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Original Article

Lateral ventricle injection of orexin-A ameliorates central precocious puberty in rat via inhibiting the expression of MEG3

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Abstract: Background: Central precocious puberty (CPP) is characterized as increasing gonadotropin-releasing hormone (GnRH) release. Orexin-A has also been shown to affect GnRH release. However, there are few reports about the effect of orexin A on the treatment of CPP. Methods: After establishing the precocious puberty model, the rats were divided into four groups: normal control, precocious puberty rats, precocious puberty rats treated with normal saline and precocious puberty rats treated with orexin-A. The vaginal opening time, second estrus cycle, ovarian index and uterus index of rats in each group were detected. qRT-PCR was performed to examine the expression of MEG3 and kisspeptin in rats. HT22 cells were transfected with pcDNA-MEG3 to detect the expression of Kisspeptin. Results: In this study, we found that orexin-A not only delayed the day of vaginal opening and regular estrus cycle days but also decreased the ovarian index and uterus index in rats with CPP. In addition, orexin-A reversed the up-regulation of MEG3 and kisspeptin in rats with CPP. In HT22 cells, the mRNA and protein level of kisspeptin were enhanced by pcDNA-MEG3. Conclusion: Our results suggest that orexin-A ameliorates central precocious puberty in rat and MEG3 might be involved in this effect, suggesting that MEG3 might be a novel target in treating central precocious puberty.

Keywords: Orexin-A, central precocious puberty, MEG3, kisspeptin, hypothalamic-pituitary-gonadal (HPG) axis

Introduction

Central precocious puberty (CPP) is defined as premature activation of the hypothalamic-pituitary-gonadal (HPG) axis, resulting in early development of secondary sexual characteristics [1]. Although the precise mechanisms triggering the onset of puberty are unclear, the earliest known biochemical change during puberty is increased production of kisspeptin in the hypothalamus. It has been shown that increased kisspeptin production results in increased gonadotropin-releasing hormone (GnRH) release, which activates gonadal axis function in the hypothalamus, then initiates gonad development and secretion of sex hormones and causes genital development [2]. Thus, a rise in kisspeptin is widely acknowledged as the seminal event that initiates HPG axis activation during puberty.

CPP has traditionally been treated with monthly injections of Gonadotropin-releasing hormone analogs (GnRHa) [3]. Orexin-A is a peptide synthesized mainly by neurons with perikarya located within and around the lateral and posterior hypothalamus, found to modulate the activity of GnRH neurons and gonadotropin-secreting pituitary cells [4]. Orexin-A has also been shown to affect GnRH release. Orexin A is involved in the regulation of sleep/wakefulness, energy homeostasis and locomotor activity [5]. It also appears to have a significant impact on the regulation of tropic hormones secretion on the level of the hypothalamus and pituitary [6]. Orexin A acts through two different G-protein coupled receptors, OX1R and OX2R. Dual-immunofluorescence labeling in rats revealed that approximately 80% of GnRH neurons express OX1R [7]. However, there are few reports about the effect of orexin A on the treatment of CPP.

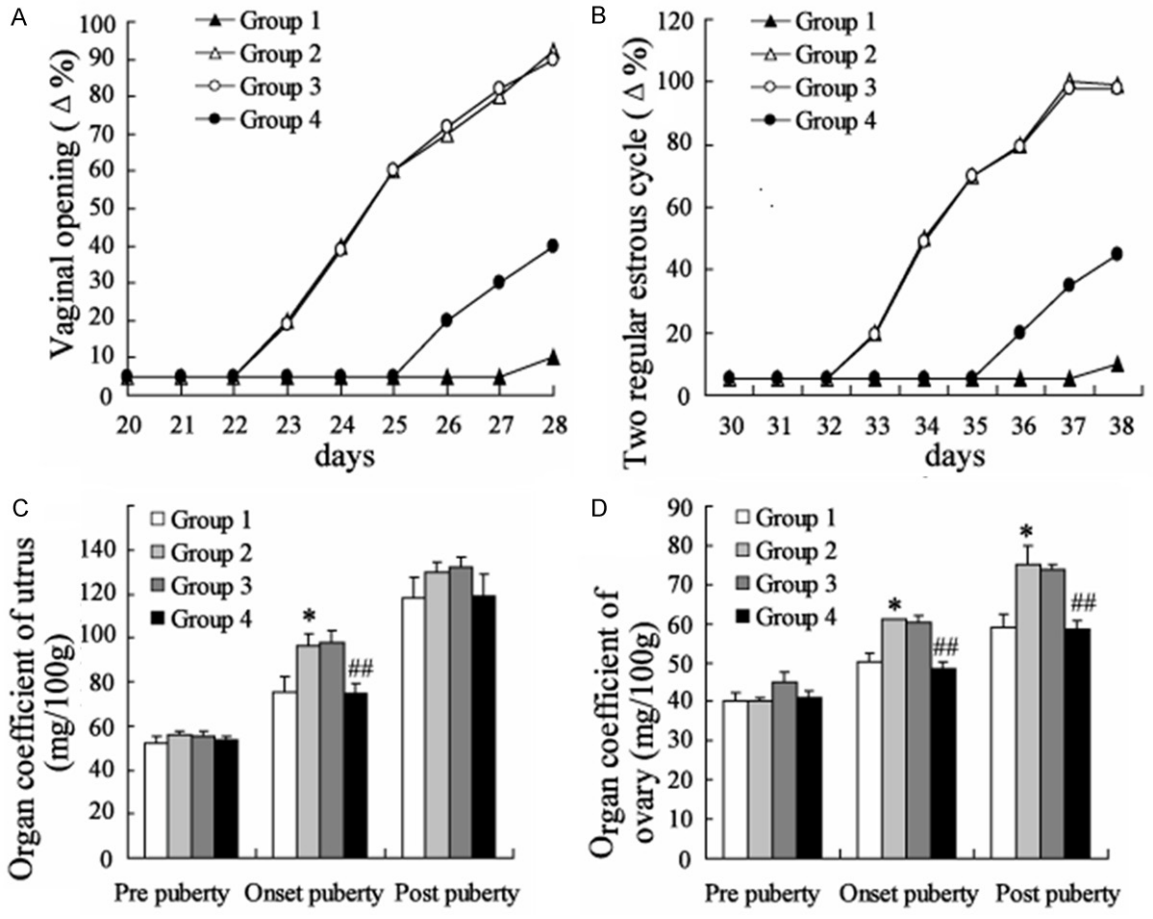

Figure 1. Orexin-A delayed the symptom of central precocious puberty. Female rats were treated with 300 μg of danazol through subcutaneous injection at 5 days after birth to establish the model of central precocious puberty. Group 1: control group; Group 2: rats with central precocious puberty; Group 3: rats with central precocious puberty and treated with normal saline; Group 4: rats with central precocious puberty and treated with 0.5 nmol/L of rexin-A for 1 time/day. A. The days of vaginal opening in study rats. B. The days of two regular estrous cycle in study rats. C. The organ coefficient of uterus in study rats at different stage of puberty. D. The organ coefficient of ovary in study rats at different stage of puberty. All values are mean ± SD. *VS Group 1, P<0.05; ##VS Group 3, P<0.05.

Recently, long non-coding RNAs (lncRNAs) were widely identified as novel regulators in normal and disease development [8]. Maternally Expressed Gene 3 (MEG3) is an imprinted lncRNA and highly expressed in the pituitary [9]. In addition, MEG3 mRNA is also detected in the brain, placenta, adrenal gland, pancreas, and ovary, suggesting its neuroendocrine-related functions [10]. Moreover, MEG3 mRNA was detected in several cell types of the normal pituitary, and loss of MEG3 expression was restricted only to clinically nonfunctioning tumors [11]. It has suggested that MEG3 is the only human gene specifically associated with gonadotroph derived clinically nonfunctioning pituitary tumors [12].

In this study, we aimed to investigate the effect of orexin-A on central precocious puberty in rat. We also examined the expression of MEG3 and detected the potential relationship between MEG3 expression and kisspeptin in hypothalamus of central precocious puberty rat treated with orexin-A.

Materials and methods

Precocious puberty rat model and animal groups

This study was approved by ethical committee for animal experiments of Ethics committee of Northern Jiangsu People's Hospital. Five-days-old specific pathogen free grade female SD

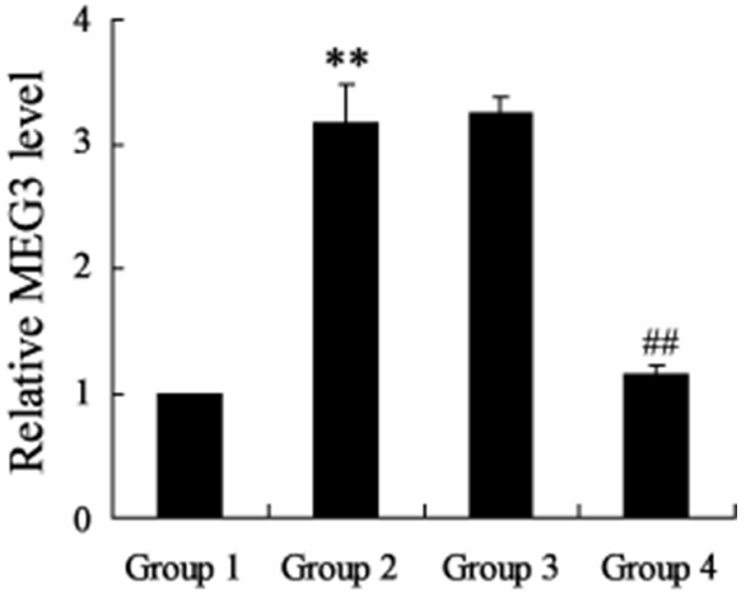

Figure 2. The expression of MEG3 in hypothalamus of study rats. All values are mean ± SD. **VS Group 1, P<0.05; ##VS Group 3, P<0.05.

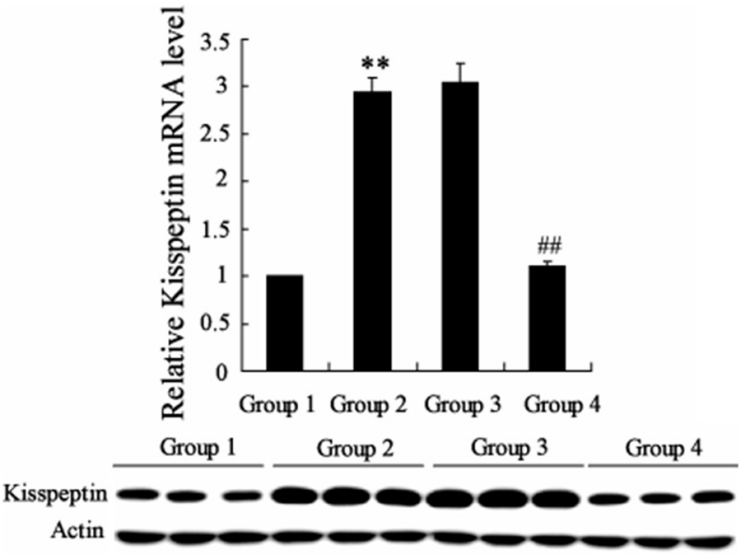

Figure 3. The expression of Kisspeptin in hypothalamus of study rats. All values are mean ± SD. **VS Group 1, P<0.05; ##VS Group 3, P<0.05.

rats, weighing (25±5) g, were purchased from Jun Nanjing Better Biotechnology Co. Ltd. After two days of adaptive feeding, rats were randomly divided into four groups: Group 1 (normal control, n=12), Group 2 (precocious puberty rats, n=12), Group 3 (precocious puberty rats treated with normal saline, n=12), Group 4 (precocious puberty rats treated with orexin-A, n=12). Each rat in experimental groups 2, 3 and 4 were subcutaneously injected with 300 μg of danazol to establish the precocious puberty model. Each rat in groups 4 were injected with orexin-A (0.5 nmol/L) for 1 time/day via lateral ventricle at 15-days-old. Each rat in groups 3 were injected with equal volumes of normal saline. When rats were 20-days-old, vaginal openings were checked and vaginal opening time (VO) was recorded. These were recorded on the 20th day if the vaginal was open at the first inspection.

For detecting the uterus index and ovary index (organ wet weight/body mass), rat in each group were sub-divided into pre puberty, onset puberty and post puberty according to the sacrificed time. Pre puberty rats were sacrificed after the first estrus cycle of vaginal opening. Then, rats in onset puberty were sacrificed at the second estrus cycle and rats in post puberty were sacrificed at the second estrus cycle after puberty. Uterus and ovary specimens were taken from all rats; and uterus index and ovary index were calculated. Hypothalamic tissues were carefully cut, removed and stored in liquid nitrogen.

Western blot analysis

Tissue samples were grinded from liquid nitrogen and lysed using RIPA. A Bio-Rad protein assay kit was used to determine the protein concentration. The total cellular and tissue protein extracts were separated on 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked and incubated with primary antibodies for Kisspeptin and Actin overnight at 4°C. Actin was used as a control. The membranes were incubated with the appropriate

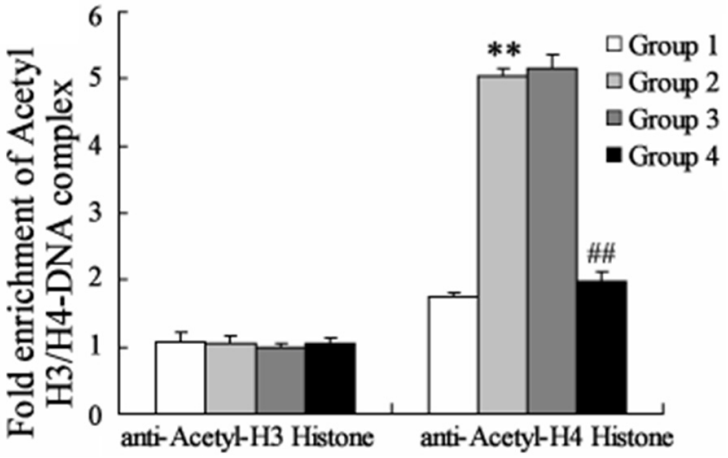

Figure 4. The binding of acetyl-H3 histone (or acetyl-H4 histone) to the promoter of Kisspeptin in study rats. All values are mean ± SD. **VS Group 1, P<0.05; ##VS Group 3, P<0.05.

horseradish peroxidase-conjugated monoclonal secondary antibody (Santa Cruz) at room temperature for 1.5 h. An ECL detection kit (ThermoIBiotech, Boston, MA, USA) was used to visualize the immunoreactive protein bands. Densitometry (Quantity One Software; Bio-Rad, Hercules, CA, USA) analysis was used to quantify the relative levels of protein expression. The experiments were repeated three times.

Quantitative real-time PCR

Total RNA was isolated from tissues using the RNeasy kit according to the manufacturer's instructions. Reverse transcription reactions were carried out with 1 μg total RNA using the PrimeScript RT reagent kit (TaKaRa BIO, Dalian, China). Real-time PCR was performed on a Bio-Rad CFX-96 real-time PCR system using SYBR Premix DimerEraser kit (TaKaRa, Dalian, China) following the manufacturer's instructions. GAPDH was used as a housekeeper gene for the qRT-PCR reactions. Each test was done in triple replication and the 2-ΔCt method was used to calculate the expression of MEG3 and Kisspeptin in tissue samples.

Chromatin immunoprecipitation assay

ChIP assays were performed using a ChIP assay kit to assess the histone acetylation status of the Kisspeptin promoter region according to the manufacturer's instructions. The precleared chromatin was incubated with anti-acetyl-H3, anti-acetyl-H4 (Upstate) or normal mouse/rabbit IgG (Southern Biotechnology Associates, Inc) as negative control. A small amount of input DNA (~2.5%) was used as positive control. Each ChIP assay was performed at least twice to ensure reproducibility.

Cell culture and transfection

HT22 cells were cultured in DMEM (Hyclone, USA) with 10% fetal bovine serum (Hyclone, USA) and were incubated in a humidified incubator with 5% CO_2 at 37°C, and were seeded on 60-mm culture dishes at 100,000 cells per dish. Cell density was maintained 80% or less confluency to attenuate excessive growth. pcDNA-MEG3 was employed to overexpress MEG3 in HT22 cells. Transfection of pcDNA-MEG3 or pcDNA was conducted using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction.

Statistical analysis

SPSS 11.5 statistical software was applied to the experimental data, and results were expressed as mean ± SD. Multiple groups of data were analyzed using ANOVA (one-way ANOVA) and t-test was used to compare two groups. *P*<0.05 was considered as statistically significant difference.

Results

Orexin-A delays the symptom of central precocious puberty

After establishing the precocious puberty model and dividing the rats into four groups: Group 1 (normal control, n=12), Group 2 (precocious puberty rats, n=12), Group 3 (precocious puberty rats treated with normal saline, n=12), Group 4 (precocious puberty rats treated with orexin-A, n=12), we monitored the day of vaginal opening to determine the maturation of the external genitalia and detected two regular estrus cycle days of study rats. Comparing to the female rats of the normal control group, the time of vaginal opening was found to be highly decreased in rats of Group 2 and Group 3. While, orexin-A delayed the day of vaginal opening in rats of Group 4 comparing to that in

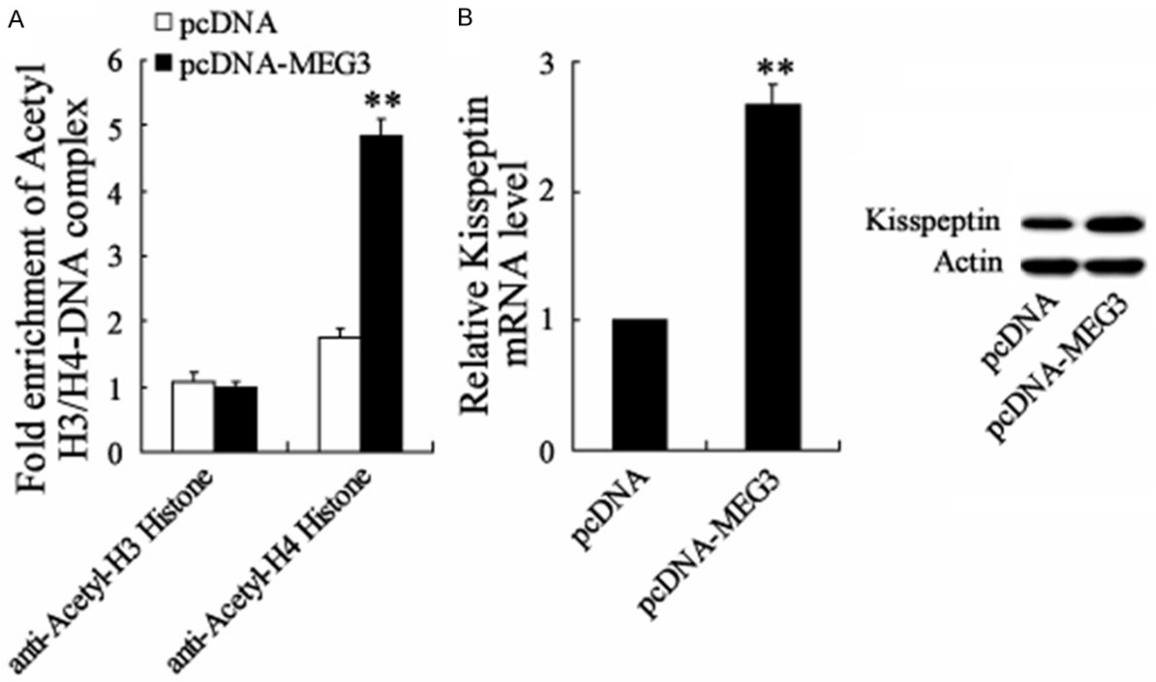

Figure 5. The effect of MEG3 overexpression on the expression of Kisspeptin in HT22 cells. A. The binding of acetyl-H3 histone (or acetyl-H4 histone) to the promoter of Kisspeptin in HT22 cells overexpressed MEG3. B. The expression of Kisspeptin in HT22 cells overexpressed MEG3. All values are mean ± SD. **VS control, P<0.05.

Group 3 (**Figure 1A**). In the respect of regular estrus cycle days, it was also postponed in precocious puberty rats injected with orexin-A (**Figure 1B**).

Then each of the groups was sub-divided into pre puberty, onset puberty and post puberty according to the sacrificed time. Uterus and ovary specimens were taken from all rats and uterus index and ovary index (organ wet weight/body mass) were calculated. As shown in **Figure 1C** and **1D**, at the stage of puberty onset, uterus index and ovary index were all increased in rats of Group 2 and 3 than that in Group 1, while orexin-A reversed this increase in precocious puberty rats. In addition, ovary index of Group 4 also decreased comparing to that in Group 3 at post puberty. These findings indicated that injection of orexin-A could ameliorate central precocious puberty in rats.

Orexin-A inhibits the expression of MEG3 in hypothalamus of rats with central precocious puberty

To elucidate the mechanism of orexin-A ameliorating the central precocious puberty, the expression of MEG3 in hypothalamus of rats were examined by real-time RCR. It has been shown that the mRNA level of MEG3 was significantly enhanced in precocious puberty rats comparing to that in normal controls. Moreover, lower mRNA level of MEG3 was observed in precocious puberty rats injected with orexin-A than that in precocious puberty rats injected with normal saline (**Figure 2**).

Expression of Kisspeptin in hypothalamus of rats with central precocious puberty

As Kisspeptin was showed to involve in pubertal development of the HPG axis, we further detected the expression of Kisspeptin in hypothalamus of rats, and the results indicated that the mRNA and protein level of Kisspeptin in hypothalamus of central precocious puberty rats were higher than that in normal controls. In addition, orexin-A reversed this dysregulation of Kisspeptin in hypothalamus of central precocious puberty rats (**Figure 3**).

Orexin-A reduces the binding of acetyl-H4 histone to the promoter of Kisspeptin in rats with central precocious puberty

We investigated the binding of acetyl-H3 histone (or acetyl-H4 histone) to the promoter of

Kisspeptin in study rats with ChIP assay. As shown in **Figure 4**, there is no change in the binding of acetyl-H3 histone to the promoter of Kisspeptin in these four groups. However, the binding of acetyl-H4 histone to Kisspeptin was highly enhanced in precocious puberty rats comparing to normal controls. Orexin-A also reversed this binding and decreased the binding of acetyl-H4 histone to Kisspeptin in precocious puberty rats (**Figure 4**).

Effect of MEG3 overexpression on the expression of Kisspeptin in HT22 cells

To investigate the role of MEG3 in regulating the expression of Kisspeptin, HT22 cells were transfected with pcDNA-MEG3 or pcDNA to overexpress MEG3. Through detecting the binding of acetyl-histone to the promoter of Kisspeptin, we found that overexpressed MEG3 enhanced the binding of acetyl-H4 histone to the promoter of Kisspeptin (**Figure 5A**). In addition, the mRNA and protein level of Kisspeptin were enhanced in HT22 cells transfected with pcDNA-MEG3 (**Figure 5B**).

Discussion

Central precocious puberty (CPP), also called GnRH-dependent precocious puberty, is based on hypothalamic-pituitary-gonadal axis activation associated with progressive pubertal development, accelerated growth rate and advancement of skeletal age. The hypothalamic-pituitary-gonadal axis regulates puberty initiation and reproduction. Kisspeptins play a central role in the modulation of GnRH secretion with peripheral factors that influence the timing of puberty, such as adipokines and endocrine disrupting chemicals [13]. CPP can compromise final adult height, cause incongruity between psychological and physical development, and may also trigger psychological problems arising from early menarche [14].

Orexin A has been proposed to regulate the HPG axis and sexual behaviors. It also have a stimulatory effect on release of GnRH from rat hypothalamic explants in vitro [15]. Despite this finding, the mechanism of action of orexin A on central precocious puberty was unclear. In this study, we have confirmed that orexin-A delayed the day of vaginal opening and decreased the uterus index and ovary index in rats with central precocious puberty, which indicated that orexin-A might relieve the symptom of central precocious puberty in rats. To verify this findings, we further detected the expression of Kisspeptins and found that orexin-A also reversed the expression of Kisspeptins in central precocious puberty rats.

Kisspeptin is neuropeptides encoded by the KiSS-1 gene and is a powerful stimulus for GnRH-induced gonadotropin secretion [16]. Intermittent kisspeptin administration to immature animals was shown to induce precocious activation of the gonadotropic axis and pubertal development [17]. Young-Jun Rhie et al. demonstrated that serum kisspeptin levels were significantly higher in girls with central precocious puberty than in their age matched pre-pubertal controls [18]. In addition, Miguel A. Sanchez-Garrido et al. reviewed that kisspeptins were the most recently identified players in the central control of puberty onset by metabolic cues [19]. Several studies have demonstrated that pharmacological blockade of Gpr54 which was the putative receptor of kisspeptins was sufficient to delay puberty onset in female rats [20]. In this study, the result of orexin-A decreasing the expression of Kisspeptins in central precocious puberty rats further verified the protective effect of orexin-A on central precocious puberty.

As recent discovery of lncRNAs being critical regulators in normal and disease development provides new clues for delineating the molecular regulation in disease development, we examined the expression of MEG3 in central precocious puberty rats. We found that orexin-A also reversed the expression of MEG3 in central precocious puberty rats. In addition, the mRNA and protein level of Kisspeptin were enhanced in HT22 cells which overexpressed MEG3. In several cancer cell lines, MEG3 expression is lost and it acts as a tumor suppressor, such as those derived from brain, bladder, breast, cervix, colon, liver and prostate [9, 10, 21]. Xun Zhang et al. reported that pituitary-derived MEG3 functioned as a growth suppressor in human pituitary adenomas [10]. MEG3 is supposed to be related to the pathogenesis of human clinically non-functioning tumors of a gonadotroph lineage.

In summary, our results suggest orexin A has important interactions in the central precocious puberty in rats. These in vitro studies

show that orexin-A ameliorates central precocious puberty in rat and MEG3 might be involved in this effect, suggesting that MEG3 might be a novel target in treat central precocious puberty. Future work will examine the effect of MEG3 on GnRH neurons in situ to prove this hypothesis.

Disclosure of conflict of interest

None.

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