Androgen Excess Increases Food Intake in a Rat Polycystic Ovary Syndrome Model by Downregulating Hypothalamus Insulin and Leptin Signaling Pathways Preceding Weight Gain

Ying Liu, Yu-Chen Xu, Yu-Gui Cui, Shi-Wen Jiang, Fei-Yang Diao, Jia-Yin Liu, Xiang Ma

The State Key Laboratory of Reproductive Medicine, Clinical Center of Reproductive Medicine, First Affiliated Hospital, Nanjing Medical University, Nanjing, China; Clinical Center of Reproductive Medicine, Xuzhou Central Hospital, Xuzhou Clinical School of Xuzhou Medical College, Xuzhou, China; Center of Reproductive Medicine, State Key Laboratory of Reproductive Medicine, Research Institute for Reproductive Health and Genetic Diseases, The Affiliated Wuxi Maternity and Child Health Care Hospital of Nanjing Medical University, Wuxi, China

Keywords
Androgen excess · Food intake · Hypothalamus insulin sensitivity · Hypothalamus leptin sensitivity · Polycystic ovary syndrome

Abstract
Background: Polycystic ovary syndrome (PCOS) is a common reproductive and metabolic disorder characterized by high androgen levels. The aim of this study was to evaluate the effects of hyperandrogenism on the hypothalamus and subsequently on the food intake and obesity in females.

Methods: A dihydroxy testosterone (DHT)-induced rat model was established to recapitulate the hyperandrogenism features of PCOS patients. Body weight and food intake of the rats were recorded. The food intake of DHT-induced rats was restricted by pair feeding to exclude possible effects of weight gain on the hypothalamus. The expression levels of relevant proteins and mRNAs in the hypothalamus and primary hypothalamic neurons exposed to DHT were analyzed by Western blotting and RT-PCR, respectively. The leptin levels in the serum and cerebrospinal fluid (CSF) were measured, and leptin was injected via the intracerebroventricular (ICV) route to test the hypothalamic sensitivity to insulin and leptin before obesity and with restricted food intake. DHT significantly reduced the leptin levels in the CSF, and ICV injection of leptin inhibited the DHT-induced increase in food intake.

Conclusions: Androgen excess increased food intake in rats and promoted obesity by downregulating insulin and leptin signaling in the hypothalamus, most likely by suppressing leptin levels in the CSF.

Introduction
Polycystic ovary syndrome (PCOS) is a common disorder of the female reproductive system characterized by anovulation, hyperandrogenism, and polycystic ovaries [1, 2]. It increases the risk of abdominal adiposity, obesity, and insulin resistance (IR) [3–6], and the current
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Materials and Methods

Human Subjects
Female participants were recruited from the Clinical Center of Reproductive Medicine, the First Affiliated Hospital of Nanjing Medical University, and the Clinical Center of Reproductive Medicine, Xuzhou Clinical School of Xuzhou Medical College, from January 2021 to March 2021. The study protocol was approved by the First Affiliated Hospital of Nanjing Medical University (approval no. 2012-SR-048). All participants signed the informed consent. PCOS was diagnosed according to the Rotterdam criteria, and hyperandrogenism was determined based on endocrine parameters. Healthy non-PCOS controls had regular menstrual cycle, normal ovarian morphology, and normal androgen levels. All participants were instructed not to take oral contraceptives, metformin, and levothyroxine; receive ovulation induction therapy; or control their weight over a 3-month period. The participants were divided into the following groups: group I – overweight/obese women with PCOS (BMI ≥ 24), group II – normal weight women with PCOS (BMI < 24), group III – overweight/obese non-PCOS controls (BMI ≥ 24), and group IV – normal weight non-PCOS controls (BMI < 24). Plasma NPY levels were measured using an ELISA kit (RayBiotech, Inc., Peachtree Corners, GA, USA). The intra- and inter-assay variation coefficients were below 10% and 15%, respectively, and the detection limit of the assay was 0.2 ng/mL.

Animal Experiments

Ethics Statement
All animal experiments were approved by the Nanjing Medical University Committee for the Use and Care of Animals (approval no. IACUC-14030124) and conducted in accordance with the Animal Research Committee Guidelines of Nanjing Medical University.

Establishment of DHT-Induced Rat Model
Female Sprague Dawley rats (21 days old) were obtained from the Charles River (Beijing, China) and housed under standard conditions and randomly divided into the DHT and control groups. DHT induction was initiated as described previously [26]. Briefly, the rats were subcutaneously implanted with silastic capsules (Dow Corning Corporation, Midland, MI, USA) containing 7.5 mg DHT. The capsule continuously released DHT over a period of 90 days at the rate of 83 μg/day. The control rats received identical capsules without DHT.

Pair feeding was applied to control the possible effect of overnutrition or obesity on the hypothalamus. All animals were housed individually 1 week after capsule implantation, and the feeding pairs were established wherein the control animals were fed ad libitum, and the DHT-treated rats were given the same daily average amount of food. Pair feeding was maintained for 7 weeks, and the food intake was measured daily.

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Patients with body weight-matched healthy women and established a dihydroxy testosterone (DHT)-induced rat model of PCOS. Our findings provide new insights into the pathogenesis of metabolic syndrome associated with PCOS, which may facilitate more effective prevention and intervention procedures.

Materials and Methods

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were fasted for 14 h and injected intraperitoneally with 2 g/kg D-ino-1.176; Bruker, Bremen, Germany) under isoflurane anesthetiza-
tion. Visceral and subcutaneous fat contents were measured and
also measured by ELISA (Clone Corp). The intra- and inter-assay
coefficients of variation were less than 10% and 12%, respectively,
respectively, for insulin. The detection limits of the assays were
0.04 ng/mL for leptin and 0.2 ng/mL for insulin. Plasma DHT was
measured using specific ELISA kits (Merck Milli-
Gene Company Cat. no. Dilution

| Gene          | Company   | Cat. no. | Dilution |
|---------------|-----------|----------|----------|
| P-JAK2 (Y1007/1008) | Abcam     | Ab32101  | 1:1,000  |
| JAK2          | CST       | 3230     | 1:1,000  |
| P-SATA3 (Y705) | CST       | 9145     | 1:2,000  |
| STAT3         | CST       | 4904     | 1:2,000  |
| P-IR (Y1361)  | Abcam     | Ab60946  | 1:500    |
| IR            | CST       | 3025     | 1:1,000  |
| P-AKT (ser 473)| CST       | 4060     | 1:1,000  |
| AKT           | CST       | 4691     | 1:1,000  |
| β-Actin       | Abcam     | ab8226   | 1:10,000 |

CST, Cell Signaling Technology.

Measurement of Body Fat
Body fat composition was evaluated by Micro-CT (SkyScan
1176; Bruker, Bremen, Germany) under isoflurane anesthetiza-
tion. Visceral and subcutaneous fat contents were measured and
compared.

Intraperitoneal Glucose Tolerance Test and Intraperitoneal
Insulin Tolerance Test
For the intraperitoneal glucose tolerance test (IPGTT), the rats
were fasted for 14 h and injected intraperitoneally with 2 g/kg D-
glucose (Sigma-Aldrich, St. Louis, MO, USA). Blood samples were
collected 0, 15, 30, 60, and 120 min later by a retro-orbital punc-
ture, and glucose levels were measured using a glucometer (AC-
cu-CHEK, Performa; Roche Diagnostics). Blood samples were
also collected for measuring insulin levels. For the intraperitoneal
insulin tolerance test (IPTTT), the animals were fasted for 6 h and
injected intraperitoneally with 1 U/kg insulin (Humulin; Lilly).
Blood samples were collected 15, 30, 60, and 120 min later from
the tail vein for measuring glucose levels.

Metabolic Cage Experiments
Whole-body metabolism was assessed in terms of food intake,
locomotor activity, VO2, and VCO2 over a period of 5 days. The
first 2 days were used for acclimatization (n = 6/group). The data
were recorded at 15-min intervals using a PhenoMaster (TSE Sys-
tems, Bad Homburg vor der Höhe, Germany).

Measurement of Metabolic Factors and Steroid Levels
Blood and cerebrospinal fluid (CSF) samples were collected af-
aer 14 h of fasting. The circulating total cholesterol, triglyceride,
and free fatty acid levels were assessed by the enzymatic colorimet-
ric method according to manufacturer’s protocols (Wako Pure
Chemical Industries, Ltd., Osaka, Japan). Plasma leptin and insu-
lus levels were measured using specific ELISA kits (Merck Milli-
Gene Company Cat. no. Dilution

| Gene          | Company   | Cat. no. | Dilution |
|---------------|-----------|----------|----------|
| β-Actin       | For/Rev   | TGGCCGATCCTTCTTCCTGTTCAACG |
| Pomc          | For/Rev   | AGATCCAGCCCTGAGACATCTGTTCAACG |
| Ucp1          | For/Rev   | TCCTCACAGAGGCTGCTTTCCCTGAGACAAAC |
| Leptin        | For/Rev   | GAAGCGTTGGTAGCTGGTAGTGGAACAGGCAAG |

and the detection limit of the assay was 13.8 pg/mL. The leptin lev-
els in the CSF were measured using the Rat Cytokine Array Q2 kit
(Raybiotech) according to the manufacturer’s protocol.

Central Leptin Sensitivity
Central leptin sensitivity was measured as previously described
[27]. Briefly, 2 weeks after implantation with silastic capsules, the
rats were placed in a stereotaxic apparatus under isoflurane inhala-
tion. A brain infusion cannula (RWD Life Science Co., Ltd, Shen-
zen, China) was positioned in the right lateral brain ventricle (1.2
mm lateral relative to the bregma, 1 mm posterior to the bregma,
and 3.5 mm below the surface of the skull). Leptin (4 μg/2 μL) or
artificial CSF (2 μL) was injected into the lateral ventricle a week
after implantation of the intracerebroventricular (ICV) cannulas.
Food intake was measured for 24 h after the leptin or aCSF injec-
tion, and p-STAT3 levels were measured 1 h after ICV injection.

ICV Administration of LY294002
To determine the specific effect of hypothalamic insulin signal-
ning on glucose metabolism, 7-week-old female rats were given ICV
injection of 10 μL and 25 μM LY294002 (a PI3K inhibitor that is
known to block the insulin regulatory pathway) dissolved in 50%
DMSO and 50% aCSF or 10 μL solvent. The IPGTT was performed
45 min later.

Cell Culture and Reagents
The primary hypothalamic neurons were isolated from the
brains of 18-day-old female Sprague Dawley rats as previously de-
scribed [28]. The animals were decapitated, and the hypothalamus
tissues were immediately isolated and immersed in ice-cold dis-
secting solution (8 g NaCl, 0.4 g KCl, 0.045 g NaHPO4·7H2O, 0.03
g KH2PO4, 7.5 g sucrose, 3 g glucose, and 2.35 g HEPES, per liter).
The tissues were minced into 1-mm3 pieces and incubated with
equal volume of 0.25% trypsin at 37°C for 10 min. The digested
tissues were washed with complete DMEM for 5 min and homog-
ennized by passing through a 1-mL pipette and a flamed tip glass
pipette 10 times each. The tubes were left undisturbed on ice, and
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the cloudy supernatant was aspirated and seeded onto collagen-coated coverslips (8 μg/mL Collagen I of the rat tail, BD falcon, USA). The cells were incubated at 37°C under 5% CO₂ in DMEM supplemented with 10% horse serum and 10% FBS. The medium was changed 6 h later to Neurobasal medium supplemented with 2% B27 and 500 μM L-glutamine.

Western Blotting
The hypothalamus tissues were lysed, and the protein contents were determined. Western blotting was performed as previously described [29]. Briefly, 60 μg proteins per sample were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The blots were incubated with primary and secondary antibodies as listed in Table 1. Positive bands were visualized by ECL, and the background-normalized densities of protein bands were evaluated with the AlphaEaseFC Imaging Software (Alpha Innotech, San Leandro, CA, USA).

Real-Time Quantitative PCR
Total mRNA was isolated from the brown adipose tissue and hypothalamus tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed with the SYBR PrimeScript RT Reagent Kit (Takara, Maebashi, Japan). Real-time PCR was performed in the StepOnePlus system (Applied Biosystems) according to the manufacturer’s instructions, using primers listed in Table 2. Relative gene expression levels were analyzed using the 2−ΔΔCT method.

Statistical Analysis
Data are presented as the mean ± standard error. All statistical analyses were conducted using the SPSS 19.0 software. Quantitative data were compared using one-way ANOVA, two-way ANOVA, Pearson’s correlation, and Student’s t test, and p < 0.05 was considered statistically significant.

Results
Serum NPY Levels Are Higher in PCOS Patients
There were 11 overweight and 15 lean PCOS patients in our cohort, along with 13 weight-matched non-PCOS controls (Table 3). As shown in Figure 1, both overweight and lean PCOS patients had significantly higher serum NPY levels compared to the weight-matched healthy women (p < 0.05 for both). The overweight control wom-

![Fig. 1. a Serum NPY concentrations in overweight PCOS, normal weight PCOS, overweight control, and normal weight control subjects. b NPY levels and plasma androgen levels were positively correlated. Data are presented as mean ± SD. a versus b p < 0.01, b versus c p < 0.05.]

![Table 3. Geographic data of human subjects (mean±SD)]

| The average of all groups | lean PCOS | overweight/obesity PCOS | lean control | overweight/obesity control |
|---------------------------|-----------|-------------------------|-------------|---------------------------|
| Number                    | 15        | 11                      | 7           | 6                         |
| Plasma androgen level, ng/mL | 0.7149±0.12183a | 0.8069±0.30288a | 0.4184±0.05241b | 0.3340±0.21376b |
| BMI, kg/m²                | 21.4647±2.35328c | 28.6164±2.74726d | 22.5974±1.21424c | 27.84±2.22818d |

a vs. b p < 0.05, c vs. d p < 0.05.
en also displayed significantly higher NPY levels compared to the normal weight healthy women ($p < 0.05$). There is no significant difference in NPY levels between lean and overweight PCOS patients. NPY levels and plasma androgen levels were positively correlated ($r = 0.433$, $p = 0.0074$).

**DHT-Induced Obesity and Impaired Lipid Metabolism in Female Rats**

The metabolic changes induced by DHT in the rat model were measured in terms of body weight, adiposity, and lipid metabolism. The body weight ($p < 0.001$; Fig. 2a) and serum DHT levels ($p < 0.05$; Fig. 2b) were markedly higher for the 7- and 16-week-old DHT rats compared to the age-matched controls. The CT scan demonstrated a significantly higher visceral fat mass in the DHT-treated versus control rats ($p < 0.05$; Fig. 2c), which indicated obesity. The circulating triglyceride levels increased significantly after 90 days of DHT treatment ($p < 0.05$; Fig. 2d), whereas the free fatty acid concentration decreased significantly ($p < 0.05$; Fig. 2f) and total cholesterol levels were unaffected by DHT treatment (Fig. 2e). Moreover, the $Ucp1$ mRNA level was significantly downregulated in the brown fat of DHT-treated rats ($p < 0.05$; Fig. 2g), indicating reduced lipolysis. In contrast, the leptin mRNA and protein levels were markedly increased.
Fig. 3. Effects of DHT on glucose tolerance and insulin sensitivity in female rats. Blood glucose (a) and blood insulin levels (b) of 8-week-old rats at different time points. Note the initial increase and subsequent decrease in insulin levels in DHT rats after glucose administration. c Results of the IPGTT showing that blood glucose levels were sharply increased in 13-week-old DHT rats compared to control rats. Note the significant decrease in glucose tolerance in 13-week-old but not in 8-week-old DHT rats. Results of the IPITT showing that blood glucose levels (d) and corresponding 0- to 120-min AUC values (e) in 8-week-old DHT and control rats and blood glucose levels (f) and corresponding 0- to 120-min AUC values (g) for 13-week-old DHT and control rats. Note the significant increase in the AUC values in DHT rats. Data are presented as mean ± SEM (n = 5–6). *p < 0.05, **p < 0.01. SEM, standard error.

Fig. 4. Basal metabolism during light and dark cycles in 6-week-old control and DHT rats (n = 6/group). Oxygen consumption (a) and carbon dioxide production (b) were unchanged with DHT treatment. c RER was increased with DHT treatment during the night. d Locomotor activity was decreased in DHT rats during the day. e Water consumption was significantly increased in DHT rats during the day. f Food intake was significantly increased in DHT rats during both day and night. Values and error bars represent the mean ± SEM. *p < 0.05; **p < 0.01. SEM, standard error; RER, respiratory exchange ratio.
Fig. 5. Effects of DHT on insulin signaling and expression of key regulators of feeding in the hypothalamus of 13-week-old rats. a, d Representative immunoblot showing protein levels in the hypothalamus tissues of DHT and control rats after 14 h of fasting with or without insulin injection, respectively. b, e Results of densitometry analysis on the ratio of p-AKT/AKT. Note the sharp reduction in the phosphorylation of AKT and GSK phosphorylation in DHT rats. c, f Results of densitometry analysis on the ratio of p-GSK/GSK. The administration of exogenous insulin could not fully alleviate the DHT-induced inhibition of AKT and GSK phosphorylation. g Blood glucose levels in the IPGTT after ICV injection of LY294002. Blocking the PI3K/AKT pathway led to decreased glucose tolerance. Experiments were performed in 3 rats for each group. Data are expressed as the mean ± SEM (n = 3). *p < 0.05. SEM, standard error.
in the white fat and blood, respectively (p < 0.05; Fig. 2h, i). Taken together, DHT-induced obesity in female rats and significantly affected lipid metabolism by stimulating leptin secretion from the white fat.

**DHT Impaired Glucose Tolerance and Insulin Sensitivity**

The effects of DHT on glucose metabolism in the 8-week-old and 13-week-old rats were analyzed by the IPGTT and IPITT. As shown in Figure 3a, the IPGTT did not show any significant difference in blood glucose levels between the DHT-treated and control rats at 8 weeks of age. However, the fasting insulin levels in the DHT-treated animals showed an initial spike compared to the control rats, followed by a sharp decrease 120 min after glucose administration (p < 0.05; Fig. 3b). Furthermore, blood glucose levels increased significantly following prolonged DHT exposure in the 13-week-old rats compared to the controls (p < 0.01; Fig. 3e), indicating impaired glucose tolerance. The IPITT showed a spike in blood glucose levels of 8-week-old DHT rats after 30 and 120 min of insulin injection compared to the controls (p < 0.05; Fig. 3c), resulting in higher AUC in the former (p < 0.05; Fig. 3d). Thus, prolonged DHT treatment impaired glucose tolerance and insulin sensitivity in a time-dependent manner.

**DHT-Induced Obesity by Increasing Food Intake**

To determine whether the DHT-induced aberrant glycolipid metabolism and obesity is related to food intake, we analyzed the effects of DHT on food intake and whole-body metabolism. The basal metabolism of weight-matched 6-week-old DHT and control rats was evaluated to determine whether the eating behavior of the DHT-treated rats was altered prior to weight gain. Interestingly, no significant differences were observed in oxygen consumption and CO2 production between the 2 groups (Fig. 4a, b). However, DHT treatment markedly increased the respiratory exchange ratio (p < 0.01; Fig. 4c), water consumption (p < 0.05; Fig. 4e), and food intake (p < 0.01; Fig. 4f) and decreased the locomotor activity compared with controls (p < 0.05; Fig. 4d). Taken together, DHT-induced weight gain was associated with an increased food intake as well as altered metabolism indices.

**DHT Inhibited Insulin Signaling in the Hypothalamus of 13-Week-Old Rats**

The hypothalamic insulin signaling pathway is the key regulator of feeding behavior. To determine whether DHT altered insulin signaling in the hypothalamus, we analyzed the in situ levels of total and phosphorylated Akt and GSK in 13-week-old DHT and control rats and detected a significant downregulation of p-Akt and p-GSK in the DHT-treated versus control rats (p < 0.05; Fig. 5a–c). To assess the effects of exogenous insulin, the animals were injected with 2 U/kg insulin after 14 h of fasting. Akt and GSK phosphorylation were similarly inhibited after insulin injection (p < 0.05; Fig. 5d–f), indicating that DHT-induced rats had impaired insulin signaling in the hypothalamus even in the presence of exogenous insulin. IPGTT results further showed that ICV injection of the PI3K inhibitor LY294002 [30] increased glucose levels after 15, 30, 60, and 120 min of glucose administration (p < 0.05; Fig. 5g), indicating that hypothalamic insulin signaling regulates glucose metabolism.

**DHT Inhibited Hypothalamic Insulin and Leptin Signaling and Upregulated NPY/Agrp Prior to Obesity Development**

Based on our observation that the 6-week-old prepubertal DHT-treated rats did not develop obesity, we hypothesized that DHT impaired insulin and leptin signaling before obesity development. As shown in Figure 6, prepubertal DHT treatment inhibited phosphorylation of IR, AKT, GSK, JAK2, and STAT3 (p < 0.05; Fig. 6a–g) and significantly increased hypothalamic production of NPY and Agrp (p < 0.05; Fig. 6h). In contrast, the expression of POMC was similar in both groups. These findings strongly suggested that DHT directly regulated the activity of the feeding center by promoting the expression of orexigenic genes, which in turn increased the food intake.

**DHT Inhibited Hypothalamic Insulin and Leptin Signaling and Upregulated NPY and Agrp under Restricted Food Intake**

To exclude the possible effects of overnutrition or obesity on the hypothalamus, feeding pairs of DHT-treated and control rats were established (see Methods). As an additional control group, DHT-treated rats were given ad libitum access to food. No significant difference was observed between the pair-fed DHT and control rats (p > 0.05; Fig. 7b), whereas the body weights of the ad libitum fed DHT rats were significantly higher than the respective controls (p < 0.01; Fig. 7a). The insulin levels were similar in the DHT and control rats in the ad libitum-fed and pair-fed groups (p > 0.05; Fig. 7c, d). Although the serum leptin levels were significantly higher in the ad libitum fed DHT-treated versus control animals (p < 0.05; Fig. 7e), this difference disappeared once pair feeding was started (p > 0.05; Fig. 7f). Thus, increased
Fig. 6. Effects of DHT on insulin and leptin signaling in the hypothalamus of 6-week-old, prepubertal, nonobese rats. a–e Representative immunoblots showing phosphorylated and total IR, AKT, GSK, JAK2, and Stat3 levels, with β-actin as the loading control. b–d, f, g Densitometry analysis and comparison of phosphorylated forms versus total amounts of different proteins. DHT treatment significantly decreased the phosphorylation levels of these signaling proteins. h Expression levels of feeding-related genes in the hypothalamus of 6-week-old DHT and control rats. Note the significant increase in the mRNA levels of NPY and Agrp genes. Experiments were performed in 3 rats for each group. Data are expressed in mean ± SEM (n = 6). *p < 0.05, **p < 0.01. SEM, standard error.
Fig. 7. Effect of DHT on the body weight and serum insulin/leptin levels after pair feeding. a, b Ad libitum fed 7-week-old DHT rats gained more weight than control rats, whereas pair feeding leveled the difference between the 2 groups ($n = 6$). c, d Insulin levels were similar in the DHT and control rats in both ad libitum and pair-fed groups ($n = 5$). e, f DHT rats had higher fasting leptin levels than control rats at ad libitum condition ($n = 5$) (e) but not at pair feeding condition ($n = 5$) (f). g mRNA levels of the feeding-related genes NPY and Agrp were significantly increased in the hypothalamus of DHT rats after pair feeding ($n = 3$). Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$. SEM, standard error.
Fig. 8. Effect of DHT on insulin and leptin signaling in the hypothalamus of 7-week-old pair-fed rats. a, f Representative immunoblots showing phosphorylated and total IR, AKT, GSK, JAK2, and Stat3 levels, with β-actin as the loading control. b–e, g–j Densitometry analysis and comparison of phosphorylated versus total amounts of proteins. DHT treatment significantly decreased the phosphorylation levels of these signaling factors after pair feeding. Experiments were performed in 3 rats for each group. Data are expressed in mean ± SEM. *p < 0.05, **p < 0.01. SEM, standard error.
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leptin secretion and the changes in body weight caused by excessive DHT could be suppressed by food restriction, indicating that DHT-induced obesity required an increased food intake. Furthermore, NPY and Agrp mRNA levels were significantly upregulated in the DHT rats compared to the pair-fed controls \((p < 0.05; \text{Fig. 7g})\), while no significant difference was seen in the expression of POMC mRNA between the 2 groups. Given the similar expression of NPY and Agrp in the ad libitum and pair-fed rats, the effect of DHT on the hypothalamic feeding center appeared to be unrelated to obesity. Consistent with this, DHT inhibited the phosphorylation of IR, AKT, JAK2, and STAT3 \((p < 0.05; \text{Fig. 8})\) under food restriction, further supporting that suppression of insulin and leptin signaling is a direct effect rather than an indirect effect of obesity.

**DHT Upregulated NPY and Downregulated POMC in Primary Hypothalamic Neurons**

The mechanistic basis of DHT action was further elucidated using primary embryonic hypothalamic neurons cultured with or without leptin. DHT had no effect on NPY, Agrp, and POMC mRNA levels in the absence of leptin (Fig. 9a) but upregulated NPY and downregulated POMC mRNA levels along with leptin \((p < 0.05; \text{Fig. 9b})\) without affecting Agrp mRNA levels (Fig. 9b). Thus, DHT upregulated the orexigenic genes and suppressed anorex-ic genes in the presence of leptin.

**ICV Injection of Leptin Inhibited the Improvement of Food Intake Caused by DHT**

DHT significantly inhibited leptin levels in the CSF \((p < 0.05; \text{Fig. 10a})\). To determine leptin sensitivity, the rats were given ICV injection of leptin, and their food intake and STAT3 phosphorylation were measured. Central injection of leptin inhibited the improvement of food intake caused by DHT \((p < 0.05; \text{Fig. 10b})\). The phosphorylation of STAT3 was also increased in the DHT rats \((p < 0.05; \text{Fig. 10c, d})\).

**Discussion**

Studies show that PCOS patients are more likely to have eating orders like bulimia nervosa or binge eating \([7–9]\), although the molecular mechanisms have not been elucidated. Hyperandrogenism plays a central role in the pathogenesis PCOS whether as an initial and independent cause \([31, 32]\) or a concurrent or secondary phenomenon of PCOS \([33, 34]\). Although hyperandrogenism is correlated with changes in neuroendocrine signaling, metabolism, feeding behaviors, and weight gain in PCOS patients, its pathological effects are ambiguous \([5]\). High androgen levels are known to impair glucose and lipid metabolism in fibroblasts, fat tissues, and the liver, which could cause obesity \([35]\). On the other hand, hyperandrogenism can also lead to weight gain by increasing food intake \([36, 37]\). In addition, obesity itself affects the insulin and leptin signaling in the hypothalamus \([38]\) and therefore food intake as well \([38]\).
In this study, we tested the serum NPY levels in lean and overweight/obese PCOS patients with hyperandrogenism, as well as weight-matched healthy women with normal androgen levels. We also investigated the relationship between androgen and NPY levels. Serum NPY levels and androgen levels were found to be positively correlated, suggesting that hyperandrogenism may alter the peripheral secretion of NPY. We also investigated whether the serum NPY level is associated with the body weight of PCOS patients and can predict weight gain and did not detect any correlation. Both overweight and lean PCOS patients had significantly higher circulating levels of NPY compared to the weight-matched healthy controls. Thus, it seems unlikely that the serum NPY would be able to directly influence the food intake in PCOS patients. This could be attributed to the different origins and roles of NPY in the nervous system and bloodstream [39, 40]. NPY is mainly secreted by neurons in the central and peripheral nervous systems [41], and regulates food intake, energy metabolism, immune responses, and emotional reactions [39]. Although the elevated serum NPY did not correlate with the weight gain in PCOS patients, studies show that peripheral NPY plays a crucial role in promoting adipocyte proliferation, differentiation, and accumulation in white adipose tissue, eventually leading to obesity [42]. Plasma NPY is also significantly elevated in patients with depression [43], which coincides with the increased prevalence of depression and anxiety symptoms in PCOS patients [44]. In a future study, we will investigate the correlation between NPY levels and emotional status of PCOS patients. It is noteworthy that NPY/AgRP-expressing neurons not only regulate food intake but also affect the secretion of gonadotropin-releasing hormone and luteinizing hormone [45], which may explain the concurrent energy imbalance and infertility that is often observed in PCOS patients.

We used the DHT-induced PCOS rat model to analyze the effect of hyperandrogenism on hypothalamic neurons and found that long-term exposure to high androgen levels increased food intake, weight gain, and fat percentage and also impaired glucose tolerance and insulin sensitivity, which are consistent with the clinical
characteristics of PCOS. The food intake and NPY levels were particularly elevated in the ad libitum fed rats and even with restricted feeding and lack of weight gain. Moreover, DHT significantly upregulated NPY and Agrp mRNA expression in the hypothalamus. DHT upregulated NPY and downregulated POMC mRNA levels along with leptin, without affecting Agrp mRNA levels. The difference between the results of in vitro and in vivo experiments may be contributed to the different complexity of experimental conditions. In vivo, the activities of both NPY/Agrp and POMC neurons are regulated by several kinds of signaling such as leptin, insulin, and ghrelin. But in vitro, only the effects of DHT on leptin-induced expression of orexigenic peptides and anorexigenic peptide were observed. Besides, the concentration of DHT and treatment time were not completely consistent with the experimental condition in vivo. We speculate that DHT regulates NPY/Agrp and POMC expressions directly through androgen receptors or indirectly by interfering with the leptin-induced modulation of NPY/Agrp and POMC. Consistent with our findings, Iwasa et al. [46] reported that exogenous administration of androgens increased food intake in ovariectomized rats. These findings highlight the significance of androgen-induced hypothalamic alterations in the pathogenesis of PCOS.

The hypothalamus is the key regulator of energy intake and expenditure [13]. The activities of both NPY/Agrp and POMC neurons are regulated by insulin and leptin [17], and hypothalamic insulin and leptin resistance can lead to food intake and metabolic disorders by affecting these neurons. In IPGTT experiments, we observed that blocking the PI3K/Akt/GSK pathway with LY29400 significantly decreased glucose tolerance similar to that observed with DHT, thus confirming the involvement of insulin pathway in glucose homeostasis. Pair feeding could not abrogate the blockade of DHT on leptin/insulin signaling in the hypothalamus. Therefore, a combination of diet control and antiandrogenic treatment may reverse hyperandrogenism-induced symptoms in PCOS patients. For patients experiencing difficulty controlling their food intake and body weight, antiandrogenic treatment could be a suitable alternative. Since the effects of DHT on the hypothalamus are mediated by suppression of the AKT pathway, AKT activators may also alleviate the hyperandrogenism-caused symptoms of PCOS.

Based on these findings, we next analyzed the effect of ICV leptin injection on food intake and found that it neutralized the DHT-induced increase in food intake. ICV injection of leptin also increased the p-STAT3/STAT3 ratio in the hypothalamus of DHT-treated rats. The leptin levels in the CSF of the DHT-treated rats were significantly lower compared to that of the control rats, whereas the serum leptin was elevated in the DHT-treated versus control rats. This could be related to the impaired transport of leptin through the blood-brain barrier (BBB) [45, 47–49]. In order to exert its effects on the central nervous system, the leptin released by adipose tissues must cross the BBB and bind to leptin receptors (ObR) on the surface and vesicles of hypothalamic neurons [50, 51]. The leptin receptor-mediated transport of leptin across the BBB controls food reward [52]. A high-fat diet increases the expression of the short form of the BBB leptin receptor [53]. However, Gonzalez-Carter et al. [54] reported that ObR is not required for efficient transport across human endothelial monolayers and showed that an ObR-neutralizing antibody (9F8) inhibited leptin-ObR interaction without preventing leptin translocation across the BBB. Recent studies have shown that hypothalamic tanyocytes shuttle circulating leptin into the hypothalamus [55, 56], and tanyctic LepRb-EGFR-mediated transport of leptin is crucial to the pathophysiology of diabetes and obesity [56, 57]. The inhibition of TSPO (translocator protein) in tanyocytes reduced food intake and elevated energy expenditure [58]. Studies show that the BBB permeability is increased in chronically testosterone-depleted male mice, and testosterone supplementation in castrated mice restored its selective permeability [59]. In our future study, we will investigate the effect of DHT on the transportation of circulating leptin to the hypothalamus, and the possible anti-obesity effect of increasing leptin levels in the CSF of PCOS patients.

The DHT-induced rat model has several limitations when used to simulate PCOS. Since PCOS is the result of multiple genetic and environmental factors, the model may oversimplify its pathogenesis by focusing only on the androgenic effects on the hypothalamus. For example, DHT could simultaneously affect the expression and functions of insulin/IGF, thyroid function, or directly affect the energy production and consumption in peripheral organs such as the liver, muscles, and brain. Second, the study did not include assays to localize the effects of androgen excess to the specific neurons in the hypothalamus. Finally, the molecular action of DHT on insulin and leptin signaling pathways requires further study. Single-cell sequencing of DHT-induced hypothalamic tissues may resolve these concerns.
In conclusion, androgen excess increased food intake and promoted obesity by downregulating insulin and leptin signaling in the hypothalamus and increasing the expression of orexigenic genes. The hyperandrogenic regulation of the hypothalamus function plays a critical role in the development of metabolic disorders in PCOS patients. These findings shed new light on the pathogenesis of PCOS and provide useful insights on clinical management of PCOS patients. Further studies are required on the target genes and regulatory pathways of DHT in the hypothalamus to better understand the molecular and cellular mechanisms of PCOS.

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Statement of Ethics

The study protocol was approved by the First Affiliated Hospital of Nanjing Medical University (approval no. 2012-SR-048) and was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. All participants signed the written informed consent. All animal experiments were approved by the Nanjing Medical University Committee for the Use and Care of Animals (approval no. IACUC-14030124) and conducted in accordance with the Animal Research Committee Guidelines of Nanjing Medical University.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author Contributions

Y. Liu, X. Ma, and J.Y. Liu conceived the study and designed the experiments. Y. Liu and Y.C. Xu performed the experiments, collected the data, analyzed the results, and wrote the manuscript. Y. Liu prepared the original draft and wrote it. Shiwen Jiang, Yuguui Cui, and X. Ma reviewed and edited the manuscript. J.Y. Liu and X. Ma got the funding and realized the project administration. J.Y. Liu and X. Ma had primary responsibility for the final content.

Data Availability Statement

All data generated or analyzed during this study are included in this published article. Online supplementary Figure S1 (see www.karger.com/doi/10.1159/000521236 for all online suppl. material) is attached.
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