank accession no. NM_019374), and Gnrh1 (position 57–360, GenBank accession no. NM_012767.2) were synthesized by in vitro transcription from the rat hypothalamic cDNA using a DIG-labelling kit (Boehringer Mannheim GmbH, Mannheim, Germany). A sense RNA probe for Kiss1, Tac3, and Pdyn was used as a negative control. We previously confirmed that no positive signal for Gnrh1 mRNA was detected in the brain sections hybridized with the corresponding sense probe [32]. Briefly, the brain sections treated with 1 μg/mL protease K and 0.25% acetic anhydride in 0.1 M triethanolamine were hybridized with 1 μg/mL DIG-labeled probes. After hybridization, the sections were treated with 20 μg/mL RNase A (Roche Diagnostics, Indianapolis, IN, USA), immersed in 1.5% blocking reagent solution (PerkinElmer, Waltham, MA, USA), and then incubated with an alkaline phosphatase-conjugated anti-DIG antibody (1:1000, Roche Diagnostics, RRID:AB_514497). The sections were treated with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate solution (Roche Diagnostics) until a visible signal was detected. After processing, the sections were mounted and examined using an optical microscope (BX50, Olympus).

2.5. Assays

Plasma LH concentrations were determined using a double antibody radioimmunoassay (RIA) with a rat LH RIA kit provided by the National Hormone and Pituitary Program (Baltimore, MD, USA), and were expressed in terms of NIDDK-rLH-RP-3. The least detectable LH level was 0.078 ng/mL and 0.156 ng/mL for the 100- (male) and 50-L (female) μL plasma samples, respectively. The intra- and inter assay coefficients of variation were 7.4% and 12.6% at 0.63 ng/mL for 50-μL plasma, respectively.

Plasma estradiol levels were determined using a commercial kit (estradiol enzyme-linked immunosorbent assay [ELISA] kit; Cayman Chemical). Plasma (100 μL) and standard samples for ELISA were extracted twice with diethyl ether and dissolved in 100 μL of ELISA buffer. The extracted samples were assayed in duplicate according to kit instructions.

Plasma testosterone levels were determined using a commercial kit (testosterone ELISA kit; Cayman Chemical). Plasma (20 μL) and standard samples for ELISA were extracted twice with diethyl ether and dissolved in 200 μL of ELISA buffer. The extracted samples (50 μL) were assayed in duplicate according to the manufacturer’s instructions.

Plasma leptin concentrations were determined using a commercial kit (rat leptin ELISA kit; Yanaihara Institute, Japan) in accordance with the manufacturer’s instructions.

Plasma triglyceride concentrations were determined using a commercial kit (triglyceride E test- Wako; Wako, Japan) in accordance with the manufacturer’s instructions.

2.6. Data and statistical analyses

The statistical differences in body weight (BW) changes between the groups were determined using two-way analysis of variance (ANOVA; with diet and time as main effects) followed by the Bonferroni test. To quantify the number of Kiss1-expressing cells, every fourth section through the ARC was counted, and one value/animal was used for the following statistical analysis. To quantify the number of Gnrh1-expressing cells, every second section in the POA region was counted and one value/animal was used for the following statistical analysis. LH pulses were identified using the PULSAR computer program [33]. Statistical differences in percent BW change; adipose tissue, testicular, epididymis, ovarian, and uterine weights; and plasma leptin, triglyceride, testosterone, estradiol, and LH levels; percentage of proestrus, estrus, and diestrus during the estrous cycle; estrous length; each LH pulse parameter; and the cell numbers expressing Kiss1, Tac3, Pdyn, and Gnrh1 mRNA between the groups were determined using the Student t test. Statistical differences in the rate of animals showing irregular estrous cycles were determined using the Pearson chi-square test.

3. Results

3.1. Development of obesity and related metabolic disorders in HFD groups

In the male rats, the BW of the HFD groups was significantly (P < 0.05, two-way ANOVA) higher than that of the normal-diet control.
group at 4 weeks and thereafter (Fig. 1A, left). After 17 weeks of the respective diets (the BW of the HFD-fed rats reached 119% of that of the control rats), we analyzed the weight of the adipose tissue and the plasma leptin and triglyceride levels. The weight of adipose tissue of the HFD-fed males was significantly greater than that of the control males (Table 2). In addition, the weight of the adipose tissue divided by the BW also showed a significant difference between the two groups (Table 2). The plasma leptin concentrations in the HFD-fed male rats were significantly (P < 0.05, Student t test) higher than that in the controls (Table 2). No significant difference in plasma triglyceride levels was found between the groups (Table 2).

In the female rats, the BW of the HFD group was significantly (P < 0.05, two-way ANOVA) higher than that of the normal-diet control group at 2 weeks and thereafter (Fig. 1A, right). HFD for 17–18 weeks (from 7 to 24–25 weeks of age) caused a statistically significant (P < 0.05, Student t test) increase in the weight of the adipose tissue and plasma leptin levels compared with the control group (Table 2). We found a significant difference in the weight of adipose tissue divided by the BW between the two groups (Table 2). The plasma leptin and triglyceride levels in the HFD-fed female rats were significantly (P < 0.05, Student t test) higher than in the control female rats (Table 2).

3.2. Testicular and epididymal functions, plasma testosterone and LH levels, and ARC Kiss1/Tac3/Pdyn expression levels in HFD male rats

Testicular and epididymal weights were comparable between the control and HFD groups, whereas significant differences in testicular and epididymal weights divided by BW was found between the control and HFD-fed male rats (Fig. 2A). Fig. 2B shows representative photomicrographs of HE-stained testes and epididymis. No obvious changes in testis and epididymis morphology were observed between control and HFD-fed male rats: normal elongated spermatids were seen in the seminiferous tubules (Fig. 2B, upper panels) and epididymal tubules were filled with numerous spermatozoa (Fig. 2B, lower panels) in both groups. Consistent with the morphology, no significant difference in plasma testosterone, LH (Fig. 2C), and estradiol levels (control: 2.76 ± 0.50 pg/mL vs. HFD: 2.06 ± 0.20 pg/mL) was found between the two male groups.

The ARC Kiss1/Tac3/Pdyn expressions were identified in the CAST rats that received testosterone. Fig. 3A shows the Kiss1-, Tac3-, and Pdyn-expressing cells in the ARC of the representative CAST + T male rats in each group. The Kiss1, Tac3, and Pdyn expressions detected using ISH were weaker in the HFD-fed male rats than in the control rats (Fig. 3A). The numbers of ARC Kiss1- and Pdyn-expressing cells in the HFD-fed male rats were significantly lower than those in the normal-fed rats (Fig. 3B). No positive signals for Kiss1, Tac3 and Pdyn mRNA were detected in the brain sections hybridized with the corresponding sense probe (Fig. 3A, right).

3.3. Irregular estrous cyclicity and suppression of plasma estrogen level in the HFD-fed female rats

Fig. 4A shows the results of the vaginal smear patterns of the representative animals in each group. Of the normal-diet controls, 84.6% of the normal-fed females and 87.5% of the HFD-fed females showed estrous cyclicity. In contrast, no estrous cyclicity was observed in the HFD-fed female rats. The plasma estrogen levels were significantly lower in the HFD-fed female rats than in the control female rats (Table 2).

### Table 2

|                      | Normal-fed Males | HFD-fed Males | Normal-fed Females | HFD-fed Females |
|----------------------|------------------|---------------|--------------------|-----------------|
| n                    | 8                | 9             | 13                 | 19              |
| BW at the beginning (g) | 195.1 ± 6.2     | 185.9 ± 6.5  | 172.7 ± 1.0        | 172.1 ± 1.0     |
| BW after 16 weeks of receiving the diets (g) | 594.6 ± 17.0  | 696.0 ± 16.8* | 326.0 ± 5.9        | 397.0 ± 12.6*   |
| Changes in BW (%)    | 307.5 ± 15.4     | 379.5 ± 19.3* | 189.0 ± 4.0        | 235.8 ± 8.8*    |
| Adipose tissue (g)   | 26.93 ± 1.91     | 44.68 ± 1.69* | 16.63 ± 1.04       | 30.59 ± 2.18*   |
| Adipose tissue (g/100 g BW) | 4.6 ± 0.3   | 6.7 ± 0.2    | 5.1 ± 0.3          | 7.7 ± 0.4*      |
| Plasma leptin (ng/mL) | 3.12 ± 0.37      | 4.99 ± 0.29*  | 1.09 ± 0.10        | 1.73 ± 0.14*    |
| Plasma triglyceride (mg/dL) | 76.80 ± 5.61 | 79.77 ± 7.73 | 57.59 ± 12.09      | 97.07 ± 10.31*  |

Data are represented as mean ± SEM from normal-fed or HFD-fed rats.

* P < 0.05 compared with normal-fed control group (Student t test).
% showed at least two consecutive 4- or 5-day estrous cycles (11/13), and the other rats showed unclear proestrus or extended estrus (2/13). Regarding the HFD groups, 57.9% of the HFD rats showed an irregular estrous cyclicity ($P < .05$, Pearson chi-square test; Fig. 4B, left). The vaginal smear showed unclear proestrus (5/19), extended diestrus (5/19), or extended estrus (1/19). Eight of the 19 HFD-fed rats showed regular estrous cycles (Fig. 4B, left). The days that showed proestrus in the HFD group were significantly ($P < 0.05$, Student $t$ test) fewer than those in the control group, and the days that showed diestrus tended to increase in the HFD group than in the control group ($P = 0.053$; Fig. 4B, middle). Consequently, the estrus cycle in the HFD group was statistically ($P < 0.05$, Student $t$ test) longer than that in control groups (Fig. 4B, right).

The plasma estrogen levels of the HFD animals at diestrus were significantly lower ($P < 0.05$, Student $t$ test) than those of the control rats (Fig. 4C), resulting in significant decreases in the uterus weights.

Fig. 2. Effect of HFD on the weights and morphology of the genital organs and plasma testosterone and LH levels in male rats. (A) The testicular and epididymal weights in each group. Considering the body weight difference between groups, it also showed the testicular and epididymal weights divided by the body weight for each animal (right). (B) Photomicrographs of testicular and epididymis sections stained by hematoxylin and eosin in a representative animal from each group. Scale bars, 100 μm. (C) Plasma testosterone and LH levels in each group. Note that the testes, epididymis, and plasma samples were obtained under anesthesia just before castration in the intact male rats (after 16 weeks of receiving the diets). The bar charts portray the mean ± SEM with the individual data points overlaid. *$P < 0.05$ (vs. normal-diet controls, Student $t$ test). The numbers in each column indicate the number of animals used.
By contrast, the proestrous estrogen levels were comparable between the groups (control, 105.59 ± 29.72 pg/mL vs. HFD, 80.01 ± 8.99 pg/mL). The HFD-fed females had significantly heavier ovaries than the control females (Fig. 4D, left), whereas no significant difference in ovarian weight divided by BW was found between the control and HFD-fed female rats (control, 14.3 ± 0.5 mg/100 g of BW vs. HFD, 14.6 ± 0.5 mg/100 g of BW).

3.4. ARC Kiss1/Tac3/Pdyn expression and pulsatile LH secretion in the HFD female rats

To identify the ARC Kiss1/Tac3/Pdyn expressions and pulsatile LH secretion, female rats were ovariectomized and received E2 tube implantation. Fig. 5A shows Kiss1-, Tac3-, and Pdyn-expressing (upper) cells in the ARC of the representative animals in each group. Few Kiss1-, Tac3-, and Pdyn-expressing cells in the ARC were found in 2 of the 9 intact HFD rats, whereas the cells were abundantly found in all other HFD- and normal-fed control rats (Supplemental Fig. 3). Fig. 5C shows the profiles of LH release in the representative OVX + E2 rats in each group. Apparent LH pulses were found in almost all the HFD-fed and normal-fed controls (Fig. 5C). The frequency of the LH pulses, mean concentration of LH, and amplitude of the LH pulses in the HFD rats were comparable with those in the normal-fed control rats (Fig. 5D). Conversely, 2 of 10 HFD rats, whose ARC KNDy gene expression was suppressed, showed a profound inhibition of pulsatile LH release (Supplemental Fig. 1). The number of ARC Kiss1-expressing cells in female rats was positively correlated ($R = 0.82, P < 0.01$, Pearson correlation) with the frequency of the LH pulses.

3.5. POA Gnrh1 expression in the HFD-fed rats of both sexes

Fig. 6 shows the Gnrh1-expressing cells in the POA region of the representative CAST + T male and OVX + E2 female rats in each group. The Gnrh1-expressing cells were comparable in terms of the distribution pattern and cell number between the HFD-fed and control groups in both sexes (Fig. 6A). No significant difference was found in the number of Gnrh1-expressing cells in the POA region between the two groups regardless of sexes (Fig. 6B).
4. Discussion

The present study demonstrates that HFD-fed male rats showed suppression of ARC Kiss1 and Pdyn gene expressions, while the number of GnRH1-expressing cells were unaffected by HFD in testosterone-primed HFD models. The testes functions and plasma testosterone and LH levels were unaffected by HFD, which suggests that ARC Kiss1 suppression is an initial pathological change in infertility associated with male DIO. A specific hormone profile, hyperestrogenism, is found in obese males owing to aromatization in the fatty tissue and negatively regulates gonadotropin and testosterone levels by the estrogen-induced feedback actions to the hypothalamic reproductive center [34,35]. In the present study, the plasma E2 levels in male rats, however, were comparable between the groups, suggesting that the HFD-induced Kiss1 suppression is not mediated through the negative feedback pathway by estrogen derived from the fatty tissue. We also found that the HFD-fed female rats exhibited lower uterine weights and prolonged estrous cyclicities. These would be induced by the reduction in endogenous E2,
because plasma E2 levels were significantly decreased in the HFD rats at diestrus. Interestingly, HFD-fed females showed irregular estrous cycles, whereas 80% of HFD-fed rats showed apparent LH pulses and ARC KNDy gene expression. Previous gonadotropin analyses in C57BL/6 J mice revealed that DIO females showed prolonged estrous cycles but no changes in LH levels, consistent with our results [36]. These symptoms in the DIO rodents are similar to infertility associated with metabolic syndrome in obese women, which showed menstrual irregularity despite normal LH level [37]. Obesity-related hyperleptinemia was suggested to be involved in the inhibition of ovarian steroidogenesis via local leptin signaling in the ovary [38]. Therefore, the declined estradiol and increased leptin levels in the HFD-fed female rats in this study might have caused gonadotropin-independent reproductive disruption via a local ovarian pathway [38]. Cumulatively, our findings indicated that obese male rats showed suppression of ARC Kiss1 expression, which may lead to the suppression of reproductive functions in the future. Despite that, the irregular estrous cycles in the obese female rats were mainly owing to a hypothalamic Kiss1-independent pathway. The present results also suggest a sex-related difference in the response to ARC Kiss1 expression in DIO in rats.

Importantly, male obesity reduced the expression of ARC Kiss1 and Pdyn, which are co-expressed in ARC kisspeptin neurons in mouse, rat, cow, goat, and nonhuman primate [18]. The present results and a previous report that showed profound suppression of ARC KNDy gene expression...
expressions in genetically obese female rats [31] indicate a possibility of cell death or transcriptional repression of the ARC KNDy neurons. Some evidence shows that obesity causes hypothalamic injury; HFD induced the expression of a neuronal injury marker protein (chaperone Hsp72) in the ARC neurons of mice, and gliosis was found in the medial basal hypothalamus of obese rodents and humans [39]. Furthermore, a histological study using the terminal deoxynucleotidyl transferase dUTP nick end labeling assay and transmission electron microscopy revealed that apoptosis was significantly increased in the hypothalamic pro-opiomelanocortin neurons of obese rats [40]. Conversely, several clinical studies revealed that obesity-related hypogonadotropic hypogonadism was rescued by weight loss [11], implying that the HFD-induced suppression of KNDy gene expression in the present study might be mediated by the reversible transcriptional suppression of KNDy neurons but not by cell death.

To the best of our knowledge, this is the first study to show that ARC Kiss1 expressions in male rats are more vulnerable to metabolic stress induced by HFD than those in females. Sex-related differences in the effects of obesity have been reported in terms of weight gains, metabolic alterations, learning deficits, and hippocampal synaptic plasticity [41, 42]. Development of HFD-induced hypothalamic inflammation is associated with declined myocardial function in male mice but not in female mice [43], which is consistent with a less development of left ventricular hypertrophy in female mice compared with that in male mice in response to obesity [44]. Furthermore, microglial activation and peripheral macrophage infiltration were induced by obesity in the hypothalamus of males, whereas females were protected from the increase in inflammatory cytokines and neither exhibited microglia morphology changes nor monocyte-derived macrophage infiltration [36]. Although little is known about the relationship between KNDy neurons and chronic hypothalamic inflammation associated with obesity, several findings imply a link between KNDy neurons and autoimmune inflammation. For instance, ARC Kiss1 expression was suppressed by peripheral administration of lipopolysaccharide, a proinflammatory agent in male rats [19]. Considering these facts, hypothalamic kisspeptin neurons in males might be more susceptible to the inflammatory response induced by HFD than those in females. Besides, a sex-related difference in synaptic inputs to the KNDy neurons from hypothalamic nuclei, which are known to be involved in metabolic stress such as that in the caudal region of the ARC and lateral magnocellular part of the paraventricular nucleus [45]. Thus, the differences in neuronal input and/or obesity-related humoral signals to KNDy neurons between the sexes might be involved in the higher responsiveness of Kiss1 expression in males than in females.

In the present study, the HFD-induced obesity in the male rats caused the downregulation of ARC Kiss1 expression, but the testicular morphology and plasma LH/testosterone levels were still normal. The finding is consistent with that of a previous study that only 30 % reduction in ARC Kiss1 expression by inducible knockdown in male mice suppressed the LH pulse frequency, whereas the mean and baseline LH levels, steroidogenesis, and spermatogenesis were maintained at the same level as that in the controls [14]. Thus, the remaining Kiss1 neurons in the HFD-fed male rats in the present study might be sufficient to maintain testosterone levels and testicular functions, although the ARC Kiss1 expression in the HFD male rats were significantly decreased by 28 % compared with that in the control animals. This suggests that the hypothalamic compensatory mechanism contributes to the maintenance of reproductive function against HFD-induced stress. It should be noted that a different protocol of HFD feeding could also have adverse effects on testes steroidogenesis. Plasma testosterone levels as well as ARC Kiss1 expression in Wistar male rats were decreased by HFD feeding from pre-puberty to adult stage even when the same HFD (45 % of the total energy from lard fat, D12451 from Research Diets Inc) was fed for the same periods (4 months, from postnatal day 23–120) as in the current study [26]. Furthermore, HFD with a higher fat content (60 % of the total energy from lard fat, D12492 from Research Diets Inc) for 10 weeks reduced plasma testosterone levels in Wistar male rats [46]. Based on these results, HFD feeding from pre-puberty and an increase in fat contents seem to inhibit testes steroidogenesis. In this context, ARC Kiss1 suppression might be an initial pathological change in hypogonadotropic hypogonadism induced by obesity in male rats.

In this study, the ARC Kiss1 gene expression was comparable levels between almost all HFD-fed female and control rats. Consistent with the results of a recent study, HFD feeding (a HFD with lard comprising 50 %
of the total energy for 13 weeks) had no effect on kisspeptin immuno-reactivity in female Wistar rats [25,27]. The Kiss1 expression was also unaffected by HFD feeding (43 % of the total energy from lipids) for 14–16 weeks in female C57BL/6J mice, whereas the HFD feeding induced a marked decrease in hypothalamic Kiss1 expressions in female DBA/2J mice [23]. These results suggested that a difference in strains have a different effect on the susceptibility of the ARC Kiss1 gene to obesity in female rats.

Interestingly, the OVX + E2 rats were divided into two subgroups; most of the HFD-fed female rats (80 %) showed normal LH pulses and ARC Kiss1 expression, whereas the smaller subgroup (20 %) showed strictly attenuated LH pulse and Kiss1 expression. Although the mechanism underlying these results remains unclear, there are reports showing a similar bimodal pattern for HFD. In outbred Sprague–Dawley (SD) rats, two phenotypes are known to be exhibited: dietary obesity-susceptible rats and obesity-resistant rats [46]. Additionally, HFD reduced reproductive capacity in dietary obesity-susceptible SD rats [49]. In outbred Wistar female rats, 50 % rats showed DIO and the remainder were HFD resistant [50]. We could not identify the relationship between the rats used in this study and their mothers to clarify their genomic background because all rats were purchased from the same supplier two or three times. Whether the bimodal results in our study are owing to genetic and/or environmental causes remain unknown; therefore, further studies are required to clarify these points.

In summary, our findings show that HFD-fed male rats showed ARC Kiss1 suppression and normal levels of plasma LH and testosterone. The Kiss1 suppression might lead to male reproductive dysfunctions in the future. Despite that, irregular menstruation was mainly induced by KNDy-independent pathways during the incipient stage of obese infertility in female rats. Thus, hypothalamic KNDy neurons in male rats may be susceptible to HFD-induced obesity compared with those in females. The notion that central Kiss1 expression is associated with obesity-induced infertility in male rats is supported by a previous clinical study that indicated that kisspeptin administration to obese hypogonadotropic men could stimulate serum testosterone and LH release [10]. Furthermore, infertility in obesity may be caused by dysfunction of the gonads and hypothalamic kisspeptin neurons, and our study would be useful for developing treatment methods for reproductive disruption in obesity.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version, at doi:https://doi.org/10.1016/j.peptides.2021.170546.

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