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ORIGINAL ARTICLE

Kisspeptin permits the sexual development of female rats with normal and precocious puberty but is not a trigger for it

Wei Song [1], Kailin Li [2], Chao Sun [2], Jiang Xue [1]

1 Department of Pediatrics, The Second Hospital of Shandong University, Jinan, Shandong Province, P. R. China
2 Department of Central Laboratory, The Second Hospital of Shandong University, Jinan, Shandong Province, P. R. China

Correspondence to: Jiang Xue
Department of Pediatrics, The Second Hospital of Shandong University
#247, Beiyuan Street, Jinan, Shandong Province 250033, P. R. China.
E-MAIL: sdxj69@163.com

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Abstract

OBJECTIVE: We inferred how KISS-1/GPR54 system to involved in precocious puberty by observing hormones level during the process of precocious puberty in model and normal rats during sexual development and the estrus cycle.
METHOD: Female rats were divided randomly into CPP and control groups; the former were injected with NMDA twice daily, and control groups were injected with saline. Blood and tissue samples were collected and measured during the stages of prepuberty, vaginal opening, estrus, proestrus and diestrus.
RESULTS: The times of onset of puberty and sexual maturity in the CPP group were significantly earlier than in the control groups. Hypothalamic levels of KISS-1 and GPR54 gene expression, kisspeptin, luteinizing hormone, and follicle stimulating hormone started to rise before puberty. In stable estrus cycles, kisspeptin levels were the lowest during proestrus, while gonadotropin-releasing hormone (GnRH) levels rose to the highest during estrus. GnRH levels increased significantly in the estrus cycle compared with the prepubertal stage, but kisspeptin levels did not change significantly.
CONCLUSION: the hypothalamic KISS-1/GPR54 system might permit the onset of puberty, but is not its primary trigger. Hormone levels were lower and gonadal maturity markers in the CPP groups were worse than in the control groups.

Abbreviations:

ICPP	- idiopathic central precocious puberty
HPG	- hypothalamic–pituitary–gonadal
GnRH	- gonadotropin-releasing hormone
LH	- luteinizing hormone
NMDA	- N-methyl-D,L-aspartate
VO	- vaginal opening
PCOS	- polycystic ovary syndrome

INTRODUCTION

Idiopathic central precocious puberty (ICPP) is defined as the onset of isosexual rapid pubertal development resulting from premature activation of the hypothalamic-pituitary-gonadal (HPG) axis (Shankar & Pescovitz 1995). ICPP is also termed gonadotropin-releasing hormone (GnRH)-dependent precocious puberty. ICPP are analogous to the normal adolescent development besides ICPP happens earlier. Their processes are dominated by growth hormone and the HPG axes hormones. CPP causes early pubertal growth spurt, advancement in skeletal development whilst early closure of epiphysial plate thereby results in short final adult stature. It also leads to some psychological and social problems. It was found in our clinical treatment that some patients of premature thelarch developed to CPP, which needs diagnosis and treatment in the early stages. GnRH stimulation test that fasting, GnRH subcutaneous injection, before and after injection, 30, 60, 90 minutes, respectively, blood sample collection, measure serum FSH and LH level is accepted as gold standard test in the evaluation of HPG axis and diagnosis of CPP. Diagnosis using a GnRH stimulation test is too complicated and low reproducibility. It is time consuming, costly and uncomfortable for patients. GnRH analogues do not completely inhibit the HPG axis, meaning that the final height is not improved in some patients. A simple and sensitive diagnostic method is needed that can lead to an effective treatment for inhibiting the onset of puberty. GnRH imbalance as a cause of ICPP is a focus of current research in the field (Shin 2016). Mutations in the *KISS-1/GPR54* genes causing precocious puberty have attracted attention to kisspeptin as a key candidate neuroendocrine peptide in controlling the HPG axis (Luan *et al.* 2007; Teles *et al.* 2008). Kisspeptin is now recognized as an indispensable factor in the neuroendocrine control of puberty and reproduction (Pinilla *et al.* 2012; Clarkson *et al.* 2010).

In 1996, Lee found that the *KISS-1* gene (located in the human chromosome 1q32-41 region) could inhibit tumour metastasis, It codes for neuroendocrine peptides, which are eventually degraded into the carboxyl-terminal ends of short peptides, members of the arginine-phenylalanine amide peptide family (Lee *et al.* 1996). *KISS-1* gene expression is highest in the hypothalamic arcuate nucleus and anteroventral periventricular nucleus in rats. The protein GPR54 is the kisspeptin receptor, which is a member of the rhodopsin 7 transmembrane protein family. Its gene is located at human chromosome 19q13(Ohtaki *et al.* 2001).

Currently, how kisspeptin is involved in precocious puberty has not been fully elucidated. One study found that kisspeptin levels were higher in cases of precocious puberty (Abaci *et al.* 2015), while another found no significant changes (Navarro *et al.* 2004b). Some reasch found that GnRH and LH were increased and the degree of increase was positively proportional to the dosage of kisspeptin after giving the mice kisspeptin (Seminara *et al.* 2003). Further research is needed to determine the links between hypothalamic *KISS-1* gene expression and serum kisspeptin levels, and the changes in serum kisspeptin levels in normal and pathological sexual development and estrus cycles. Therefore, the aim of this study was to determine how kisspeptin might govern reproduction via pulse and surge modes of GnRH/luteinizing hormone (LH) secretion, and participate in the process of GnRH dependent precocious puberty.

MATERIALS AND METHODS

Animals

Forty-five 25-day-old specific pathogen-free Sprague Dawley female rats weighing 50±3 g were purchased from Peng Yue Breeding Laboratory Animals Ltd. (Jinan, Shandong, P. R. China, license number 370181589911068). Animal care was conducted in accordance with the Animal Research Committee Guidelines of Shandong University. All the experimental procedures were approved by the Institutional Ethics Committee of Second Hospital of Shandong University. The rats were maintained in a 12-h light, 12-h dark cycle and fed a standard rodent diet. The temperature was 20–26 °C. Water and food were available *ad libitum*. Rats were randomly housed in nine groups of five per cage. Five cages of rats were used as the control groups and four were used as CPP groups (model group). The CPP groups received sub-cutaneous (s.c.) injections of 40 mg/kg N-methyl-D,L-aspartate (NMDA, purchased from Solarbio, Shanghai, P. R. China) dissolved in 0.2 ml saline, twice a day, until their vaginas were open. NMDA is a glutamate agonist that stimulates GnRH release (Mahachoklertwattana *et al.* 1994). The control groups received s.c. injections of 0.2 mL sterile 0.9% NaCl, twice a day.

Experimental design: Experiment 1, observing the estrus cycle

The estrus cycle was observed and determined by taking vaginal smears daily after vaginal opening (VO). The control groups were divided into five groups based on vaginal smear results: prepubertal group (before puberty); VO group; proestrus group; estrus group; and diestrus group with the lowest LH level. The CPP groups were divided into four groups based on vaginal smears: VO CPP group in rats with precocious puberty; proestrus/estrus CPP group; estrus CPP group and diestrus CPP group. When any rats in VO CPP Group showed VO, the same numbers of animals in prepubertal group were weighed and euthanized by spinal dislocation on the same day. After two consecutive regular estrus cycles, the remaining groups in their corresponding periods (VO, estrus, proestrus and diestrus) were weighed and euthanized; blood and tissue samples were collected and analysed. The CPP groups was compared

with the corresponding normal development of the rats, The CPP group of VO was compared with control group of prepubertal, it also was compared with control group of VO.

Experiment 2: measurement of blood samples

Blood samples were collected from the angular vein by removing the animal's eyeball before it was decapitated. The sera were separated from the fresh blood by a pipette after natural coagulation. The sera were stored at –80 °C until measurement.

Experiment 3: measurement of tissue samples

After the rats had been decapitated, the ovaries were taken immediately, weighed and embedded in paraffin wax for histology. The tissues were sectioned at 2 mm thickness, and sections were stained with haematoxylin and eosin. Endometrial thickness was measured microscopically using an eyepiece micrometer. The total numbers of *corpora lutea* were counted in ovary sections microscopically. The organ index, defined as wet organ weight/body weight, was used to measure the stage of development of uteri and ovaries.

Experiment 4: KISS-1 and GPR54 gene expression in the hypothalamus

The mRNA levels of *KISS-1* and GPR54 were evaluated in hypothalamic samples. The rats were euthanized and decapitated at the stages of prepuberty, VO, estrus, diestrus, and proestrus and their hypothalami were dissected immediately, as described (Quennell *et al.* 2011). The hypothalamic samples were snap-frozen in liquid nitrogen and stored at –80 °C until RNA analysis.

Measurement of serum LH, kisspeptin and GnRH levels

The serum LH, kisspeptin and GnRH concentrations were measured using commercial rat enzyme-linked immunosorbent assay kits (CUSABIO**,** Wuhan, Hubei, P. R. China) after all samples had been collected and stored, according to the manufacturer's recommendations. The sensitivity of the LH assay was 0.001 mIU/ml; that of the GnRH assay was 0.001 pg/ml; and that of the kisspeptin assay was 0.001 ng/ml. The intra-and inter-assay coefficients of variation were <5%.

RNA analysis by real-time reverse-transcription polymerase chain reaction (RT-qPCR)

RT-qPCR was conducted using a slight modification of a previously described method (Li *et al.* 2010). Total RNA was isolated from hypothalamic samples using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and 1 mg RNA samples from each rat were reverse-transcribed into cDNA. The primers used for the assessment of hypothalamic genes were as follows: *KISS-1* Forward: 5'-TGATCTCGCTGGCTTCTTGG-3' and Reverse: 5'-AGGCATTAACGAGTTCCTGGG-3' *GPR54* Forward: 5'-TTCGTTATCTGCCGCCACAA-3' and Reverse: 5'-AGACCTGCTGGATGTAGTTGAC-3'

The *KISS-1* product size is 138 bp and the GPR54 product size is 181 bp. The nucleotide sequences for these genes were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and based on National Center for Biotechnology Information rat reference sequences. The primers were synthesized commercially by Invitrogen Life Technologies. The final reaction mixture contained 1 ml cDNA, 10 pmol each forward and reverse primers, 0.4 ml ROX reference dye, and 10 ml SYBR Premix Ex Taq DNA polymerase in a total reaction volume of 20 ml. The PCR conditions were 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Quantitative analysis of gene expression levels was carried out using a StepOnePlus Real-Time PCR System and StepOne Software v. 2.1 according to the recommendations of the manufacturer (Applied Biosystems, Foster City, USA).

Statistics

All data are expressed as the mean ± standard error of the mean for each group. One way analysis of variance was used to assess variations among groups. Pearson correlation analysis was used to analyse any correlations. The significance level was set at $p<0.05$. All analyses were performed with IBM SPSS Statistics software (v. 23, IBM Corp., Armonk, NY, USA).

RESULTS

Timing of onset of puberty and sexual maturity

NMDA significantly advanced the time of VO in the CPP groups to 35±1 d compared with control groups (41±0.2 d) ($p=0.03$). The vaginas of all groups had opened after 42 days of age. The estrus cycle was determined using daily vaginal smears (Figure 1). The CPP groups had established two regular estrus cycles before 42 days of age, but at this point the control groups had not yet established regular cycles.

Comparisons of estrus cycle serum levels, genital organ weights and gene expression levels

The uterine index differed significantly among different estrus cycle phases in the CPP and control groups. In control rats, it was 2.25 times higher than for the CPP rats at proestrus (Figure 2A).

Endometrial thickness differed significantly among different estrus cycle periods in both the CPP and control groups. Endometrial thickness in control rats was significantly higher than that for the CPP rats in estrus and proestrus (Figure 2B).

The VO of CPP groups showed abundant growing follicles (Figure 3A and C), as well as fresh *corpora lutea* (Figure 3B, C). The numbers of *corpora lutea* differed significantly among different estrus cycle phases in both the CPP and control groups. The number of *corpora lutea* in control rats was 13 times more than in the CPP rats at estrus, and 60% of CPP rats at proestrus (Figure 2C).

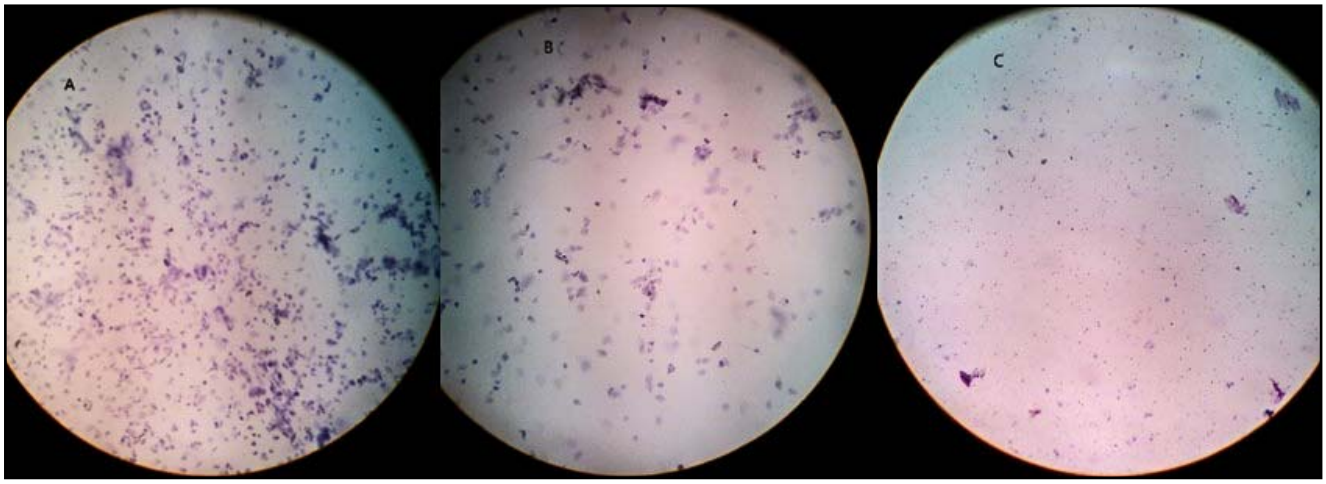

Fig. 1. Vaginal smear histology. In proestrus, vaginal smears show oval-shaped flat epithelial cells. (B) Estrus vaginal smears show clusters of cornified epithelial cells. (C) Vaginal smears in diestrus show many leukocytes.

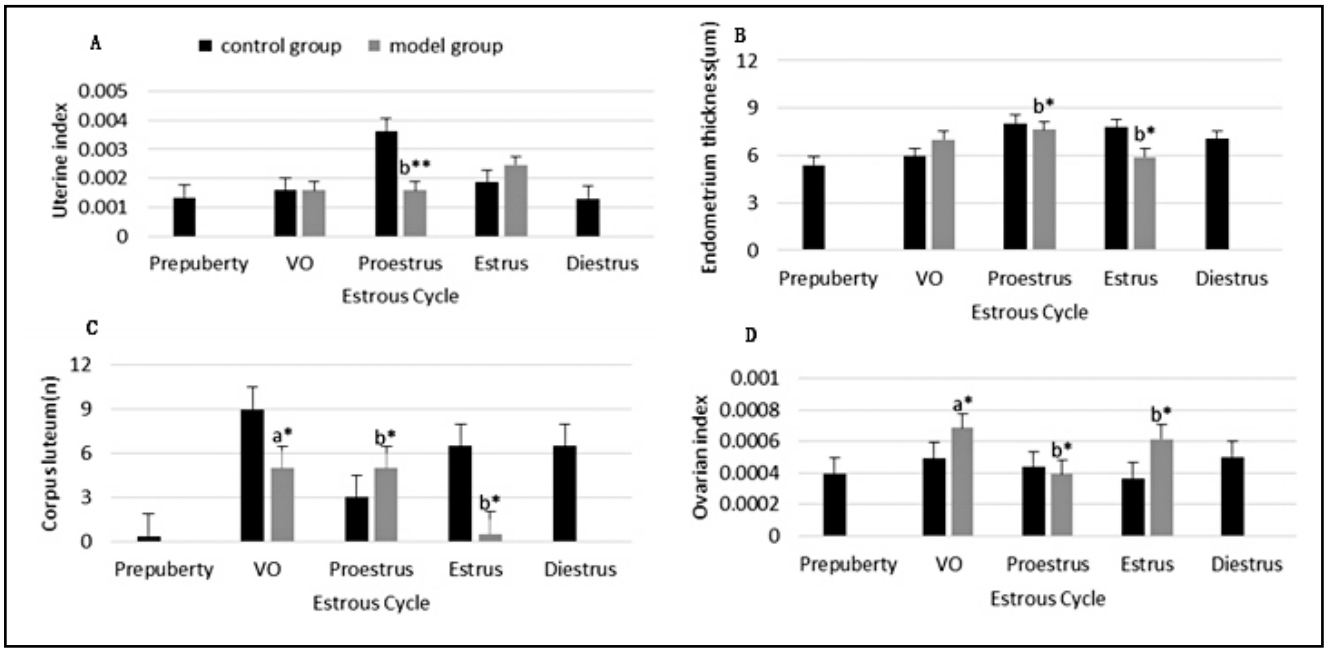

Fig. 2. Somatic measures in female rats. Uterine index (A), endometrial thickness (B), *corpora lutea* counts (C), ovarian index (D) in control and CPP rats at prepuberty, VO stage, proestrus, estrus, and diestrus. Values are expressed as the mean ± SEM a* $p<0.05$ vs prepubertal control values. b*$p<0.05$ and b**$p<0.01$ vs corresponding control values.

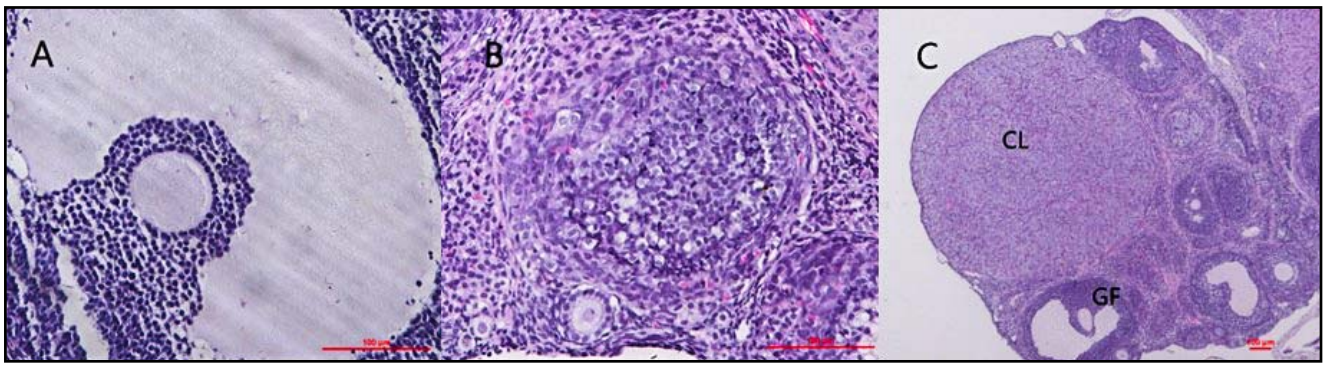

Figure 3. Ovarian ultrastructure in CPP rats with precocious puberty. This shows series of representative ovarian sections stained with haematoxylin and eosin. Control rats showed normal cycling ovaries with growing follicles (GF) (A and C), and a corpus luteum (CL) (B and C). Scale bars=100 mm.

The control rats took longer to reach given estrus stages than the CPP rats, but were compared at the same stages. The ovarian index differed significantly among different estrus cycle phases in both the CPP and control groups. In control rats, it was significantly higher than in the CPP rats during proestrus, and lower than in the CPP rats during estrus (Figure 2D).

The serum kisspeptin levels differed significantly among different estrus cycle period in the control groups, but did not differ significantly in the CPP groups. The serum kisspeptin levels in control rats were 13% of those for the CPP rats at proestrus (Figure 4A).

The serum LH levels in control rats were 2.33 times as much as in the CPP rats at proestrus. The LH levels did not differ significantly among different estrus cycle period in either the CPP or control groups (Figure 4B).

The serum GnRH levels in control rats were 1.5 times higher than for the CPP rats at proestrus. However, the GnRH levels did not differ significantly among different estrus cycle periods in the CPP or control groups (Figure 4C).

The mRNA expression levels of *KISS-1* differed significantly among different estrus cycle phases in the control groups, but did not differ significantly in the CPP groups. The *KISS-1*mRNA values in control rats were twice as much as in the CPP rats at proestrus (Figure 4D).

The mRNA expression levels of *GPR54* did not differ significantly among different estrus cycle periods in either the CPP or control groups, and did not differ significantly between the CPP and control groups at similar estrus cycle stages (Figure 4E).

Comparison of CPP and control groups on the same day

The numbers of *corpora lutea* were markedly elevated in VO CPP group (VO stage of the precocious puberty group) compared with prepubertal group. The ovarian index was higher in prepubertal group compared with VO CPP group. No significant differences were observed in the uterine indices, endometrial thickness, or the levels of LH, kisspeptin, GnRH, *KISS-1*mRNA, or GPR54 mRNA between prepubertal group and VO CPP group. (Fig. 2C, D).

Correlation studies

The levels of *KISS-1* mRNA and kisspeptin peaked at diestrus, but the changes in *KISS-1* mRNA levels were not synchronous with those of kisspeptin during sexual development and the estrus cycle stages. The concentration of GnRH peaked during estrus. The numbers of *corpora lutea* and weight of ovaries were positively correlated (Pearson's r=0.673; p=0.012), and the uterine index and endometrial thickness were also positively correlated (Pearson's r=0.577; p=0.039). The uterine index and kisspeptin levels showed a negative correlation (Pearson's r=–0.736; p=0.004), and the endometrial thickness and *KISS-1* mRNA levels showed a positive correlation (Pearson's r=0.673; p=0.017). Finally, the ovarian index was negatively correlated with LH level (Pearson's r=–0.649; p=0.016).

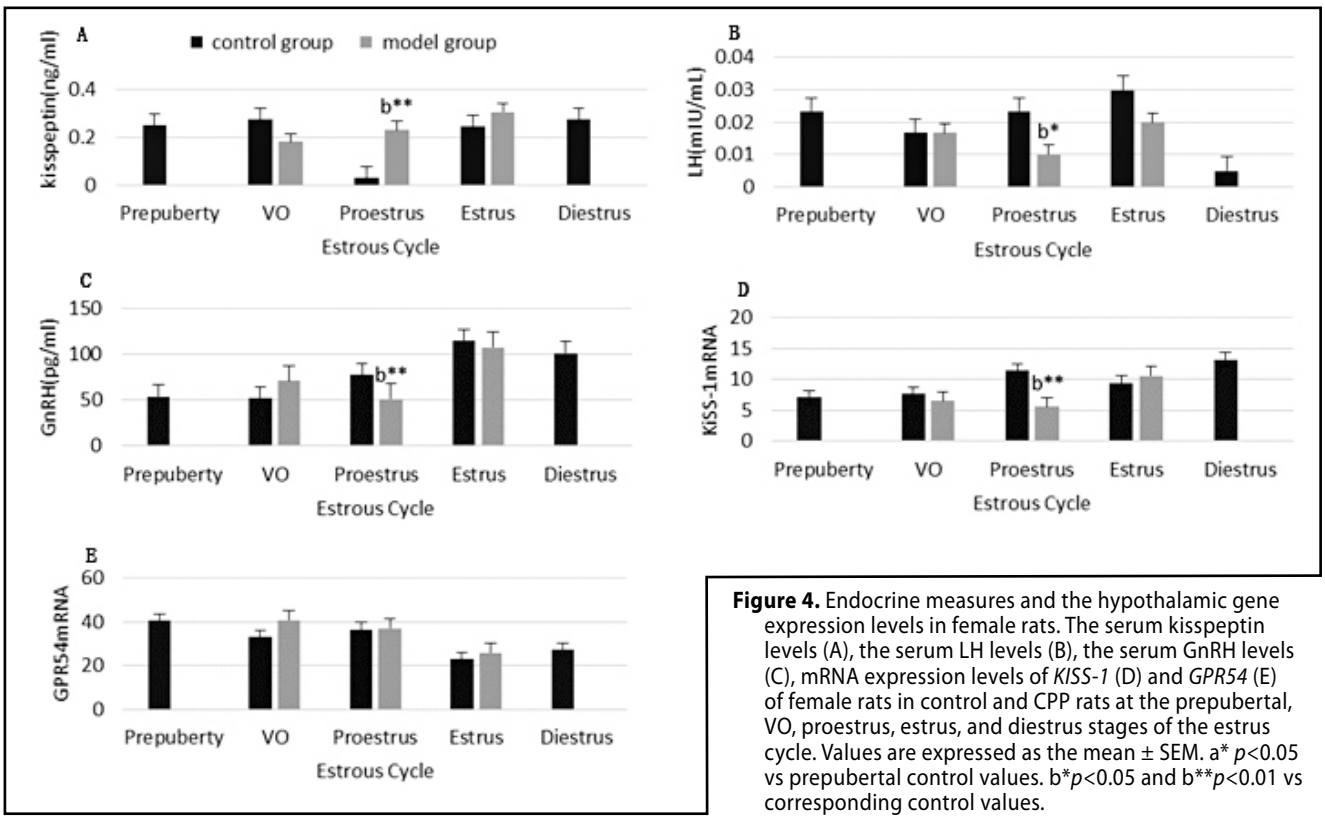

Figure 4. Endocrine measures and the hypothalamic gene expression levels in female rats. The serum kisspeptin levels (A), the serum LH levels (B), the serum GnRH levels (C), mRNA expression levels of *KISS-1* (D) and *GPR54* (E) of female rats in control and CPP rats at the prepubertal, VO, proestrus, estrus, and diestrus stages of the estrus cycle. Values are expressed as the mean ± SEM. a* p<0.05 vs prepubertal control values. b*p<0.05 and b**p<0.01 vs corresponding control values.

DISCUSSION

In this study, we investigated how kisspeptin levels might control precocious puberty and the onset of estrus cycle phases in rats. Puberty is regulated by the HPG axis, and if one part of the axis is aberrant, it can cause abnormal puberty. GnRH is the initiating and core factor in the HPG axis, which is influenced by many neurotransmitters and neuropeptides. Luan et al. found mutations in the *KISS-1/GPR54* genes causing precocious puberty (Teles *et al.* 2008; Luan *et al.* 2007). other previous research suggested that the *KISS-1/GPR54* signalling pathways of GnRH-regulating neurons play important roles at the onset of puberty (Muir *et al.* 2001). VO is a sign of the initiation of puberty in rats. and two regular estrus cycles can be considered as indicating sexual maturity. NMDA stimulates GnRH release, thus leading to precocious puberty. Here, the CPP groups were injected with NMDA twice a day until puberty. The uterine index, endometrial thickness, ovarian size, and numbers of *corpora lutea* were all increased significantly in rats showing precocious puberty compared with prepubertal rats at the same age. The LH levels peaked during proestrus in the control groups, and were minimum at diestrus, which met with the needs of our design. Therefore, the classification of estrus cycles using vaginal smears are appropriate. NMDA not only advanced VO, but also accelerated the development of reproductive organs and tissues. This model of precocious puberty has been validated (Shehata 1974) .

Counts of *corpora lutea*, the uterine and ovarian indices and endometrial thickness were minimum before puberty in the control groups. The uterine and ovarian indices were significantly increased after establishing stable estrus cycles. Changes in the uterine index and increasing endometrial thickness were synchronized with proestrus, estrus, and diestrus, as in previous research.

The uterine development of rats with precocious puberty was worse than in the control groups. The uterine index in these rats was also lower than the control group during proestrus. After sexual maturation and regular cycling, endometrial thickness was less than in the control group at various stages, and the uteri of control groups developed better. The uterine index continued to increase after puberty in the CPP rats, even though uteri did not reach a mature weight. From these observations, we speculate that this might be the reason why precocious puberty in humans is associated with reduced fecundity and increased rates of miscarriage. The ovarian index in the CPP groups was higher than in the control groups during estrus, This could infer that women with precocious puberty are more vulnerable to polycystic ovary syndrome (PCOS), as described previously (Lehman *et al.* 2010). The mechanism needs to be addressed in future experiments.

GnRH and kisspeptin are secreted in a pulsatile manner (Choe *et al.* 2013),and in primates, repetitive administration of kisspeptin increased the plasma LH levels before puberty (Plant *et al.* 2006) . We observed that the kisspeptin level of CPP group was markedly higher than control group at the proestrus stage, this may be caused by the low levels of GnRH and LH in proestrus. Thereby, we assume that the frequency and amplitude of GnRH pulses influence LH secretion, thus influencing ovarian function, oestrogen secretion, ovulation, endometrial thickness. In the previous study, an increased frequency of GnRH pulses is implicated in the pathogenesis of PCOS, and higher kisspeptin levels could be associated with 1 phenotype of PCOS (Osuka *et al.* 2017; Thompson & Kaiser 2014). After removal of NMDA , the estrus cycle of CPP group existed and orderly, there should be no impact on decreasing of GnRH secretion and regression of reproductive organs. Smyth showed that in NMDA-induced rats the LH response to NMDA is less than in the control group (Smyth & Wilkinson 1994). It may be that CPP groups developed not very well (van Gool *et al.* 2007). Thus, the estrus cycle might be mainly regulated by GnRH, and kisspeptin might only permit this regulation or act upstream of the HPG axis.

It has been reported that *KISS-1* and *GPR54* gene knockout mice failed to go through puberty at the correct time; however, some studies showed evidence of gonadotrophic activity after several months (Colledge 2009; Chan *et al.* 2009). It's probably work in hypothalamus, not necessarily in blood. This hypothesis needs to be proved in further studies. Hypothalamic GPR54 gene expression had already increased before puberty in rats with normal sexual development. An increasing trend of hypothalamic *GPR54* expression was observed during diestrus, proestrus, estrus, but this did not reach statistical significance in the control groups. The expression was relatively stable. GPR54 might not play a leading role in the *KISS-1/GPR54* system. The hypothalamic *KISS-1* expression had increased before puberty. The changes from high to low were linked with diestrus, proestrus, and estrus, as in previous research (Navarro *et al.* 2004a). Therefore, we assume that the expression levels of *GPR54* and *KISS-1* have already been determined before or at birth. However, male and female rats might be different in this regard, and this needs to be assessed. The kisspeptin levels were not consistent with *KISS-1* gene expression levels, so we speculate that kisspeptin might be stored after being synthesized, until secretion or it's probably only work in hypothalamus, not necessarily in blood. This hypothesis needs to be proved in further studies.

In conclusion, gonadal development and hormone levels of the rats with precocious puberty were worse than in normal control rats. Precocious puberty might make women more vulnerable to PCOS and lead to subfertility. Hypothalamic *KISS-1*/*GPR54* gene expression levels in rats with precocious puberty increased before

puberty. These might permit the onset of puberty but not be its specific trigger.

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Conflict of Interest: *All authors declare that there is no conflict of interest.*

Compliance with Ethical Standards: *All applicable international, national, and institutional guidelines for the care and use of animals were followed. This article does not contain any studies on human participants performed by any of the authors.*

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