

Effects of Nourishing “Yin”-Removing “Fire” Chinese Herb Mixture on the Differential Expression of GABA_A Receptor α Subunits in Hypothalamus of Precocious Puberty Female Rats*

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ABSTRACT

GABAergic input to Gonadotropin-releasing hormone (GnRH) neurons is necessary to initiate the onset of puberty and its action mainly depends on GABA_A receptor of which the subunit composition, properties and consequently function varies during this period. Nourishing “Yin”-Removing “Fire” Chinese herb mixture, a Chinese herb-based formulation, has been proved that it may retard the initiation of pubertal development in female precocious puberty rats. Our objective is to investigate the effects of Nourishing “Yin”-Removing “Fire” Chinese herb mixture on the expression of GABA_A receptor α subunits in hypothalamus. Female Sprague-Dawley rats were divided into normal (N), precocious puberty model (M) induced by danazol, model exposed to saline (MS) and model exposed to Chinese herb mixture (CHM) groups. All rats were administered by the Chinese herb mixture from P15 on. Coefficients of reproductive organs and serum gonadotropins and estradiol levels in M were significantly enhanced while they were significantly decreased in CHM. The hypothalamic GnRH mRNA was also significantly increased in M and in CHM, as well as ER α mRNA. At the mean time, the hypothalamic GABA_A receptor $\alpha 1$ and $\alpha 3$ subunits mRNA were more significantly decreased in M than those of N, while they were more significantly enhanced in CHM than those in M ($p < 0.01$), the protein expression of which in hypothalamus had the same trend as the mRNA expression. The evidence suggests that Nourishing “Yin”-Removing “Fire” Chinese herb mixture could significantly retard the sexual development of the precocious rats, and up-regulate the expressions of hypothalamic GABA_A receptor $\alpha 1$ and $\alpha 3$ subunits. Our result indicated that GABA_A receptor $\alpha 1$ and $\alpha 3$ subunits might involve in the effective treatment of herb mixture on idiopathic precocious puberty.

Keywords: GABA; GABA_A Receptor α Subunit; Precocious Puberty; Nourishing “Yin”-Removing “Fire” Chinese Herb Mixture; Rat

1. Introduction

Precocious puberty is defined as the onset of puberty before age of 8 years in girls and 9 years in boys. Precocious puberty in general is more frequent in girls, of which the majority have idiopathic precocious puberty whose etiology is unknown [1]. It has been well established that pulsatile GnRH release played a crucial role in triggering the onset of puberty [2]. GABA is one of

the primary inhibitory neurotransmitters in the regulatory network of GnRH and contributes a great deal modulating pubertal progress via its receptors in the hypothalamus [3,4]. GABA receptors are widely distributed throughout the central nervous system where they predominantly mediate inhibitory neurotransmission. Specific to the hypothalamus, studies supported that GABA, particularly through its action on GABA_A receptor, regulated firing of pubertal GnRH neurons [5].

GABA_A receptor includes six α , three β , three γ , one δ , one ϵ , one π , one θ and three ρ subunits, of which each

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composition form involves at least one α subunit [6]. It is revealed that the pattern of each GABA_A receptor subunit is complex and differs from phase to phase and region to region in mammal species, which ensures the functional heterogeneity of GABA input throughout the life span [7]. There was a normal switch from some GABA_A receptor α subunits highly expressed during development ($\alpha 2$, $\alpha 3$, $\alpha 4$) to those subunits highly expressed in adulthood ($\alpha 1$) in rats model [8]. And compared with adult subunits mRNA expression in GnRH neurons of female mice, juvenile GnRH neurons expressed a much more heterogeneous population of GABA_A receptors that $\alpha 5$, $\beta 1$, and $\gamma 2$ were the most frequently co-expressed except $\alpha 4$ and $\gamma 1$ [9]. In addition, it has been reported that $\alpha 2$ subunit expression is very high before birth to shortly after birth, and decreases gradually to adult levels, whereas $\alpha 1$ expression is minimal before birth and increases after birth until adulthood [10,11]. Examination of GABA_A receptor subunit expression in GnRH neurons of hypothalamus supports this trend of age-associated going on during pubertal development [5].

Nourishing "Yin"-Removing "Fire" Chinese herb mixture has been successfully used for the management of idiopathic precocious puberty for a long time. It is reported that it could significantly alleviate the symptoms on precocious patients whose serum gonadotropins and estrogen reduce and secondary sexual characteristics subside. And a series of experiments on female precocious puberty rats model supported that Chinese herb mixture could regulate the function of hypothalamus-pituitary-gonadal axis (HPGA) through modulating the gene and protein expressions of GnRH, kisspeptin, GABA and glutamate [12,13]. However, the mechanism of GABA receptor subunits participates in the Chinese herb mixture curative effects of precocious puberty is merely mentioned. We hypothesized that GABA_A receptor subunit composition might involve the onset of precocious puberty. The present work was to observe the effects of Nourishing "Yin"-Removing "Fire" Chinese herb mixture on the expression of GABA_A receptor α subunit mRNA in Danazol induced female precocious puberty rats and to explore the possibility of GABA_A receptor participating in advance GnRH release in precocious puberty.

2. Methods

2.1. Animal

Female Sprague-Dawley rats at 3 days of age were purchased from Medical Experimental Animals Center of Chinese Academy of Sciences (Shanghai, China). Animals were housed under laminar flow in an isolated room with controlled temperature of about 22°C under a 12-h light/dark cycle with lights on from 7:00 am to 7:00 pm. The model litters at P5 were given a single subcutaneous

injection of 300 μ g of danazol (Hualian Pharm Ltd., Shanghai, China) dissolved in 25 μ l of propylene glycol-ethanol (1:1, v/v), and allowed to grow without further treatment, the vehicles were administered with the same volume of propylene glycol-ethanol (1:1, v/v). Animals were weaned on P21, and then vaginal opening was examined daily afterwards, and the daily vaginal smears were examined. All experiments procedures involving the use of animals were conducted in accordance with NIH Guidelines and were reviewed and approved by Animal Use and Care Committee for the Fudan University.

All rats were randomly divided into normal (N), precocious puberty model (M), model exposed to saline (MS) and model exposed to Nourishing "Yin"-Removing "Fire" Chinese herb mixture (CHM) four groups. From P15, rats in CHM and MS were fed with CHM (including crude drug 3.3 g per ml) or the same volume normal saline every day. The intragastric dose increased by about 0.1 ml per day as the rat grew.

At the day of vaginal opening in M, rats of all four groups were decapitated with blood and hypothalamus tissue collected, the uterus and ovaries were dissected out of the surrounding fat, and the organ coefficients (mg/100g) were evaluated.

2.2. Hormone Measurement by RIA

At the time of decapitate, the blood of all rats was collected. The serum was separated by centrifugation and stored at -80°C until assayed. The gonadotropins and oestradiol levels were measured by double-antibody RIA kits purchased from the Beijing Sinouk Institute of Biological Technology (Beijing, China). The samples were assayed in duplicate, and all the subjects' samples were assayed together. The sensitivity for the E2 was less than 5 pg/ml; the intra- and inter-assay coefficients were less than 10% and 15.2%, respectively. For LH, the assay sensitivity was 0.2mIU/ml, and the intra- and inter-assay coefficient of variation was 2.0% - 2.4% and 4.2% - 7.5%, respectively. For FSH, the assay sensitivity was 0.25 mIU/ml, and the intra- and inter-assay coefficient of variation was 2.2% - 2.5% and 3.7% - 8.7%, respectively.

2.3. Tissue Collection and Total RNA Preparation

The rat brains in every group (n = 5) were rapidly removed and hypothalamus were separated immediately and frozen in liquid nitrogen. The target regions, including mediobasal hypothalamus and the suprachiasmatic-preoptic areas were dissected. Total hypothalamic RNA was extracted by "Trizol Regent" (Invitrogen Inc., America) according to the manufacturer's instructions. The

concentration of RNA was estimated by spectrophotometry with UV absorbance at 260 nm and 280 nm. The purity and integrity of the RNA were checked spectroscopically and by gel electrophoresis before carrying out the analytical procedures.

2.4. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The total RNA from each tissue was reverse transcribed using M-MuLV reverse transcriptase (Superscript II, Promega) and 2.5 mM oligo dT (BioDev-Tech) in the presence of 250 mM of deoxynucleotides triphosphate in a final volume of 12.5 ml at 37°C for 1 h. The reaction mixture was denatured for 5 min at 95°C and stored at -70°C. The transcribed DNA for each subunit was amplified by PCR in a thermal cycler using specific primers for each subunit. The primers were designed in order to amplify DNA sequences (ER α , sense: 5'-AATTCTGACAATCGACGCCAG-3'; antisense: 5'-GTGCTTCAACATTCTCCCTCCTC-3'. β -actin, sense: 5'-AAGCAGGAGTATGACGAGTCCG-3', Antisense: 5'-GCCTTCATACATCTCAGGTTGG-3'). The amplification mixture contained 1.5 U of Taq PCR Master Mix (Shanghai Lifefeng Biotechnology) and 20 μ M of specific primer pairs in a final volume of 20 ml. The mixture was amplified for 30 cycles. Each amplification cycle consisted of 5 min of denaturation at 95°C to activate the enzyme and 1 min denaturation at 95°C then 1 min at 58°C for annealing and 1 min at 72°C. The final extension step was 15 min at 72°C. The relative abundance of each product was estimated by the visual intensity of ethidium bromide stained bands under UV light.

2.5. Real-Time Reverse Transcriptase-PCR (qRT-PCR)

Prior to conducting real-time reverse transcriptase-PCR (qRT-PCR), the total RNA was digested with RNase-free DNase I (Invitrogen, Carlsbad, CA), by which possible contamination of genomic DNA. The SuperScript III reverse transcription system (Invitrogen Corp., Carlsbad, CA, USA) was used for reverse transcription with 2 μ g of total RNA according to the manufacturer's specifications. The primers used for GABA $_A$ receptor α subunits and GnRH were designed and synthesized by Invitrogen with standard purity. To determine the sensitivity and efficiency of the amplification, PCR assay linearity ranges were previously established for each gene cDNA. Quantitative Real-Time PCR was carried out in IQ5 Real-time PCR system (Bio-Rad). The amplification protocol was as follows: an initial denaturing step at 95°C for 2 min followed by 40 cycles of a 95°C for 10 sec, 60°C for 30 sec, and 72°C for 30 sec. Following amplification, a dissociation curve analysis was performed to insure purity of PCR products. All real-time experiments

were run in triplicate and a mean value was used for the determination of mRNA levels. The relative linear quantity of the target gene was calculated using the formula $2^{-\Delta\Delta Ct}$. Therefore, the data were expressed as n-fold change in gene expression normalized to a reference gene (β -actin) and relative to a calibrator sample. The primer sequences and realtime PCR conditions for GABA $_A$ receptor α subunits and GnRH mRNA were listed in Table 1.

2.6. Western Blot

Each of six rats in four groups was used to investigate GABA $_A$ receptor $\alpha 1$ and $\alpha 3$ subunits protein expression by western blot with a standard procedure. For total protein extraction, hypothalamus was homogenized in lysate (BioDev-Tech. Co., Ltd) and protease inhibitors using a polytron homogenizer. After homogenization, samples were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant fraction was the total tissue lysate. Protein concentration in the lysate was determined by a total protein measurement kit from BioDev-Tech. Protein samples were denatured in loading buffer and separated on a sodium dodecyl sulfate-polyacrylamide gel after loading equal amount of protein in each lane. Separated proteins were transferred to PVDF membrane at 100 V for 110 mins in 25 mM Tris-glycine buffer, pH 8.3, 10% methanol using a Transblot apparatus (Bio-Rad Laboratories, Inc.). Following the transfer, the membranes were rinsed five times with TTBS (20 mM Tris, 0.1% Tween-20) for 10 min each rinse and then incubated with 5% BSA for 4 h at 4°C to block non specific/unbound surface. The membrane was incubated overnight with polyclonal rabbit anti-rat (1:200; Millipore). The membrane was then washed with TTBS to remove unbound antibody, followed by incubation with secondary HRP-conjugated sheep anti-rabbit IgG (1:2000, Millipore) for 1 h at room temperature. The signal was detected using an ECL detection kit (GE Healthcare) and the membranes were exposed to ImageQuant LAS 4000 mini (GE Healthcare).

2.7. Statistics Analysis

All data are presented as means \pm SEM. Statistical analysis was performed on raw data using one-way analysis of variance (ANOVA), with the significance concentrations of $P < 0.05$ in two-tailed testing chosen. Comparisons among groups were made using the Student's t-test.

3. Results

3.1. Effects of Nourishing "Yin"-Removing "Fire" Chinese Herb Mixture on the Timing of Vaginal Opening

On P22 at the beginning of observation, one third of the

Table 1. Primer sequences of GABA_A receptor α 1-6 subunits and GnRH in realtime PCR.

β -actin	Forward: 5'-GACCTCTATGCCAACACAGTGC-3' Reverse: 5'-CTGCTGGAAGGTGGACAGTGA-3'
GABA _A R α 1	Forward: 5'-AGGGTAAGGTGAGGCTGTCATTGT-3' Reverse: 5'-TCTGTGTTAGGCTGGCTCATCTCC-3'
GABA _A R α 2	Forward: 5'-GAGGATGGGCTTGGGATGGAAAG-3' Reverse: 5'-GCTGGCTTGTCTCTGGCTTCTT-3'
GABA _A R α 3	Forward: 5'-TCCTGCTGAGACCAAGACCTACAA-3' Reverse: 5'-GGTTGCTGTGCTGCCACTATTATCT-3'
GABA _A R α 4	Forward: 5'-AGATGTCAACAGCAGAACTGAGGTG-3' Reverse: 5'-GCGATGCGGCAGACGAAAGA-3'
GABA _A R α 5	Forward: 5'-GGGACTGGGAATGCTGTGGGTA-3' Reverse: 5'-CGATCTTGCTGATGCTGTGTAGGT-3'
GABA _A R α 6	Forward: 5'-GCACTCTGACTCCAAGTACCATCTG-3' Reverse: 5'-GGGCGTTCTACTGAGGACTTGC-3'
GnRH	Forward: 5'-GCCGCTGTGTTCTGTTGACTGT-3' Reverse: 5'-ATCCTCCTCCTTGCCCATCTCTTG-3'

rats in M and MS had showed vaginal opening, while few in N and CHM did. The discrepancy in the proportion of vaginal opening rats in groups sustained from then on till P44 when all rats had steady-state sexual cycles (**Figure 1**.)

3.2. Effects of Nourishing “Yin”-Removing “Fire” Chinese Herb Mixture on Serum E2, LH and FSH Levels

The concentration of serum E2, LH and FSH in M were significantly higher than those of N ($p < 0.01$). And the serum E2 and LH levels were significantly lower in CHM than those in M ($p < 0.01$, respectively), and FSH level were also lower in CHM than that in M ($p < 0.05$). There were not statistically significant differences observed between the M and MS (**Figure 2**.).

3.3. Effects of Nourishing “Yin”-Removing “Fire” Chinese Herb Mixture on Reproductive Organs’ Coefficients

Table 2 shows changes in reproductive organs’ coefficients of the different rat groups. On P22 the uterus coefficient of M were 80.18 ± 2.19 mg/100g which was significant increased than those of N (59.78 ± 1.30 mg/100g), and the uterus coefficient of CHM were significantly decreased to 58.62 ± 1.72 mg/100g ($p < 0.01$, respectively). Meanwhile the ovaries coefficient of M were 5.87 ± 0.38 mg/100g which were also increased significantly compared to those of N, and the coefficients of CHM were decreased to 4.36 ± 0.22 mg/100g ($p < 0.05$, respectively). These reproductive organ coefficients on P26 were similar to those at P22 ($p < 0.05$, respectively).

3.4. Effects of Nourishing “Yin”-Removing “Fire” Chinese Herb Mixture on Hypothalamic Expression of GnRH mRNA by Realtime PCR

Effects of Nourishing “Yin”-Removing “Fire” Chinese herb mixture on the expression of GnRH mRNA were detected by realtime PCR. Relative mRNA levels for GnRH were determined by the $2^{-\Delta\Delta Ct}$ method and normalized to the β -actin mRNA. Briefly, the mean ΔCt of the N was used as an internal calibrator when comparing the mRNA quantities of GnRH in other groups. Hypothalamic GnRH mRNA expression of M was significantly elevated compared with that of N ($p < 0.01$). Hypothalamic GnRH mRNA expression was down-regulated in CHM compared to M ($p < 0.01$). And there were no significant differences between M and MS groups (**Figure 3**).

3.5. Effects of Nourishing “Yin”-Removing “Fire” Chinese Herb Mixture on the Expression of GABA_A Receptor α 1-6 Subunits by Realtime PCR

Effects of Nourishing “Yin”-Removing “Fire” Chinese herb mixture on expression of GABA_A receptor α 1-6 subunits mRNA were detected by realtime PCR. Relative mRNA expression levels for GABA_A receptor α 1-6 subunits were determined by the $2^{-\Delta\Delta Ct}$ method and normalized to the β -actin mRNA. Briefly, the mean ΔCt of the N was used as an internal calibrator when comparing the mRNA quantities of GABA_A receptor α 1-6 subunits in other three groups... RT-PCR analysis showed that of all six α subunits of GABA_A receptor only α 1 and α 3 mRNA

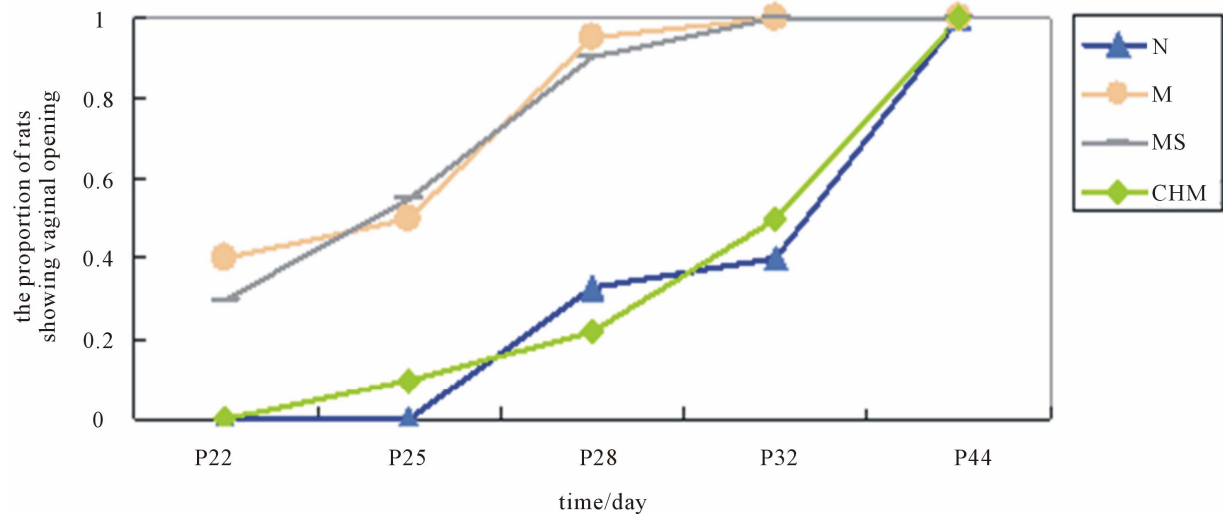


Figure 1. Effects of Nourishing “Yin”-Removing “Fire” Chinese herb mixture on the timing of vaginal opening. There were less rats in N and CHM showing vaginal opening than in M and MS at the same time since P22. The discrepancy in the proportion of vaginal opening rats in groups sustained from then on till P44 when all rats had steady-state sexual cycles.

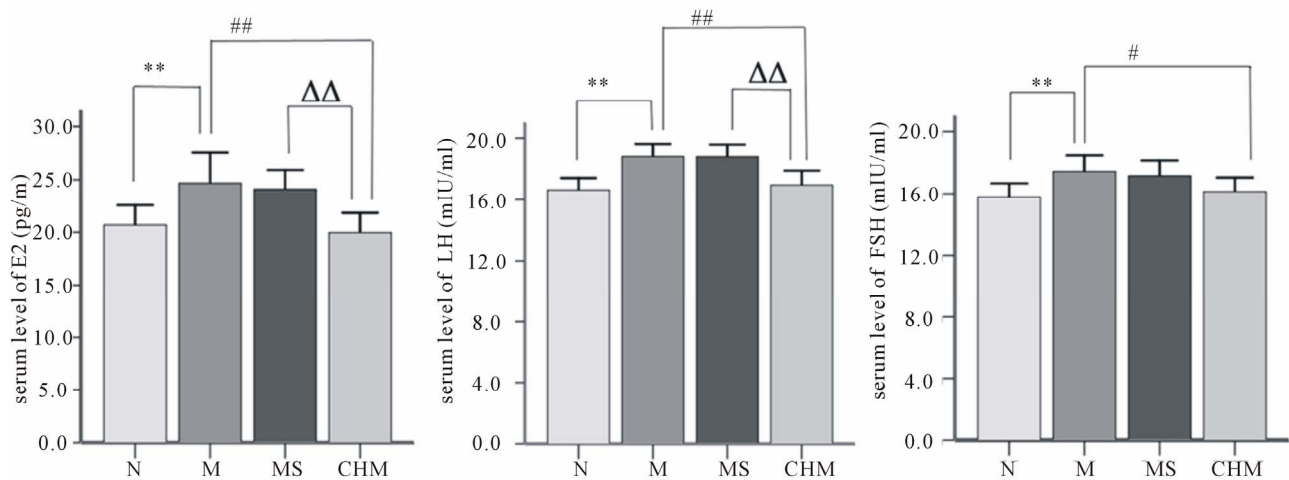


Figure 2. Effects of Nourishing “Yin”-Removing “Fire” Chinese herb mixture on serum E2, LH and FSH levels. The serum level of E2 ($p < 0.01$), LH ($p < 0.01$) and FSH ($p < 0.05$) significantly increased in M compared with N, and they were decreased in CHM. N: normal, M: model, MS: saline and CHM: Chinese herb mixture. ** $p < 0.01$ vs N; # $p < 0.05$ vs M; ## $p < 0.01$ vs M; $\Delta\Delta p < 0.01$ vs MS.

Table 2. Effects of Nourishing “Yin”-Removing “Fire” Chinese herb mixture on uterus and ovary coefficient.

Day 22					Day 26			
N	VO%	Uterus coefficient mg/100g	Ovary coefficient mg/100g	n	VO%	Uterus coefficient mg/100g	Ovary coefficient mg/100g	
N	11	0%	59.783 ± 1.307	4.847 ± 0.138	6	0%	81.870 ± 4.719	4.861 ± 0.256
M	7	100%	80.179 ± 2.192**	5.861 ± 0.386*	5	100%	107.965 ± 1.031*	5.766 ± 0.332*
MS	7	100%	88.101 ± 5.269	5.231 ± 0.429	4	100%	108.612 ± 5.971	5.280 ± 0.449
CHM	9	0%	58.627 ± 1.726 ^{##ΔΔ}	4.369 ± 0.228 ^{#Δ}	5	0%	80.434 ± 6.169 ^{#Δ}	4.745 ± 0.135 [#]

The results indicated that Chinese herb mixture had a significant effect on precocious puberty female rats especially regulating the uterus growth. N: normal, M: model, MS: saline and CHM: Chinese herb mixture. * $p < 0.05$ vs N; ** $p < 0.01$ vs N; # $p < 0.05$ vs M; ## $p < 0.01$ vs M; $\Delta p < 0.05$ vs MS; $\Delta\Delta p < 0.01$ vs MS; VO%: the percentage of vaginal opening rats.

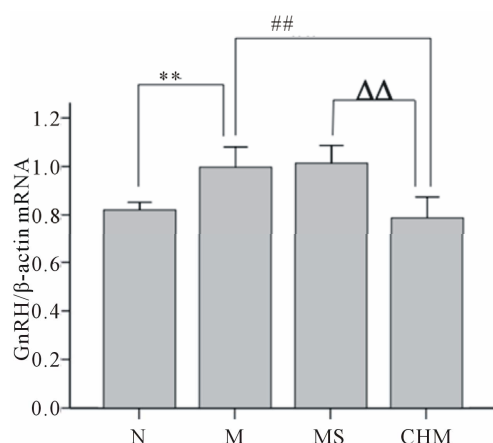


Figure 3. Effects of Nourishing “Yin”-Removing “Fire” Chinese herb mixture on GnRH mRNA expression. The expression of GnRH mRNA was decreased in M, and increased in CHM rats. N: normal, M: model, MS: saline and CHM: Chinese herb mixture. ** $p < 0.01$ vs N; ## $p < 0.01$ vs M; ΔΔ $p < 0.01$ vs MS.

in hypothalamus decreased significantly in M compared to those of N; and also only $\alpha 1$ and $\alpha 3$ subunits mRNA expression were significantly increased in CHM compared to those in M. there were no statistical differences of other subunits mRNA expression among four groups (Figure 4).

3.6. Effects of Nourishing “Yin”-Removing “Fire” Chinese Herb Mixture on the Expression of GABA_A Receptor $\alpha 1$ and $\alpha 3$ Subunits by Western Blot

According to Figure 5, $\alpha 1$ and $\alpha 3$ subunits protein expression by western blot in hypothalamus decreased significantly in M compared to N; and increased in CHM compared to those in M ($p < 0.01$, respectively). There were no disparities between M and MS.

3.7. Effects of Nourishing “Yin”-Removing “Fire” Chinese Herb Mixture on the Expression of ER α mRNA by RT-PCR

The expression of ER α mRNA in the hypothalamus of precocious rats increased compared to N ($p < 0.05$) and after the administration of Nourishing “Yin”-Removing “Fire” Chinese herb mixture that of CHM had shown a significant decrease compared to M ($p < 0.05$). The up-regulation of ER α mRNA refers to activation of negative-feedback regulation here (Figure 6).

4. Discussion

Puberty refers to the process of physical changes between childhood and adulthood during which the capacity to reproduce is obtained. Pubertal development occurs

before the age of 8 years in girls and the age of 9 years in boys is defined as precocious puberty. Precocious puberty has a profound impact on growth, development and psychosocial well-being of the patients. As is generally accepted, GnRH neurons play an original role in the onset of puberty that represented the final output of the neuronal network controlling sexual maturation in all mammals [14,15]. The regulation of GnRH secretion is associated with a complex interplay with excitatory and inhibitory neurotransmitters and hormones within the hypothalamus included, among which GABA was a major inhibitory neurotransmitter in the hypothalamus [16]. The roles of GABA and GABA_A receptor in stimulating GnRH pulsatile release triggering the onset of puberty have been in a general sense studied and established [17]. It has been recognized that the GABA_A receptor subunits compositions undergo a series of developmental changes through the life time as well. But how the GABA_A receptor subunits participate in this transition remains unclear, which has been rarely studied in a systematic study. Here we focus on the role of GABA_A receptor α subunits in the puberty initiation and the effects of herb mixture on precocious puberty.

It has been documented that GABA_A receptor subunits composition changed resulting in function heterogeneity at the onset of puberty [18], so we hypothesized that α subunits may undergo important changes in this period. In general sense the day of vaginal opening is taken as the token of puberty onset, and uterus and ovaries coefficients as the tokens of sexual development as well as serum levels of estrogens. According to the time of vaginal opening, hormone levels and sexual organ coefficients we supposed that after the regulation of Nourishing “Yin”-Removing “Fire” Chinese herb mixture, precocious rats got remission on the pathological process. The expression of GnRH and ER α mRNA showed significant increase in M compared to N and then decrease in CHM compared to M. What’s important, of all six α subunits, only $\alpha 1$ and $\alpha 3$ subunits were down-regulated in M when compared to those in N, while up-regulated in CHM. These changes showed in both mRNA expression and protein expression of GABA_A receptor $\alpha 1$ and $\alpha 3$ subunits. We may conclude that GABA_A receptor α subunits, especially $\alpha 1$ and $\alpha 3$ subunits may participate in the initiation of puberty and the regulation of Nourishing “Yin”-Removing “Fire” Chinese herb mixture. In summary, the down-regulation of GABA_A receptor $\alpha 1$ and $\alpha 3$ subunits in precocious rat hypothalamus led to the change of affinity between GABA and GABA_A receptor, that affected the inhibition process of GABA to GnRH. As a result the increasing level of GnRH referred to precocious puberty. After a period of Nourishing “Yin”-Removing “Fire” Chinese herb mixture administration all the pathological process above got reversed, and the

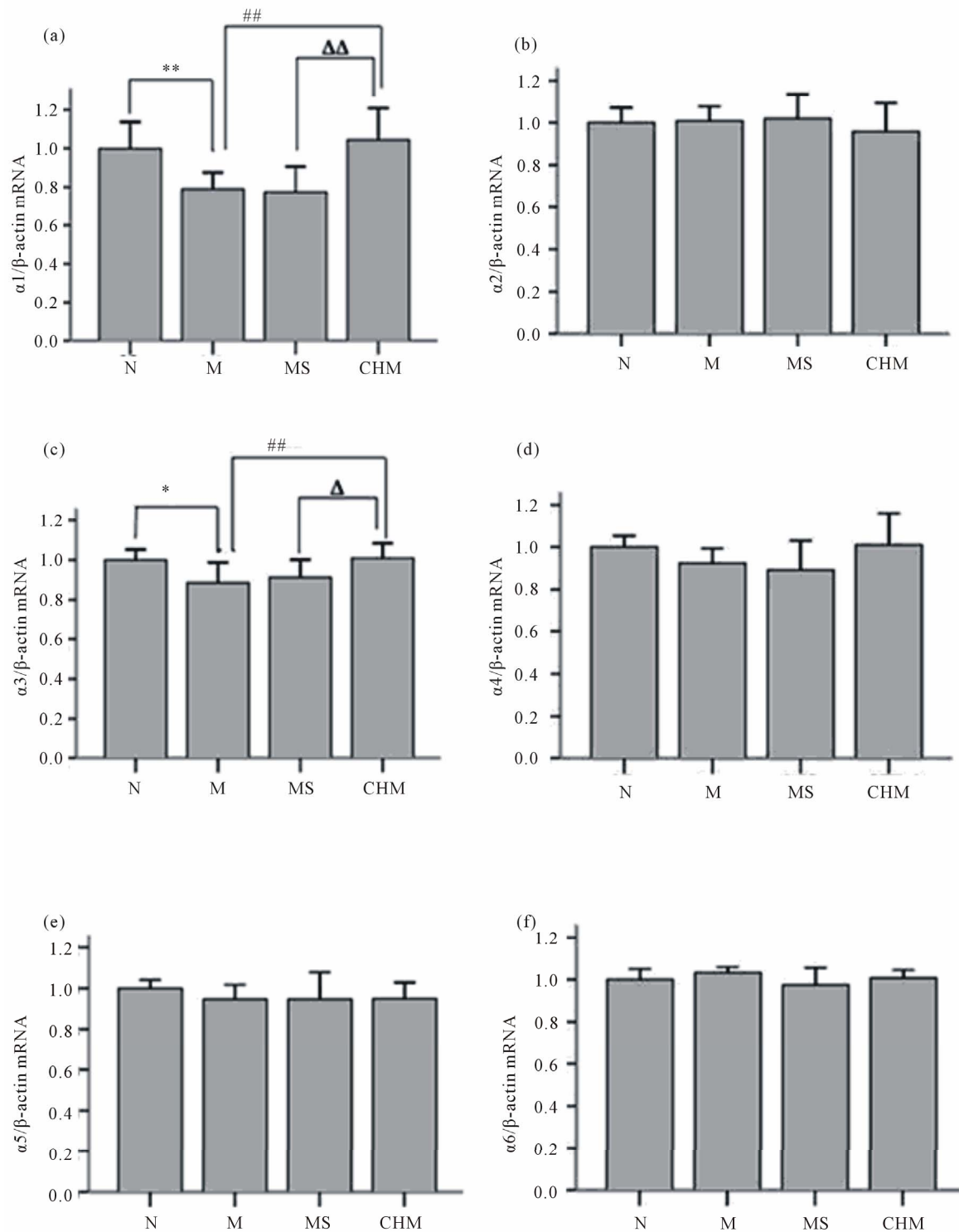


Figure 4. Effects of Nourishing “Yin”-Removing “Fire” Chinese herb mixture on GABA_A receptor α subunit mRNA expression by realtime PCR. Of all six subunits only $\alpha 1$ and $\alpha 3$ mRNA undergo changes between four groups. $\alpha 1$ and $\alpha 3$ expression in M both showed increased compared to N, and after the intervention of herb mixture that in TCM decreased compared to M. There were no statistical differences of the two mRNA expressions between M and MS. (a) GABA_A receptor $\alpha 1$ subunit; (b) GABA_A receptor $\alpha 2$ subunit; (c) GABA_A receptor $\alpha 3$ subunit; (d) GABA_A receptor $\alpha 4$ subunit; (e) GABA_A receptor $\alpha 5$ subunit; (f) GABA_A receptor $\alpha 6$ subunit. N: normal, M: model, MS: saline and CHM: Chinese herb mixture. *p < 0.05, vs N; **p < 0.01 vs N; ###p < 0.01 vs M; Δ p < 0.05 vs MS; $\Delta\Delta$ p < 0.01 vs MS.

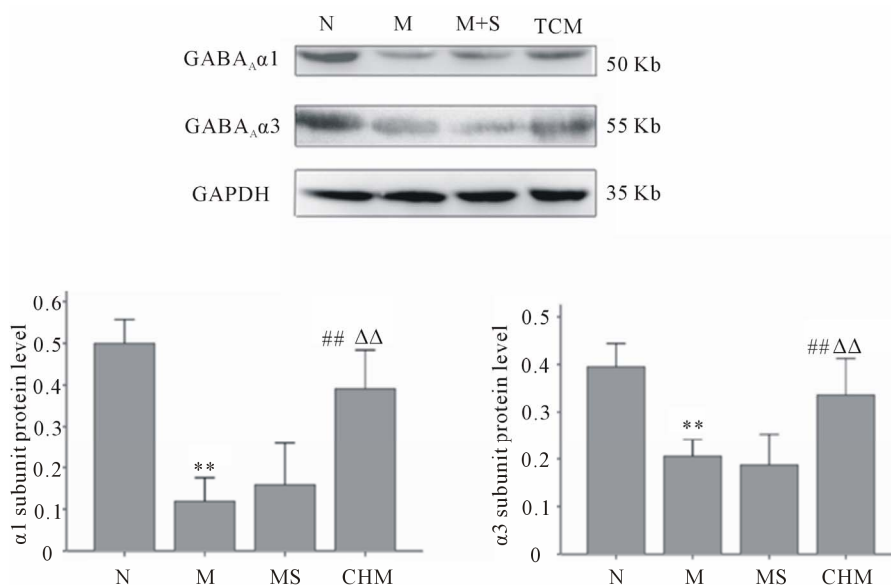


Figure 5. Effects of Nourishing “Yin”-Removing “Fire” Chinese herb mixture on GABA_A receptor $\alpha 1$ and $\alpha 3$ subunits protein expression by Western Blot. The subunits participated the regulation at onset of puberty including only $\alpha 1$ and $\alpha 3$ according to Figure 4. $\alpha 1$ ($p < 0.01$) and $\alpha 3$ ($p < 0.05$) were down-regulated in PP and up-regulated under the influence of Chinese herb mixture. N: normal, M: model, MS: saline and CHM: Chinese herb mixture. ** $p < 0.01$ vs N; ## $p < 0.01$ vs M; $\Delta\Delta p < 0.01$ vs MS.

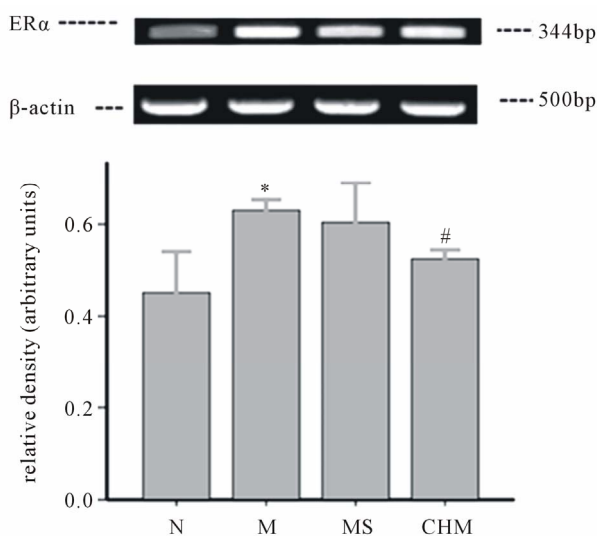


Figure 6. Effects of Nourishing “Yin”-Removing “Fire” Chinese herb mixture on ER α mRNA of female precocious pubertal rats. The expression of ER α mRNA increased in M ($p < 0.05$) compared to N, and that in TCM decreased compared to M ($p < 0.05$). N: normal, M: model, MS: saline and CHM: Chinese herb mixture. * $p < 0.05$ vs N; # $p < 0.05$ vs M.

symptoms relieved.

Nourishing “Yin”-removing “Fire” Chinese herb mixture was used for the management of IPP since last 80s’ [19,20]. And experiments on precocious puberty rats also achieved great progresses on the mechanism of Nourishing “Yin”-Removing “Fire” Chinese herb mixture regu-

lating precocious puberty. It was reported that Chinese herb mixture might regulate the abnormal function of HPGA in precocious puberty through down-regulate the hypothalamus kiss-1/PGR54 expression, and inhibit GnRH expression and release [21,22]. Another paper showed that the Chinese herb mixture could regulate the gonadotrophic and somatotrophic functions of HPGA through modulating the neuroendocrine regulation and the gene expressions of GnRH in hypothalamus, growth hormone, FSH and LH in pituitary, and IGF2 I in metaphysis that may be the mechanisms of herb mixture in modulating the course of pubertal development and ameliorating the skeletal development in precocious puberty children [23,24]. Different from the experimental results about the GABA_A receptor α expression changing during the puberty, now our experiment concluded that the GABA_A receptor $\alpha 1$ and $\alpha 3$ subunits may take part in the mechanism of the Nourishing “Yin”-Removing “Fire” Chinese herb mixture on precocious puberty. All those studies could help us to know more about how the Chinese herb medicine takes effects on precocious patients.

5. Conclusion

The nourishing “Yin”-Removing “Fire” Chinese herb mixture may significantly delay the sexual development of the precocious puberty rat, through up-regulated the expression of GABA_A receptor $\alpha 1$ and $\alpha 3$ subunits in precocious puberty rat. Based on above, GABA_A receptor might be involved in the effective treatment of herb mixture on IPP.

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