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# Mechanism of leptin-NPY on the onset of puberty in male offspring rats after androgen intervention during pregnancy

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**Objectives:** The time of onset of puberty has been increasingly earlier, but its mechanism is still unclear. This study aimed to reveal the mechanism of leptin and NPY in the onset of puberty in male offspring rats after androgen intervention during pregnancy.

**Methods:** Eight-week-old specific pathogen-free (SPF) healthy male Sprague–Dawley (SD) rats and 16 female SD rats were selected and caged at 1:2. The pregnant rats were randomly divided into the olive oil control group (OOG) and testosterone intervention group (TG), with 8 rats in each group. Olive oil and testosterone were injected from the 15th day of pregnancy, for a total of 4 injections (15th, 17th, 19th, 21st day). After the onset of puberty, the male offspring rats were anesthetized with 2% pentobarbital sodium to collect blood by ventral aorta puncture and decapitated to peel off the hypothalamus and abdominal fat. Serum testosterone (T), free testosterone (FT), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), sex hormone binding globulin (SHBG), and leptin were detected by ELISA, and then the free androgen index (FAI) was calculated. The mRNA levels of androgen receptor (AR), estrogen receptor  $\alpha$  (ER $\alpha$ ), NPY, leptinR, and NPY2R in the hypothalamus and abdominal fat were detected by RT–PCR. Protein expression levels of AR, ER $\alpha$ , NPY, leptinR, and NPY2R in the arcuate nucleus (ARC) of the hypothalamus were detected by immunohistochemistry.

**Results:** The time of onset of puberty was significantly earlier in the TG than in the OOG ( $P < 0.05$ ) and was positively correlated with body weight, body length, abdominal fat, and leptinR mRNA levels in adipose tissue in the OOG ( $P < 0.05$ ), while it was positively correlated with serum DHT and DHEA concentrations and FAI and AR mRNA levels in the hypothalamus in the TG ( $P < 0.05$ ). The NPY2R mRNA level and protein expression levels of ER $\alpha$ , NPY2R, and leptinR in the TG were significantly higher than those in the OOG, while the protein expression levels of AR and NPY in the TG were significantly lower than those in the OOG ( $P < 0.05$ ).

**Conclusions:** Testosterone intervention during pregnancy led to an earlier onset of puberty in male offspring rats, which may render the male offspring rats more sensitive to androgens, leptin, and NPY at the onset of puberty.

KEYWORDS

leptin, NPY, puberty onset, androgen, GnRH

## 1 Introduction

Puberty is defined as the “first reproductive period”, which marks the maturity of the genitals, the development of secondary sexual characteristics, the acceleration of linear growth, emotional changes, and the occurrence of first spermatorrhea or menarche (1). The onset time of puberty has shown an increasing trend of being earlier worldwide (2). In the past 150 years, the average age of menarche of European children has declined from approximately 16–17 years in the middle of the 19th century to 12 years in the middle of the 20th century (3, 4), which is estimated to be advanced by 3 years every 100 years (5). A cohort study of Swedish boys showed that the starting age of puberty was 1.5 months earlier for every decade increase in birth year (6). Moreover, the average age at which Danish boys initiated puberty was 3 months earlier from 2006 to 2008 than before (7), and that of Chinese urban boys was 2 years earlier from 2003 to 2005 than 1979 (8). The early onset of puberty not only affects mental health in children and adolescents but also increases the risk of certain diseases in adulthood, such as metabolic syndrome, cardiovascular disease, osteoporosis, and testicular cancer (9, 10), which has developed into a serious public health problem.

With the development of ‘Fetal Origin of Adult Disease’ (FOAD) and ‘Development Origin of Health and Disease’ (DOHAD) (11, 12), an increasing number of studies have linked the endocrine environment during pregnancy to the long-term effects on future generations (13–16). With lifestyle changes and the effects of exogenous endocrine disruptors (17), the proportion of women with high androgen levels during pregnancy was significantly increased (18–20). Exposure to prenatal high androgen environments may lead to changes in hormone levels and pubertal development in offspring (21).

Leptin is a protein product of the obesity (Ob) gene and plays an important role in pubertal development and reproduction (22). Recent studies have found that leptin can regulate the synthesis and release of GnRH in the hypothalamic arcuate (ARC) nucleus (23). GnRH secretion is insufficient in mice lacking the leptin gene (24). Studies have directly shown that leptin can trigger the beginning of puberty in boys (25). However, studies have found that there is no leptin receptor expression or very little expression in ARC nucleus GnRH neurons (26). Therefore, leptin cannot directly act on GnRH neurons, but it is involved in regulating puberty onset (27, 28). However, leptin may indirectly affect the activity of GnRH neurons by affecting NPY neurons (29). NPY was identified as the main regulator of GnRH

pulse secretion (30), which is the ‘gatekeeper’ of adolescence (31). As a regulator, NPY neurons are located in the ARC (32), which can directly exert an inhibitory effect on GnRH neurons (33). This effect is achieved by the direct binding of NPY to NPY1R on GnRH neurons (34). Moreover, recent studies have demonstrated that leptin receptors exist in NPY neurons (24), which indicates that leptin can directly regulate NPY neurons (35) and thus play an important role in regulating reproduction (36).

However, whether high androgen exposure during pregnancy can participate in puberty onset by affecting the levels of leptin and NPY in offspring has rarely been reported. Therefore, this study administered androgen intervention to maternal rats during pregnancy to shed light on the mechanism of leptin and NPY in puberty onset of offspring male rats after androgen intervention during pregnancy, which provides a powerful clue for exploring the relationship between prenatal androgen and the onset of puberty in male offspring.

## 2 Participants and methods

### 2.1 Rats, diet, and experimental procedures

All animal care and experimental procedures were approved by the Ethics Committee of Bengbu Medical College ([2018] No.032). A total of 16 eight-week-old healthy SD female rats (200–250 g) and 8 male rats (300–330 g) were purchased from Jinan Peng Yue Experimental Animal Reproduction Co., Ltd. Animals were raised in a clean animal room with relative temperature ( $25 \pm 2^\circ\text{C}$ ), relative humidity (40–70%), free light, and free access to water and diet. After a week of adaptive feeding, the female and male rats were caged at 9 o'clock every night at a ratio of 1:1, and vaginal plugs were observed at 9 o'clock the next morning to determine whether the rats were pregnant. Then, the pregnant female rats were randomly divided into the olive oil control group (OOG) and testosterone intervention group (TG), with 8 rats in each group. The pregnant female rats were treated with subcutaneous injection on the back of the neck starting from the 15th day of pregnancy, for a total of 4 injections (15th, 17th, 19th, 21st day). TG was given 2 mL of 2.5 g/ml testosterone solution (dissolved in olive oil), and OOG was given the same dose of olive oil. After the birth of the offspring, 36 male offspring rats were taken, which were raised to 21 days (PND21) and then weaned. From PND21, the body weight of the rats was recorded daily, and the genitals were observed. The foreskin separation of the male rats was used as a sign of the onset of puberty.

The number of days taken to initiate puberty was recorded in all experimental rats. Twelve rats (TG : OOG = 1:1) were used for the detection of body shape indicators, serum hormone levels, and the mRNA of hypothalamic neuroendocrine function genes. Then, 6 rats (TG : OOG = 1:1) were used to detect the expression of hypothalamic neuroendocrine functional proteins by immunohistochemistry.

## 2.2 Sample collection

After anesthesia with 2% pentobarbital sodium solution at a dose of 3 ml/kg, we punctured the abdominal aortas of the rats to collect blood, and the blood was centrifuged to obtain the upper serum, which was then stored at -80 °C. We decapitated the rats, stripped their hypothalamus and abdominal fat, immediately placed them in liquid nitrogen and transferred them to the refrigerator at -80 °C at the end of the experiment. The above samples were all operated on ice.

## 2.3 Body shape indices and wet weight of abdominal fat in rats

When signs of puberty onset were observed, the rats' body weight, body length, anal-genital distance (AGD), abdominal fat, and abdominal fat coefficient were measured. The body weight was measured with an electronic scale and was accurate to 0.1 g; body length refers to the distance from the tip of the nose to the superior margin of the anus, which was measured accurately to 0.1 cm; the AGD was measured by a Vernier caliper after anesthesia in rats and was accurate to 0.01 mm. Finally, abdominal fat was measured using an analytical balance and was accurate to 0.01 g; abdominal fat coefficient (%) = [abdominal fat weight (g)/body weight (g)] × 100%.

## 2.4 Detection of serum biochemical indices

The concentrations of T, FT, DHT, DHEA, SHBG, and leptin in the male rat serum were determined using ELISA with rat T (cat.

no. CSB-E05100r, CUSABIO, Wuhan, China), rat FT (cat. no. CSB-E05097r, CUSABIO), rat DHT (cat. no. CSB-E07879r, CUSABIO), rat DHEA (cat. no. CSB-E08227r, CUSABIO), rat SHBG (cat. no. CSB-E12118r, CUSABIO) and rat leptin ELISA kits (cat. no. CSB-E07433r, CUSABIO).  $FAI = [T \text{ (ng/ml)}/SHBG \text{ (ng/ml)}] \times 100$ .

## 2.5 Isolation of RNA and real-time PCR

Total RNA was extracted from the hypothalamus and abdominal adipose tissue by the TRIzol method (Thermo Scientific, Shanghai, China). For the detection of RNA concentration, we used 1 µg total RNA reverse transcription cDNA according to the instructions of the kit manufacturer (Thermo Scientific), and the mRNA levels of AR, ER $\alpha$ , NPY, leptinR, and NPY2R in the above tissues were detected by quantitative real-time PCR. The gene-specific primer sequences were designed by Shanghai Generay Biotech Co., Ltd., and all the primer sequences are shown in Table 1. The polymerase chain reaction mixture was 15 µl, and the reaction system contained 7.5 µl of 2×qPCR Mix, 1.5 µl of 2.5 µM gene primer, 2.0 µl of reverse transcription product, and 4.0 µl of ddH<sub>2</sub>O. The three-step PCR amplification protocol was as follows: predenaturation, 95°C 10 min; cycle, 95°C 15 s → 60°C 60 s, a total of 40 cycles; melting curve, 60°C → 95°C, heating 0.3°C every 15 s.

## 2.6 Immunohistochemical analysis

Hypothalamic tissue was fixed with 10% formalin and embedded in paraffin. The hypothalamus paraffin blocks were cut into 4 µm thick sections and placed in a repair box filled with citric acid antigen repair buffer (PH 6.0) for antigen retrieval in a microwave oven. After natural cooling, the slides were placed in PBS (PH 7.4), washed on a decolorized shaker 3 times for 5 minutes each time and then incubated in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase. Subsequently, 3% BSA was added to cover the tissue uniformly and sealed at room temperature for 30 min. The blocking solution was removed, and

TABLE 1 PCR primer sequences.

Primer name (-S upstream primer; -A downstream primer)	Primer sequence (5'-3')
AR-S	TGGGACCTTGGATGGAGAACT
AR-A	GGCACATAGATACTTCTGTTTCCC
ER $\alpha$ -S	GTTTGCTCCTAACTTGTCTTGG
ER $\alpha$ -A	TCAAGGTGCTGGATAGAAATGTG
NPY-S	CTCTGCGACACTACATCAATCTCA
NPY-A	CCTTGTCTGGGGGCATTT
Leptin Receptor-S	GGAAACACAGGGGTCCA
Leptin Receptor-A	TAGCAGCATCAACACCGA
NPY Receptor-S	ACAGTGAACCTTCTCATAGGCAACC
NPY Receptor-A	CAGAAGTACACACATTGAAGGAAC

PBS was dropped in a certain proportion of the primary antibody (rabbit polyclonal antibody, Servicebio, Wuhan, China) on the slice. The samples were then placed in a wet box and incubated overnight at 4°C. Then, the cells were incubated with a biotinylated secondary antibody (rabbit polyclonal antibody, Servicebio, Wuhan, China) and placed at room temperature for 50 min. A DBA kit was used to observe the immune response at room temperature for 2 minutes. Finally, hematoxylin staining was used for microscopic examination. Integrated optical density (IOD) was used to describe the relative expression of AR, ER $\alpha$ , leptinR, NPY, and NPY2R proteins. The IOD was repeated three times for each sample using Image-Pro Plus software, and the average value was taken.

## 2.7 Statistical analysis

The statistical analysis was performed in SPSS 23.0 software. First, because of the inconsistency of the sampling time of onset of puberty, we standardized the data according to the sampling time [standardized index = (index before standardization/time of onset of puberty)  $\times$  average time of onset of puberty] and carried out logarithmic conversion of the data. Measurement data are described as the mean  $\pm$  standard deviation. The differences in morphological development indices, sex hormones, and mRNA between TG and OOG were analyzed by independent-sample *t* test, while the differences in immunohistochemical indices between TG and OOG were analyzed by the mixed linear model. Pearson correlation analysis was used to analyze the correlation.  $P < 0.05$  was considered statistically significant.

## 3 Results

### 3.1 Effect of testosterone intervention during pregnancy on the time of onset of puberty in offspring male rats

Figure 1 shows that the time of onset of puberty of male offspring in the TG ( $44.11 \pm 2.14$  days;  $n = 18$ ) was significantly lower than that

in the OOG ( $46.89 \pm 2.14$  days;  $n = 18$ ), which suggested that testosterone intervention during pregnancy may lead to earlier time of onset of puberty of male offspring ( $P < 0.05$ ).

### 3.2 Effects of testosterone intervention during pregnancy on morphological development and blood circulating sex hormone levels in offspring male rats

Table 2 shows that there were no significant differences in body weight, body length, abdominal fat, abdominal fat coefficient, T, FT, DHT, DHEA, SHBG, FAI, or leptin between TG and OOG ( $P > 0.05$ ). The results of correlation analysis showed that the time of onset of puberty in the OOG was positively correlated with body weight, body length, and abdominal fat ( $P < 0.05$ ), while in the TG, the time of onset of puberty was positively correlated with serum DHT and DHEA concentrations and FAI ( $P < 0.05$ ) and negatively correlated with SHBG concentration ( $P < 0.05$ ). See Table 3 for details.

### 3.3 Effects of testosterone intervention during pregnancy on mRNA levels of AR, ER- $\alpha$ , leptinR, NPY, NPY2R

Testosterone intervention during pregnancy resulted in markedly elevated mRNA levels of NPY2R in the hypothalamus compared with the OOG, whereas the mRNA levels of leptinR in the fat were significantly downregulated ( $P < 0.05$ ; Table 4). There was a statistically positive correlation between the time of onset of puberty and leptinR mRNA levels in adipose tissue in the OOG ( $R = 0.840$ ,  $P < 0.05$ ) but not with other indicators (Table 5). Nevertheless, after testosterone intervention, there was only a statistically positive correlation between the time of onset of puberty and the level of AR mRNA in the hypothalamus ( $R = 0.883$ ,  $P < 0.05$ ; Table 5).

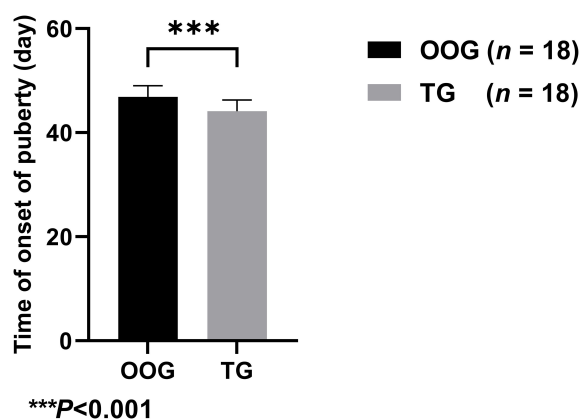


FIGURE 1

Comparison of the time of onset of puberty between OOG and TG.

TABLE 2 Comparison of body shape indices and blood circulating sex hormone indices in male offspring between OOG and TG ( $\bar{x} \pm s$ ).

Indices	OOG ( <i>n</i> = 6)	TG ( <i>n</i> = 6)	<i>t</i>	<i>P</i>
S Body weight (g)	194.762±15.998	191.768±38.97	0.17	0.87
S Body length (cm)	18.775±0.459	18.768±1.951	0.01	0.99
S Abdominal fat (g)	1.388±0.492	1.023±0.382	1.43	0.18
S Abdominal fat coefficient	0.700±0.208	0.520±0.138	1.77	0.11
Sln T (ng/ml)	1.303±0.854	0.651±0.503	1.61	0.14
Sln FT (pg/ml)	2.360±0.785	1.662±0.465	1.88	0.09
Sln DHT (pg/ml)	5.699±0.691	5.031±0.323	2.15	0.06
Sln DHEA (ng/ml)	0.158±0.762	-0.475±0.618	1.58	0.15
Sln SHBG (ng/ml)	6.592±0.250	6.679±0.578	-0.34	0.74
Sln Leptin (ng/ml)	-2.264±1.290	-2.979±0.264	1.33	0.21
Sln FAI	-0.716±0.820	-1.365±0.712	1.46	0.17

TABLE 3 Correlation between body shape indices, serum sex hormone levels and the time of onset of puberty in male offspring of OOG and TG.

Variables	Body weight	Body length	Abdominal fat	T	FT	DHT	DHEA	SHBG	FAI	Leptin
<b>OOG</b>										
Puberty onset time	0.821*	0.891*	0.844*	0.414	0.364	0.398	0.394	0.695	0.286	-0.299
Body weight	1	0.819*	0.985**	0.47	0.384	0.455	0.454	0.448	0.432	0.258
Body length		1	0.764	0.753	0.710	0.740	0.744	0.763	0.655	-0.148
Abdominal fat			1	0.353	0.264	0.338	0.333	0.389	0.308	0.204
T				1	0.987**	1.000**	0.998**	0.429	0.984**	0.077
FT					1	0.987**	0.981**	0.468	0.970**	0.067
DHT						1	0.998**	0.413	0.986**	0.078
DHEA							1	0.424	0.983**	0.066
SHBG								1	0.301	-0.347
FAI									1	0.230
<b>TG</b>										
Puberty onset time	-0.747	-0.718	-0.595	0.789	0.800	0.815*	0.812*	-0.887*	0.951**	0.029
Body weight	1	0.908*	0.830*	-0.277	-0.282	-0.252	-0.286	0.936**	-0.624	0.278
Body length		1	0.957**	-0.307	-0.305	-0.339	-0.262	0.790	-0.583	0.204
Abdominal fat			1	-0.162	-0.17	-0.203	-0.092	0.679	-0.427	0.007
T				1	0.998**	0.954**	0.981**	-0.467	0.919**	0.238
FT					1	0.953**	0.981**	-0.48	0.921**	0.278
DHT						1	0.955**	-0.465	0.877*	0.192
DHEA							1	-0.518	0.924**	0.155
SHBG								1	-0.776	0.188
FAI									1	0.067

TABLE 4 Comparison of the content of functional gene mRNA in male offspring between OOG and TG ( $\bar{x} \pm s$ ).

Indices	OOG ( <i>n</i> = 6)	TG ( <i>n</i> = 6)	<i>t</i>	<i>P</i>
<b>Hypothalamus</b>				
S AR	1.006±0.609	0.809±0.415	0.653	0.529
S ER- $\alpha$	1.015±0.997	0.485±0.436	1.195	0.272
S LeptinR	0.990±0.153	1.248±0.252	-2.149	0.057
S NPY	0.995±0.486	1.442±1.010	-0.978	0.351
S NPY2R	0.997±0.315	2.719±1.070	-3.779	<0.05
<b>Fat</b>				
S Leptin	0.997±0.498	0.671±0.372	1.283	0.228
S LeptinR	0.981±0.300	0.650±0.093	2.584	<0.05

TABLE 5 Correlation between hypothalamic functional gene mRNA levels and the time of onset of puberty in offspring of OOG and TG.

Variables	Hypothalamic AR	Hypothalamic ER $\alpha$	Hypothalamic LeptinR	Hypothalamic NPY	Hypothalamic NPY2R	Fat Leptin	Fat LeptinR
<b>OOG</b>							
Puberty onset time	-0.411	-0.546	0.42	-0.092	-0.555	-0.209	0.840*
Hypothalamic AR	1	0.960**	-0.446	-0.103	0.72	-0.013	-0.168
Hypothalamic ER $\alpha$		1	-0.365	0.137	0.609	0.085	-0.409
Hypothalamic LeptinR			1	0.568	-0.521	0.538	0.202
Hypothalamic NPY				1	-0.58	0.208	-0.505
Hypothalamic NPY2R					1	0.176	-0.065
Fat Leptin						1	-0.223
<b>TG</b>							
Puberty onset time	0.883*	-0.098	0.155	0.037	0.727	-0.256	0.377
Hypothalamic AR	1	-0.073	0.193	0.043	0.807	-0.278	0.044
Hypothalamic ER $\alpha$		1	0.544	0.911*	-0.419	0.745	-0.361
Hypothalamic LeptinR			1	0.838*	0.245	0.388	0.282
Hypothalamic NPY				1	-0.164	0.652	-0.06
Hypothalamic NPY2R					1	-0.285	0.441
Fat Leptin						1	-0.100



### 3.4 Effects of testosterone intervention during pregnancy on the expression of AR, ER $\alpha$ , NPY, NPY2R, and leptinR

The expression of AR, ER $\alpha$ , NPY, NPY2R, and leptinR in the ARC of the offspring male rats was observed by quantitative analysis of immunohistochemical images. The results showed that the protein expression levels of ER $\alpha$ , NPY2R, and leptinR in the TG were significantly increased compared with those in the OOG, while the expression levels of AR and NPY in the TG were significantly lower than those in the OOG ( $P < 0.05$ ; Table 6). Moreover, the results based on immunohistochemical sections further revealed that the expression of NPY2R and leptinR in the TG was higher than that in the OOG, while the expression of NPY in the TG was lower than that in the OOG. See Figure 2 for details.

## 4 Discussion

The early onset of puberty has become a trend. The role of the intrauterine environment during pregnancy in the onset of puberty in offspring has received increasing attention. In the current study, we found that testosterone intervention in pregnant rats leads to an early time of onset of puberty in male offspring rats, a positive association between the time of onset of puberty and AR mRNA, an increase in hypothalamic NPY2R and leptinR protein and a decrease in AR and NPY protein expression. This showed that male offspring rats after androgen intervention during pregnancy might be more sensitive to androgens, leptin, and NPY, thus leading to early puberty.

It has become a consensus that puberty onset is correlated with body shape indicators (37); that is, puberty begins when body weight reaches a certain threshold. In this study, we found positive associations between the time of onset of puberty and weight, body length, and abdominal fat in OOG, which was consistent with previous studies. However, there were positive correlations between the time of onset of puberty and serum DHT, DHEA levels, and FAI in TG, which showed that offspring male rats after androgen intervention during pregnancy might be more sensitive to androgens. At the same time, the protein expression of AR decreased in the TG, which also suggested that the time of onset of puberty of male offspring rats was more sensitive to androgen after testosterone intervention during pregnancy. It follows that the control of puberty onset of male offspring may no longer be the feedback of body shape changes but

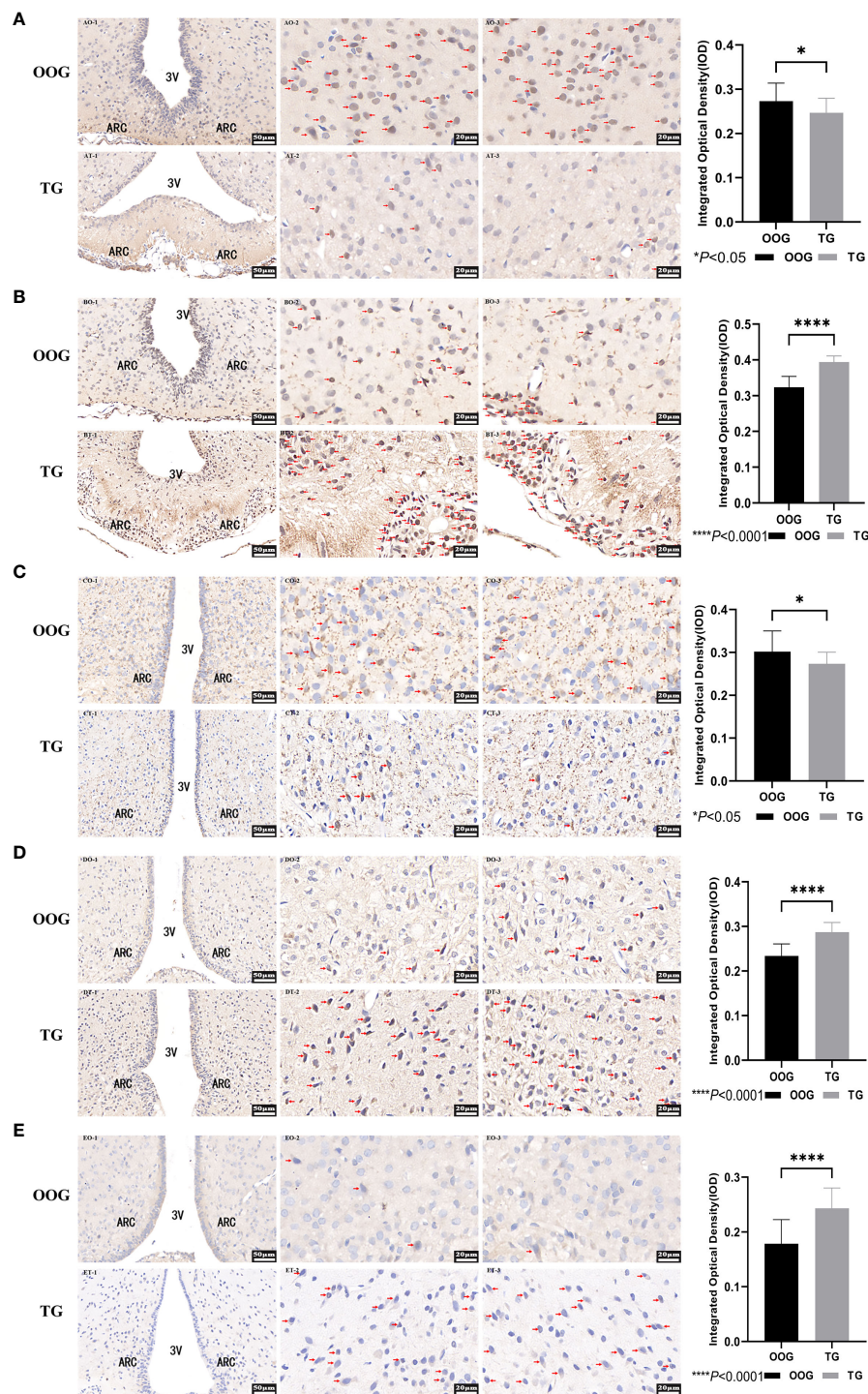
more through the feedback regulation of sex hormones in offspring male rats after androgen intervention during pregnancy (38).

It is well known that leptin sends a negative feedback signal to the brain center when the body has sufficient energy storage, thereby maintaining the body's energy balance by reducing food intake and increasing energy expenditure (39). Moreover, related studies have shown that androgen can inhibit the expression of leptin (40). Likewise, in a study on adipocytes of 3T3-L1 mice, it was found that dihydrotestosterone (DHT) significantly decreased leptin transcription and protein expression (41). In normal men, leptin levels may be limited by androgen, resulting in lower leptin levels than in women (42), and if serum leptin levels are above a certain threshold, leptin will in turn inhibit testicular function (43). Taken together, these signs suggest that androgen has an inhibitory effect on leptin. It was found that testosterone intervention reduced the protein expression of AR and upregulated the protein expression of leptinR at the onset of puberty in male offspring rats, which may be caused by the weakening of the inhibitory effect of low levels of androgens on leptin after testosterone intervention during pregnancy. In addition to its role in regulating energy balance, leptin also plays an important role in puberty onset by stimulating gonadotropin expression by upregulating the mRNA levels of GnRHR, activin, and FSHB (44). In this study, the expression of hypothalamic leptinR in the TG was higher than that in the OOG, indicating that the binding of hypothalamic leptin to leptinR may be enhanced after testosterone intervention, which affects the onset time of puberty.

Interestingly, related studies (45) have shown that there is no leptinR protein expression on GnRH neurons; therefore, by which mediator is leptin's regulation of puberty onset time achieved? NPY, a 36-amino acid orexin-producing protein, is a key feeding center in the hypothalamic arcuate nucleus (46) and binds to its receptors Y1 and Y2 to regulate reproduction and food intake, respectively (47), in which binding to NPY1R can directly inhibit the release of GnRH (28). Moreover, kisspeptin and POMC neurons are also regulated by leptin (48, 49). However, a relevant study showed that increased Kiss1 mRNA levels were detected only after the onset of puberty (50), and Kiss1 does not express substantial amounts of leptin receptors (51), indicating that kisspeptin neurons may play a role after puberty. Another study also noted that the leptin-POMC pathway has sex specificity in terms of reproductive control, that is, it is difficult to play a role in male mice (52). However, for NPY neurons, related studies (29, 53) have provided evidence that serum leptin and hypothalamic leptin receptors are involved in the regulation of GnRH release by NPY and that NPY levels are

TABLE 6 Comparisons of proteins levels between OOG and TG in the hypothalamus of male offspring ( $\bar{x} \pm s$ ).

Indices	OOG	TG	F	P
AR	0.273 $\pm$ 0.040	0.247 $\pm$ 0.032	4.538	<0.05
ER $\alpha$	0.323 $\pm$ 0.030	0.394 $\pm$ 0.017	76.953	<0.05
NPY	0.302 $\pm$ 0.048	0.273 $\pm$ 0.027	4.695	<0.05
NPY2R	0.234 $\pm$ 0.027	0.287 $\pm$ 0.022	43.167	<0.05
LeptinR	0.179 $\pm$ 0.044	0.243 $\pm$ 0.037	22.574	<0.05

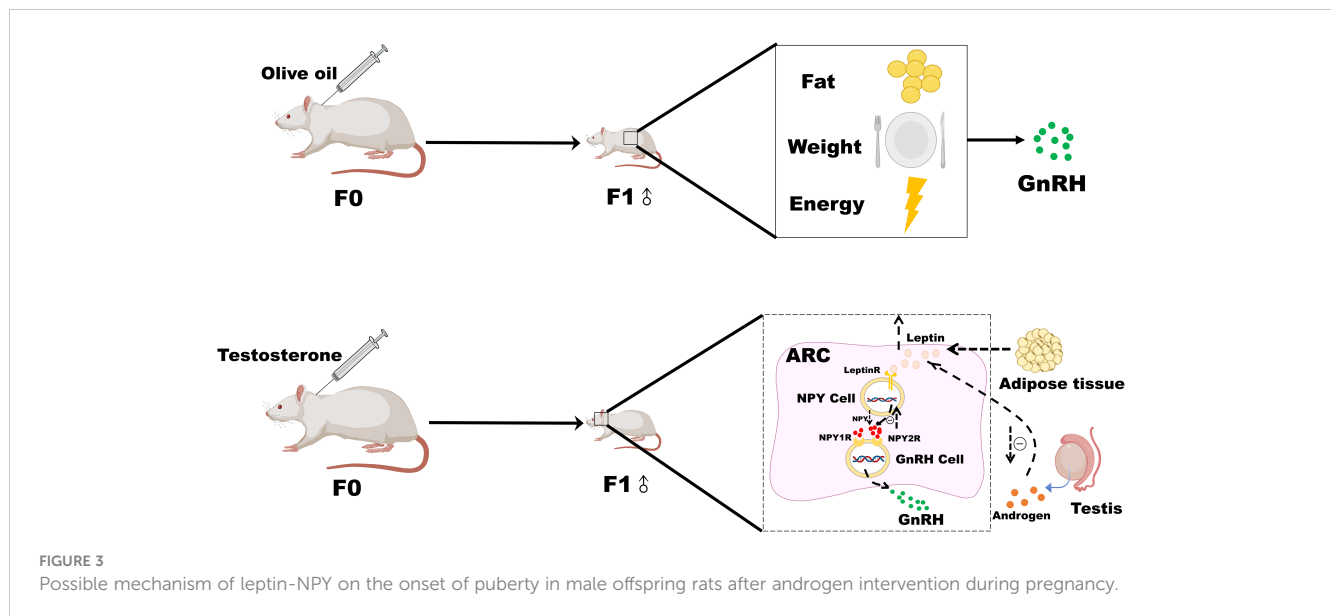


**FIGURE 2** Protein expression of AR, ERα, NPY, NPY2R, and LeptinR in the hypothalamus. (A), the immunohistochemical slice of AR; (B), the immunohistochemical slice of ERα; (C), the immunohistochemical slice of NPY; (D), the immunohistochemical slice of NPY2R; (E), the immunohistochemical slice of LeptinR. \*P< 0.05, \*\*\*\*P< 0.0001.

elevated in leptin-deficient (ob/ob) mice (54). As shown in the results of this study, at the onset of puberty, the protein expression of leptinR and NPY2R in the hypothalamus increased, whereas the expression of NPY protein decreased in the TG, which may be due to the increased sensitivity of offspring male rats to leptin, thus suggesting that the binding of leptin to the leptin receptor was

enhanced in the hypothalamus. The signal was transmitted to NPY neurons to inhibit NPY mRNA encoding the NPY protein, resulting in a decrease in NPY protein expression. Furthermore, in the environment of increased expression of NPY2R protein, the binding ability of NPY2R to NPY is enhanced, which mainly plays a role in energy expenditure and locomotion (55); thus, the





inhibition of GnRH may be weakened followed by GnRH increasing rapidly during puberty; therefore, puberty starts earlier.

## 5 Limitations

In this study, we detected only NPY2R, not NPY1R, so we cannot draw specific conclusions directly through the binding of NPY and NPY1R. Moreover, the intervention of testosterone was only in the third trimester of pregnancy, and it did not reflect the effect of testosterone on the puberty onset of offspring during pregnancy. At the same time, we sampled at the onset of puberty and reflected the differences between groups in the form of standardization, which may reduce the effectiveness of reflecting differences between groups. Finally, the association results were based on too few samples, which had limitations in the interpretation of associations.

## 6 Conclusion

Testosterone intervention in pregnant rats led to an earlier time of onset of puberty in male offspring rats. There were positive correlations between the time of onset of puberty and serum DHT, DHEA levels, and FAI, increased NPY2R mRNA, leptinR, and NPY2R protein expression, and decreased AR and NPY protein expression in male offspring rats after testosterone intervention during pregnancy. These results indicate that high androgen during pregnancy leads to early puberty onset in male offspring, which may be related to the decreased protein expression of AR, the increased expression of leptinR in the hypothalamus, and the enhanced inhibition of NPY neurons, as well as the enhanced binding of NPY2R and NPY. This could render male offspring after testosterone intervention during pregnancy more

sensitive to androgens, leptin, and NPY at the onset of puberty. Therefore, the inhibition of GnRH may be weakened, and puberty onset occurs earlier (Figure 3).

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animal study was reviewed and approved by Ethics Committee of Bengbu Medical College.

## Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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