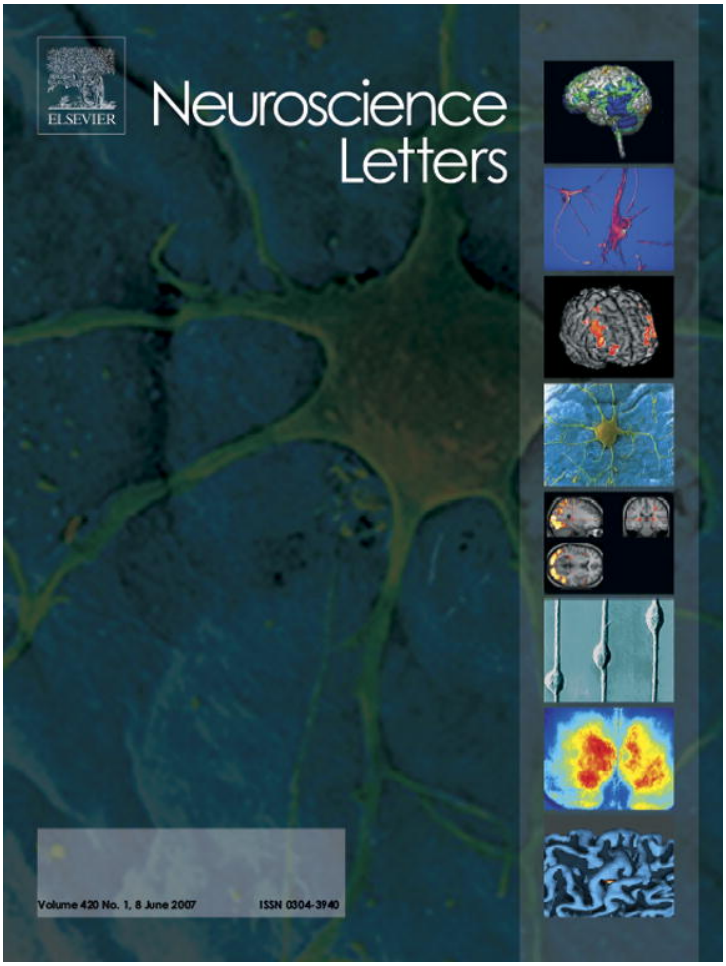

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Characteristic of hypothalamic kisspeptin expression in the pubertal development of precocious female rats

Yan Sun [a,1], Zhanzhuang Tian [a,1], Hong Zhao [a,b,*], Stephen T.C. Wong [b], Boying Chen [a]

[a] *Department of Neurobiology and Integrative Medicine, Shanghai Medical College of Fudan University (Formerly Shanghai Medical University), P.O. Box 291, 138 Yi-Xue-Yuan Road, 200032 Shanghai, PR China*
[b] *HCNR Center for Bioinformatics, Harvard Medical School and Functional and Molecular Imaging Center, Department of Radiology, Brigham and Women's Hospital, Boston, MA 02115, USA*

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Abstract

To investigate the potential role of kisspeptin in the advance onset of puberty in precocious puberty, model rats induced by danazol were used to study the developmental expression of hypothalamic kisspeptin. Kisspeptin immunoreactive cells were observed in the arcuate nucleus (ARC), periventricular nucleus (PeN) and preoptic area (POA) in model rats on the day of onset-puberty. On the day of post-puberty, however, the number of kisspeptin immunoreactive cells in ARC and PeN decreased while the number of those cells in POA increased. Kisspeptin immunoreactive cells were not detected in hypothalamus in both normal and model rats at their pre-puberty stages. Furthermore, the expression of hypothalamic Kiss-1 mRNA reached top on the day of onset-puberty in both of the normal and model rats, and the expression of Kiss-1 mRNA increased significantly in the model rats compared with those in the normal ones. Our results indicated that kisspeptin might involve in the advance onset of puberty in danazol induced female precocious model rats.

Keywords: Kisspeptin; Precocious puberty; Gonadotropin releasing hormone; Danazol; Rat

Sexual precocity is one of the most popular endocrine disorders in children, with the incidence of 0.6% throughout the world and is ten times more common in girls than in boys [3]. Though central nervous system tumors, such as hamartomas and astrocytomas, may cause true precocious puberty, most cases have no organic disease [16]. It is well known that the pathogenesis of early pubertal maturation in idiopathic precocious puberty (IPP) finally appears the higher-pulsatile release of hypothalamic gonadotropin releasing hormone (GnRH) [23]. But what actually triggers the advance release of GnRH and the neurobiological mechanism underlying the advance puberty onset remains poorly understood.

Danazol, an isoxazol derivative of 17α-ethinylestoster-one, is known to have effects on the reproductive system. It binds to androgen and progesterone receptors and possesses weak androgenic activity in rats. Our previous studies [21,22] and Morishita et al. [9] have reported that neonatal treatment with danazol may induce the true precocious puberty, but danazol has no influence on the serum levels of estradiol and testosterone at adulthood of the rat.

The Kiss-1 gene derived peptide kisspeptin and its receptor, the G protein-coupled receptor 54 (GPR54), have emerged as indispensable factors for pubertal development [2,17]. The Kiss-1 mRNA expresses in different regions of hypothalamus [4,7]. Both male and female rats show an obvious increase in Kiss-1 mRNA levels coinciding with the onset of puberty [10], central and peripheral administration of kisspeptin stimulates a dose-dependent rise in serum levels of luteinizing hormone (LH) [12] and follicle-stimulate hormone (FSH) [11] in adult rats. Moreover, kisspeptin elicited GnRH secretion in rats [1,20], repeated administration of kisspeptin-10 to immature female rats was able to induce precocious vaginal opening and early activation

* Corresponding author at: Department of Neurobiology and Integrative Medicine, Shanghai Medical College of Fudan University (Formerly Shanghai Medical University), P.O. Box 291, 138 Yi-Xue-Yuan Road, 200032 Shanghai, PR China. Tel.: +86 21 54237693.
E-mail address: zh2000h@hotmail.com (H. Zhao).
[1] These authors contributed equally to this work.

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of the gonadotropic axis [13]. Because kisspeptin plays such an important role in GnRH release and pubertal development, it is conceivable that the pathological advance release of GnRH in IPP is intervened by high levels of Kiss-1 expression. The present work aims to observe the expression of hypothalamic Kiss-1 in danazol induced female precocious rats and to explore the possible participation of kisspeptin in precocious puberty.

Eighty-four female Sprague-Dawley rats of 3 days age with the mothers were purchased from Medical Experimental Animals Center of BK Co. (Shanghai, China). The model litters at postnatal day 5 were given a single subcutaneous injection of 300 μg of danazol (Hualian Pharm Ltd., Shanghai, China) dissolved in 25 μl vehicle of propylene glycol–ethanol (1:1,v/v) [9,21]. The animals were weaned on day 21 and then were examined daily for vaginal opening and vaginal smears afterwards. All experimental procedures involving the use of animals were conducted in accordance with NIH Guidelines and were reviewed and approved by Animal Use and Care Committee of Fudan University.

In the Experiment 1, thirty rats were used to observe the day of vaginal opening and the establishment of two regular estrous cycles, which were divided into normal, model and vehicle group ($n = 10$, respectively). Vaginal smears were checked daily until two consecutive regular 4- or 5-day estrous cycles were established, then the rats were killed after deep anesthesia and decapitation. The uterus and ovaries were dissected out of the surrounding fat and weighed for evaluating the organ coefficients (mg/100 g). In the Experiment 2, eighteen rats were used to observe the hypothalamic expressions of GnRH and GnRH mRNA, which were divided into normal, model and vehicle group ($n = 6$, respectively). Hypothalamic samples were obtained as we previously described [21,22], the GnRH and GnRH mRNA detected were based on our previous experience [21].

In the Experiment 3, thirty-six rats were used to detect the hypothalamic expressions of kisspeptin and Kiss-1 mRNA, which were divided into normal and model group ($n = 18$, respectively). Animals in the both groups were sacrificed, respectively, on the day of pre-puberty (postnatal 25 d and 20 d, $n = 6$), onset-puberty (the day of vaginal opening, $n = 6$), and post-puberty (the day of establishment of two regular estrous cycles, $n = 6$). Following anesthesia, animals were perfused with sterile normal saline. One hemi-brain was removed, post-fixed for >48 h in 4% paraformaldehyde in 30% sucrose/PBS. Coronal sections were sliced at 35 μm thickness on a vibratome microslicer. Slices were washed three times with PBS, and then blocked with blocking buffer (5% normal goat serum, 1% BSA, 0.1% Triton X-100 in PBS) for 30 min at room temperature. Immunohistochemistry was performed using polyclonal antibodies to kisspeptin-10 (Kiss-1(112-121)/metastin(45–54) 1:1000, Phoenix Pharmaceuticals Inc., USA) for 48 h at 4 °C. We performed detection of primary antibodies by ABC kit (Sino-American Technology Company, China) and diaminobenzidine/H_2O_2 reagent as substrate.

Specificity of kisspeptin staining was determined by preincubation of antiserums with the metastin peptide (45–54)-NH_2 (10 μg/ml, Phoenix Pharmaceuticals Inc.) overnight, as well as omitting primary antibody to identify non-specific staining. Rat placenta (from 13.5-day pregnancy) was used as positive control for kisspeptin immunolabeling.

The other half hypothalamus including media-basal hypothalamus and the suprachiasmatic-preoptic area (limited anteriorly by the optic chiasm, laterally by the hypothalamic fissure, posteriorly by the mammilary body and in depth by the subthalamic sulcus) was used to study the expression of Kiss-1 mRNA. Total RNA was extracted using "Trizol Regent" (Biobasic Inc., Canada). The purity and integrity of the RNA were checked spectroscopically and by gel electrophoresis before carrying out the analytical procedures. Tissue RNA (2 μg) was reversed transcribed in a final volume of 20 μl, using 200 IU M-MLV reverse transcriptase in the presence of 25 pmol oligo (dT) 18 primers, 0.5 mM deoxy-NTP and 20 IU Rnasin for 60 min at 42 °C, then heat denatured for 5 min at 95 °C. Five microlitres cDNAs were further amplified by PCR using previously defined primer (Sangon Inc.) and conditions for Kiss-1 [10]. PCR was performed during 30 cycles (96 °C for 30 s, 62.5 °C for 30 s, 72 °C for 1 min) in the presence of Taq DNA polymerase (3 U per tube) and 2.2 mM magnesium chloride in a final volume of 50 μl. The final extension cycle of 72 °C for 10 min was included. In addition, 2 μg of total RNA without M-MLV reverse transcriptase was performed to check the presence of DNA contamination RT-PCR. An internal control, β-actin, was performed to account for procedural variations. For each sample, 5 μl of PCR amplification products were analyzed on 1.5% agarose gels and visualized by ethidium bromide staining. Standard DNA (100 bp DNA ladder, Promega) was run to provide the appropriate size marker. The RT-PCR products were extracted and purified from agarose gel by Golden Beads Gel Extraction kit and sequenced using the radioactive dideoxychain terminating method (Sangon Inc., China). Quantification of the intensity of the bands was evaluated by Image Master Software (SYDR-1990, SYNGENE, USA).

Results were expressed as mean ± S.E.M. (the standard error of the mean). Statistical analysis was performed using one-way analysis of variance (ANOVA), with the significance concentrations of $P < 0.05$ in the two-tailed testing chosen.

In female rats, vaginal opening is a marker of puberty [19]. In the Experiment 1, the day of vaginal opening and establishment of two regular estrous cycles were significantly advanced in the model rats than those of normal and vehicle ones ($P < 0.05$, respectively). The organ coefficients of uterus and ovary of the model rats were increased significantly comparing with the normal and vehicle ones ($P < 0.05$). There were no differences between the normal and vehicle rats (Table 1).

In the Experiment 2, GnRH cells bodies, which were abundant in rostral medial septum (MS), Broca diagonal band nucleus (DBB), and medial preoptic nucleus (MPOA) were calculated. The number of GnRH cells in model rats was less than those in normal and vehicle ones ($P < 0.05$, respectively) on the day of vaginal opening, but there was no difference between the normal and vehicle rats (Fig. 1). The negative control using antiserum after pre-absorption with excessive antigens and omission of primary antibody showed no GnRH immunolabeling as expected.

Table 1
Day of vaginal opening, two regular estrous cycles and organ coefficient of uterus and ovary of the rats

	Group		
	Normal ($n = 10$)	Model ($n = 10$)	Vehicle ($n = 10$)
Day of vaginal opening	42.67 ± 6.81	31.83 ± 5.07*	41.20 ± 3.85
Two regular estrous cycles	54.23 ± 1.28	48.75 ± 0.50*	53.50 ± 2.28
Organ coefficient of uterus	64.27 ± 1.18	75.89 ± 2.36*	65.30 ± 2.25
Organ coefficient of ovary	52.65 ± 1.19	78.49 ± 1.17*	53.50 ± 1.70

* $P < 0.05$, model vs. normal and vehicle.

Densitometric analysis of the mRNA levels showed the ratio of GnRH to β-actin in the model group increased significantly compared with that in the normal and vehicle ($P < 0.05$, respectively). There was no significant difference between the normal and vehicle ones (Fig. 1).

In the Experiment 3, there were not positive kisspeptin-ir neurons in hypothalamus on the day of pre-puberty in both the normal and model rats. The kisspeptin-ir cells were only appeared in PeN on the day of onset-puberty in normal ones, and the cell number were increased on the day of post-puberty. Kisspeptin-ir cells in ARC and POA in the normal rats were detected on the day of post-puberty. In the model rats, a number of kisspeptin-ir cells were observed in ARC, PeN and POA on the day of onset-puberty, and the kisspeptin-ir cell numbers in ARC and PeN decreased on the day of post-puberty ($P < 0.05$, compared with those of the day of onset-puberty). However, the kisspeptin-ir cell number increased significantly in POA on the day of post-puberty than those on the day of onset-puberty ($P < 0.05$). The negative control showed no kisspeptin immunolabeling. In rat placenta, cytoplasmic kisspeptin immunostaining was detected in the giant trophoblast cells (Fig. 2).

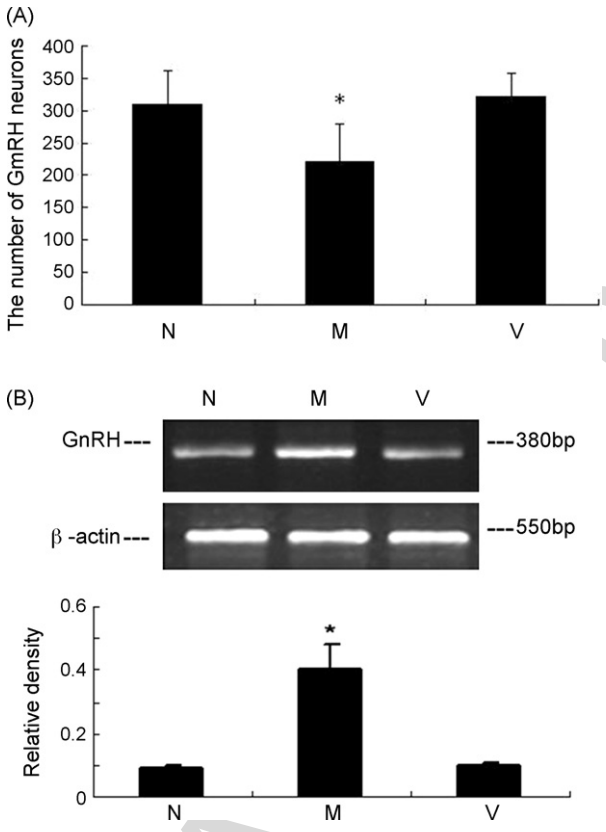

Fig. 1. The expression of hypothalamic GnRH and GnRH mRNA in the rats by immunohistochemistry and RT-PCR. (A) Total GnRH cells in the MS, DBB, and MPOA of the rats. The number of GnRH cells on the day of onset-puberty in model ones was less than those in normal and vehicle ($n = 6$ per group) group. N: normal; M: model; and V: vehicle. *$P < 0.05$, M vs. N and V, respectively. (B) The upper picture shows the gel electrophoresis of the RT-PCR products for the GnRH. Densitometric analysis of the mRNA concentration using GnRH/β-actin expressed as the mean with S.E.M. bar ($n = 6$) in each column indicated in the lower panel. The ratio of GnRH to (-actin in the model increased significantly compared with those in normal and vehicle. N: normal; M: model; and V: vehicle; * $P < 0.05$, M vs. N and V, respectively.

Comparison of the amplified PCR fragment with rat Kiss-1 mRNA sequence revealed 100% homology (data not shown). Densitometric analysis of Kiss-1 mRNA showed the ratio of Kiss-1 to β-actin reached the peak value on the day of vaginal opening in normal and model rats, and the ratio increased significantly in the model rats compared with that in the normal ones ($P < 0.05$). There was no significant difference between the model and the normal rats on the day before vaginal opening (Fig. 3).

Mammalian sexual maturation is centrally controlled by a subset of neuroendocrine neurons that produce GnRH. It has become apparent that advance onset of puberty in IPP mediated by premature increasing secretion of GnRH. Thus, the question of "what triggers the onset of puberty in IPP?" becomes rather the question of "what triggers the advance increase in GnRH release?"

To ascertain the potential action of kisspeptin and the developmental changes of Kiss-1 expression in IPP, kisspeptin and Kiss-1 mRNA levels in hypothalamus of precocious rats were studied. The most interesting finding in our study is that a number of kisspeptin-ir cells were observed in ARC, POA, and PeN since the day of onset-puberty in precocious model rats. But no kisspeptin-ir cells were detected in hypothalamus in both of the model and normal rats on the day of pre-puberty. Kisspeptin-ir cells were only detected in the PeN on the day of onset-puberty and there were much kisspeptin-ir positive neurons in ARC and POA on the day of post-puberty in the normal rats. ARC, POA, and PeN are important neuroendocrine areas in which GnRH neurons locate and GnRH fibers project to pituitary [18]. Interconnection of GnRH neurons in these areas is thought to facilitate coordinate firing of the cells for pulsatile discharge of GnRH [14]. These evidences supported that the abundant expression of kisspeptin in ARC, POA and PeN on the day of onset-puberty in precocious rats might participate in GnRH

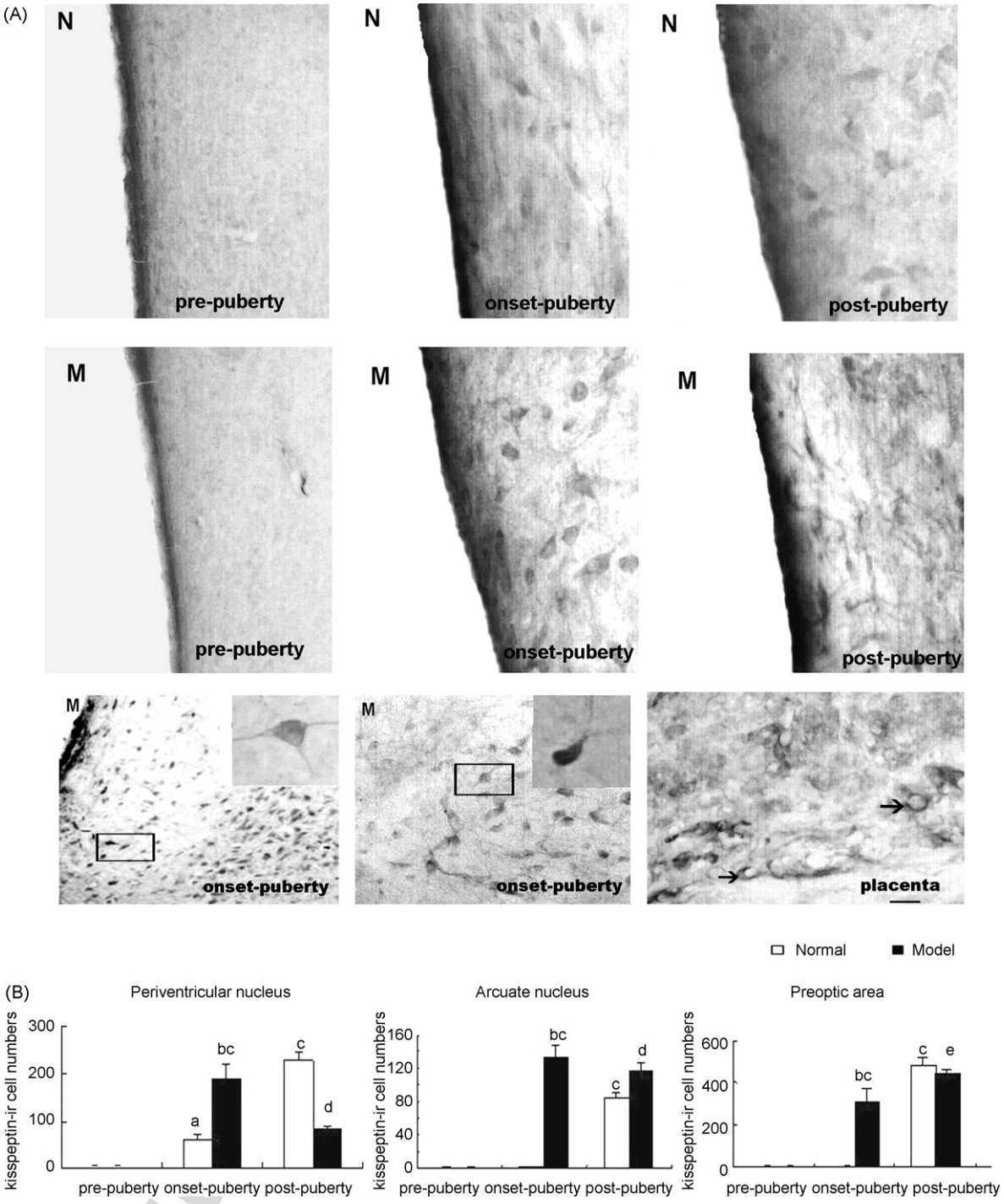

Fig. 2. The expression of hypothalamic kisspeptin in the rats by immunohistochemistry. (A) Representative microscopic images of the PeN demonstrating an increase in kisspeptin expression on the day of onset-puberty in the model rats compared with that in the normal rats (×40). The left in the lowest panel shows the expression of kisspeptin-ir neurons in the ARC on the day of onset-puberty in the model rats, and the middle shows those in the POA (Low magnification ×10, high magnification ×40). The positive control immunostaining in the rat placenta (from 13.5 d-pregnancy) was also shown in the right (high magnification ×20). The black arrow indicates the cytoplasmic kisspeptin immunostaining in the giant trophoblast cells. (B) Calculated number of the kisspeptin neurons in the PeN, ARC and POA in the normal and model rats. Twelve observations per animal and six animals per group, all observations from individual animal averaged for that animal, and then collapsed into a single value for that animal. These single numbers of each animal used to calculate the group mean. N: normal; M: model; $^{a}P < 0.01$, onset-puberty in N vs. pre-puberty in N; $^{b}P < 0.01$, onset-puberty in M vs. pre-puberty in M; $^{c}P < 0.01$, respectively, onset-puberty in M vs. onset-puberty in N and post-puberty in N vs. onset-puberty in N; $^{d}P < 0.05$, respectively, post-puberty in M vs. post-puberty in N and onset-puberty in M; $^{e}P < 0.05$, post-puberty in M vs. onset-puberty in M.

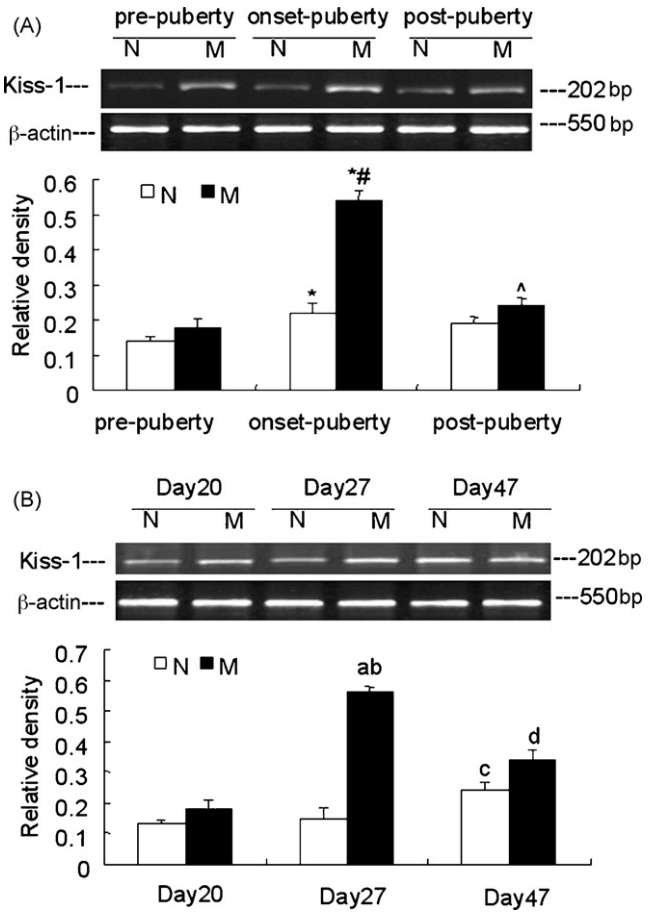

Fig. 3. The expression of hypothalamic Kiss-1 mRNA of the rats by RT-PCR. (A) Hypothalamic Kiss-1 mRNA expression on the day of pre-puberty, onset-puberty, and post-puberty of the rats. Densitometric analysis used the ratio of Kiss-1/(-actin ($n=6$ per group) expressed as the mean with S.E.M. N: normal; M: model. *$P<0.05$, onset-puberty in M vs. pre-puberty in M, $^{\#}P<0.05$, onset-puberty in M vs. onset-puberty in N, $^{\wedge}P<0.05$, post-puberty in M vs. onset-puberty in M. (B) Comparison of different pubertal development periods of Kiss-1 mRNA expression in model with normal on the same postnatal age. N: normal; M: model. $^{a}P<0.05$, D27 in M vs. D27 in N, $^{b}P<0.05$, D27 in M vs. D20 in M, $^{c}P<0.05$, D47 in N vs. D27 in N, $^{d}P<0.05$, D47 in M vs. D27 in M.

coordinate activity. Exogenously administered kisspeptin would increase the secretion of GnRH/LH in pre-pubertal, adult female rats [6,13,12] and the release of GnRH in sheep [8]. Moreover, kisspeptin and GnRH have been observed co-localized in diagonal band of Broca (DBB)/POA in ovine brain [15], kisspeptin neurons act as gatekeepers to awakening of reproduction at puberty [2]. These observations further raise the possibility that much kisspeptin expression in the precocious rats might stimulate the advance GnRH release and the premature onset-puberty. The super-release of hypothalamic GnRH in the precocious rats might contribute to the decreased number of the GnRH neuron.

Noticeably, the developmental characteristic of kisspeptin-ir cell is not the same among all the observed hypothalamic nuclei in precocious rats. The cell numbers in ARC and PeN increased to a high level on the day of onset-puberty and decreased on the day of post-puberty, whereas the kisspeptin expression in POA increased to maximum on the day of post-puberty. We hypothesize that kisspeptin in anterior hypothalamic nuclei, such as POA, might have more effects on the synthesis of the GnRH while kisspeptin in posterior nuclei including ARC and PeN might have potent effects on the release of GnRH from the median eminence.

Our results also showed that Kiss-1 and GnRH mRNAs reached the peak level on the day of onset-puberty in the model rats as well as the normal rats, and the Kiss-1 and GnRH mRNAs expression are higher in the model rats compared with those in normal rats. The high level of Kiss-1 mRNA in precocious rats seems likely to reflect increased kisspeptin activity on the advance onset of puberty. For kisspeptin is able to induced *c-fos* expression in 85% GnRH neurons [5], it is very possible that the increased Kiss-1 mRNA has a potential role in advance GnRH expression in the precocious rats. Furthermore, it is compelling that GPR54 mRNA, a gene that encoding kisspeptin receptor, distributes in POA and ARC [6] and it co-localize with GnRH neurons [5,8], whether the kisspeptin activate the advance GnRH surge through GPR54 and the exact role of kisspeptin in GnRH regulation in precocious rats remains to be studied.

It is generally accepted that the rat hypothalamus is immature at birth and the maturation process is probably between post-birth day 1 and 10 [9]. From this viewpoint, it is possible that the neonatal rats administered with danazol may affect the hypothalamic pituitary axis with the rapid rate of maturation, producing a true precocious puberty.

In conclusion, we analyzed that characteristic of kisspeptin expression in POA, ARC, and PeN in the precocious female rats. This suggested that hypothalamic kisspeptin might involve in the advance pulsatile of GnRH in the precocious puberty rats while kisspeptin in the different hypothalamic nuclei acted distinct roles in the regulation of GnRH release. Hopefully, the mechanism of advance GnRH releasing in precocious puberty will be unmasked more in future studies so as to bring benefits to children patients.

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