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# Morphological changes of gonadotropin-releasing hormone neurons in the rat preoptic area across puberty

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## Abstract

Gonadotropin-releasing hormone (GnRH) neurons in the preoptic area may undergo morphological changes during the pubertal period when their activities are upregulated. To clarify the regulatory mechanism of puberty onset, this study aimed to investigate the morphological changes of GnRH neurons in the preoptic area of GnRH-enhanced green fluorescent protein transgenic rats. Under confocal laser microscopy, pubertal GnRH neurons exhibited an inverted Y distribution pattern. Prepubertal GnRH neurons were generally unipolar and bipolar, and were distinguished as smooth type cells with few small processes or irregular type cells with many spine-like processes in the proximal dendrites. The number of GnRH neurons in the preoptic area and spine-like processes were increased during the course of reproductive maturation. There was no significant difference between male and female rats. Immunofluorescence staining revealed synaptophysin punctae close to the distal end of GnRH neurons, indicating that some presynaptic terminals may form a synaptic linkage with these neurons.

***Key Words:*** *nerve regeneration; preoptic area; gonadotropin-releasing hormone; neurons; pubertal period; luteinizing hormone; transgenesis; bipolar neurons; neural regeneration*

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## Introduction

Gonadotropin-releasing hormone (GnRH) (also known as luteinizing hormone-releasing hormone or luliberin), is a trophic peptide hormone responsible for the release of follicle-stimulating hormone and luteinizing hormone from the anterior pituitary. GnRH is a decapeptide synthesized in GnRH neurons of the medial forebrain (Arroyo et al., 2011; von Wolff et al., 2011; Sun et al., 2013). Cell bodies of GnRH neurons in rodents are mainly scattered in the medial septum, diagonal band of Broca, and preoptic area, at the level of the organum vasculosum of the lamina terminalis (Arroyo et al., 2011). The preoptic area of the hypothalamus is a key region for the production of GnRH. This area contains most of the GnRH-secreting neurons. GnRH neurons originate in the nasal region and migrate to the brain. These neurons are scattered throughout the medial septum and hypothalamus and form a network through their very long dendrites (> 1 mm long). This interwoven network therefore receives a shared synaptic input. This process allows these neurons to synchronize the release of GnRH. GnRH neurons in the preoptic area are considered to directly regulate sexual maturation and reproductive behavior (Kinoshita et al., 2005; Clarkson and Herbison, 2006b; Adachi et al., 2007). Axons of preoptic GnRH neurons extend to the median eminence of hypothalamus and release GnRH into the hypophyseal portal blood (Yin et al., 2010). Once in the anterior pituitary, GnRH controls the synthesis and secretion of gonadotropins, luteinizing hormone, and follicle-stimulating hormone (Debruyne et al., 2010; Farkas et al., 2010; Gentil et al., 2012).

GnRH neurons originate from the olfactory placode, and during embryonic development, these neurons migrate from this region to the brain along olfactory neuronal axons (Wray, 2002; Yu et al., 2011). GnRH neurons synthesize the various hormones well before puberty. Indeed, endogenous secretion of GnRH has been shown to occur during late fetal development or early neonatal development (Ko et al., 2010; Pandit and Saxena, 2010). However, the activity of GnRH neurons before puberty remains at a low level, which may be due to a central inhibition of GnRH secretion (Monje et al., 2010) or a shift in sensitivity resulting from the feedback of gonadal steroids (Shaw et al., 2012). Either response increases the release of GnRH and triggers the pubertal process (Shaw et al., 2012; Tada et al., 2013). Previous studies in different animal species have suggested that the activation of GnRH neurons in late postnatal development initiates puberty (Monje et al., 2010; Shaw et al., 2012; Tada et al., 2013). However, the mechanism underlying this effect remains debatable across these different species.

The morphology of GnRH neurons is altered following the onset of puberty. In the mouse, prepubertal GnRH neurons have been reported to appear as a relatively complex network of dendritic branches compared with those at postpuberty

(Cottrell et al., 2006). During postnatal development, the soma and dendritic spine density in GnRH neurons of mice are significantly increased, which reflects an abundance of excitatory inputs to GnRH neurons at the adult stage (Cottrell et al., 2006). Whether a similar morphological characterization of GnRH neurons occurs in the rat remains to be determined. Postnatal morphological changes of GnRH neurons in the rat have been reported (Sharif et al., 2013). However, their observations were only made by immunocytochemistry, and this method does not provide extensive labeling of dendritic arborizations and spine-like processes of neurons. Detailed morphological studies are important to better understand the regulatory mechanism of puberty onset.

Transgenic mice expressing green fluorescent protein (GFP) or enhanced GFP (EGFP) in GnRH neurons have recently been established to visualize living GnRH neurons (Teixeira et al., 2010; Wojniusz et al., 2011). The present study aimed to investigate the morphological changes of GnRH neurons in the preoptic area using GnRH-EGFP transgenic rats.

## Materials and Methods

### Animals

For the morphological investigations of the preoptic GnRH neurons, male and female Wistar rats ($n$ = 16 for each sex) aged 3 weeks (prepuberty) and 49–84 days (postpuberty; adult) were used to express EGFP under the control of the GnRH promoter, according to Teixeira et al. (2010). All rats were provided by the Experimental Animal Center of College of Basic Medical Science, Jilin University, China (license No. SCXK (Ji) 2007-0003). Animals were maintained under a 14-hour light and 10-hour dark cycle with *ad libitum* access to a standard rodent chow diet and water. The stage of the estrous cycle in adult female rats was determined by vaginal smear histology, and diestrus female rats were used for the analysis of synaptic inputs to the GnRH neurons (Teixeira et al., 2010). All experiments were performed with the approval of the Beihua University Animal Care Committee, China.

### Tissue preparation

Rats were anesthetized by intraperitoneal injection of 5 mg/kg pentobarbital sodium (Dainippon Sumitomo Pharma, Osaka, Japan) and then perfused through the left ventricle with 0.9% saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB; pH 7.4). Rats were then quickly decapitated. The brains were removed from the cranium and post-fixed in a fresh solution of the same fixative at 4°C for 24 hours. The post-fixed brains were immersed in 30% sucrose in PB at 4°C for 3 days and then frozen rapidly by immersing them in n-hexane at –60°C. Serial frontal sections were cut (100 μm and 30 μm in thickness) on a cryostat (Leica Microsystems, Wetzlar, Germany) from the diagonal band of Broca to the caudal extent of the mammillary bodies (Yu et al., 2011). Sections were collected in three sets, transferred to individual wells in a 6-well plate containing 0.1 mol/L PBS (pH 7.4), and stored at 4°C until further use. Sections at 100 μm and 30 μm thickness were used for GnRH neuronal morphology and immunocytochemistry, respectively.

### Morphological analyses of GnRH neurons

Prepubertal (four males and four females) and postpubertal (four males and four females) GnRH-EGFP transgenic rats were used for morphological analyses. Floating sections 100 μm thick were soaked in PB, mounted on gelatin-coated glass slides, and then covered with coverslips with Gel/Mount Aqueous Mounting Medium (Biomeda Corporation, Hayward, CA, USA). Detailed morphological analysis of EGFP-expressing GnRH neurons in the preoptic area was observed by a confocal laser scanning microscope (Digital Eclipse C1 TE2000-E, 3D; Nikon Insteck, Tokyo, Japan) with a green argon laser (excitation: 488 nm, emission: 515/530 nm). Fluorescence images were acquired and analyzed using a Nikon Plan Fluor 10 × objective (numerical aperture, 0.3) and 100 × oil immersion objective (numerical aperture, 1.45) lens with the confocal microscope Digital Eclipse C1 control software EZ-C1 (Version 2.30; Nikon Insteck). The confocal image represented the maximal projection of the optical image stack, reconstructed from a series of images at 400-nm intervals. A three-dimensional image was obtained using the high-performance three-dimensional imaging software, Volocity Visualization (Version 3.6.1; Improvision, Lexington, MA, USA). The brightness and contrast of the images were adjusted in Adobe Photoshop CS. Morphological data obtained from each rat were counted and averaged as a single observation for statistical analyses.

### Immunofluorescence staining of synaptophysin

GnRH-EGFP transgenic rats at prepuberty (four males and four females) and postpuberty (four males and four females) were used for synaptophysin staining. Twenty-five prepubertal and 40 postpubertal GnRH-EGFP neurons in male and female rats, respectively, were used for the analysis of synaptic inputs to GnRH neurons. Floating sections (30 μm thick) were rinsed in PBS and incubated with 5% normal goat serum in 0.1 mol/L PBS containing 0.3% Triton X-100 (PBST; pH 7.4; Sigma, St. Louis, MO, USA) for 2 hours at room temperature to reduce nonspecific binding. Sections were incubated with the primary antibody, mouse anti-rat synaptophysin monoclonal antibody (1:200 in PBST; Clone SVP-38, Sigma) for 48 hours. After washing (three times for 10 minutes) in PBST, sections were then incubated for 2 hours with the secondary antibody, Alexa Flour 568 goat anti-mouse IgG (1:1,000 in PBST; Molecular Probes, Eugene, OR, USA). Sections were then washed, mounted on gelatin-coated glass slides, and covered with a coverslip. All procedures were performed at room temperature and in darkness. Images were acquired and processed as described for morphological analyses of GnRH neurons. The Z-series stack of confocal images at 400-nm intervals was collected using the 100 × oil immersion objective (numerical aperture, 1.45) with a green argon laser exciting at 488 nm (for EGFP) and a red helium-neon laser exciting at 543 nm (for synaptophysin). The number of

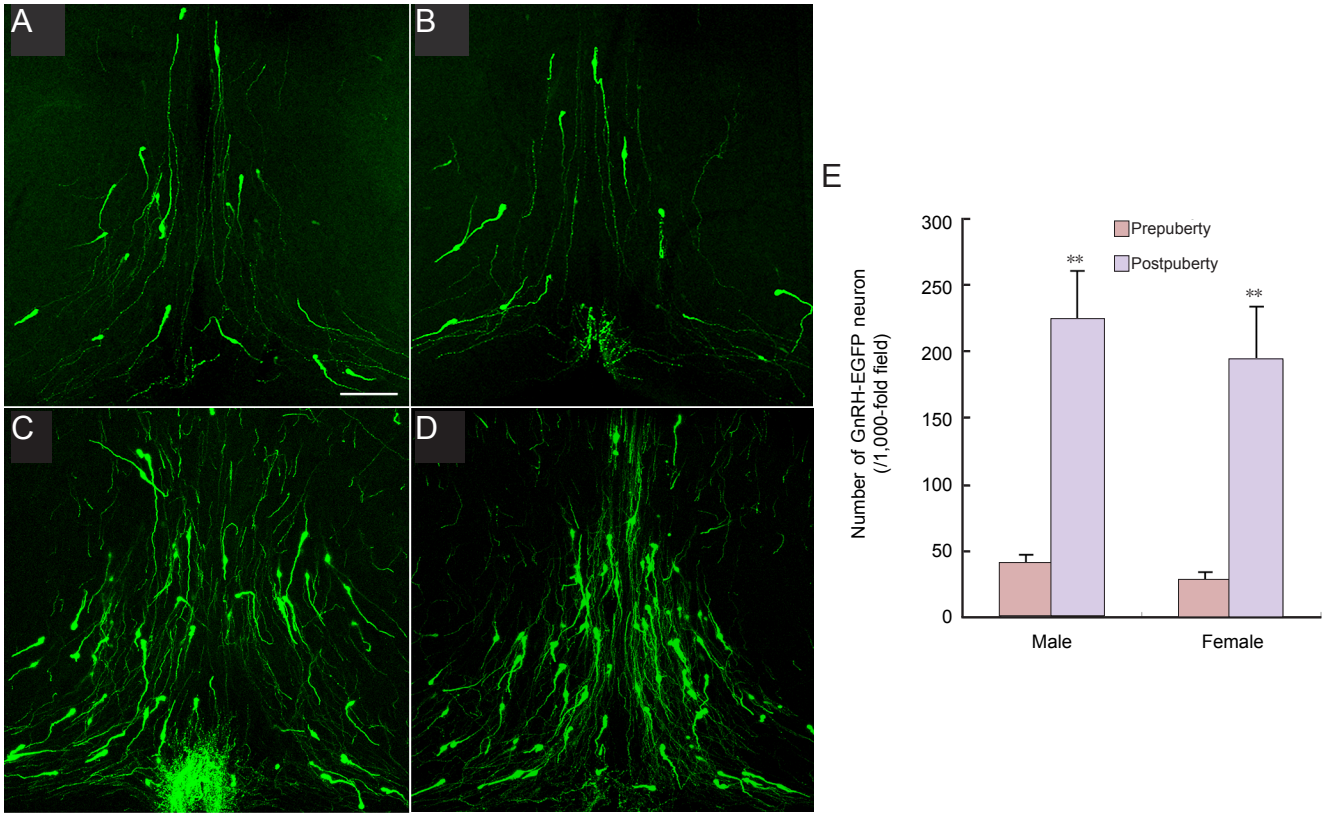


**Figure 1 The effect of prepuberty and postpuberty on the number of GnRH-EGFP neurons in the preoptic area.**
(A–D) Confocal laser scanning images of GnRH-EGFP neurons in the preoptic area showing an inverted Y distribution of these neurons. GnRH neurons in prepubertal (A) male and (B) female rats. GnRH neurons in postpubertal (C) male and (D) female rats. (E) Counts of GnRH-EGFP neurons in prepubertal and postpubertal rats. $**P < 0.05$, *vs.* prepuberty. GnRH: Gonadotropin-releasing hormone; EGFP: enhanced green fluorescent protein. Scale bar: 200 μm.

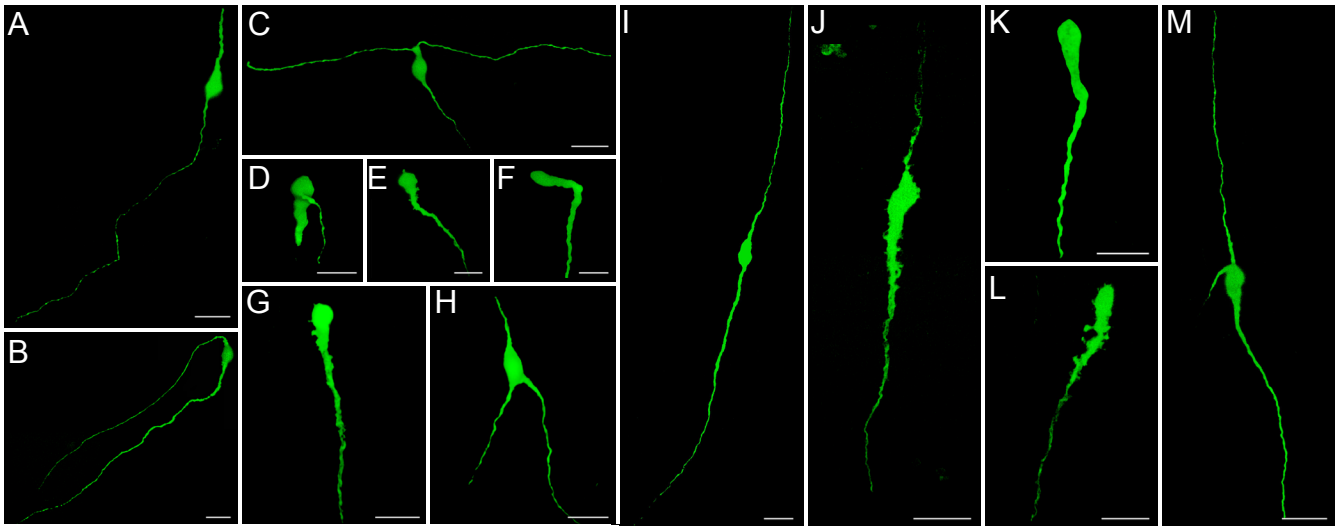


**Figure 2 The effect of prepuberty and postpuberty on the morphology of GnRH-EGFP neurons in the preoptic area.**
(A–H) Confocal laser scan images of GnRH-EGFP neurons in prepubertal rats. (A–D) Smooth type of bipolar GnRH neurons. The bipolar neurons show complex dendritic morphologies as follows: (A) normal smooth bipolar GnRH neurons, (B) differing smooth bipolar GnRH neurons with a unidirectional extension of both dorsal and ventral dendrites, (C) dendritic bifurcation, and (D) two dendrites of different thickness originating from the basal region of the soma. (E) Irregular type of the bipolar neuron. (F) Smooth type of the unipolar GnRH neuron. (G) Irregular type of the unipolar neuron. (H) Multipolar GnRH neuron. (I–M) GnRH-EGFP neurons in postpubertal rats. (I) Smooth type of the bipolar GnRH neuron. (J) Irregular type of the bipolar neuron. (K) Smooth type of the unipolar GnRH neuron. (L) Irregular type of the unipolar neuron. (M) Multipolar GnRH neuron. Scale bars: 25 μm. GnRH: Gonadotropin-releasing hormone; EGFP: enhanced green fluorescent protein.

spines in the cell bodies and initial 50-μm length of dendrites were measured in reconstructed three-dimensional images obtained using the software, Volocity Visualization (Version 3.6.1, Improvision). The number of synaptophysin-positive puncta directly opposite to GnRH-EGFP soma and dendrites were counted and combined to provide mean values for each neuron. The initial 50-μm length of dendrite from the soma was designated for analyzing the number of synaptophysin appositions in the dendrites.

**Statistical analysis**

All data are expressed as mean ± SEM and were analyzed by paired or unpaired Student's *t*-tests using SPSS 19.0 software (IBM Corporation, Armonk, NY, USA). Significance was reached at values of $P < 0.05$.

## Results

**The effect of prepuberty and postpuberty on the number of GnRH-EGFP neurons in the preoptic area**

In the preoptic area of EGFP transgenic rats, EGFP was localized in all GnRH neurons. Furthermore, cell boundaries, including dendrites and spine-like processes, were clearly visible, which are observations that have not been adequately identified with immunocytochemistry (Kato et al., 2003; Farkas et al., 2010; Wojniusz et al., 2011; Sharif et al., 2013; Srivastava et al., 2014). GnRH-EGFP neurons in both the prepubertal and postpubertal preoptic area possessed a similar inverted Y distributional pattern (**Figure 1A–D**). The number of GnRH neurons in the preoptic area was significantly ($P < 0.05$) higher in postpubertal rats compared with prepubertal rats (**Figure 1A–D**). In the preoptic area of male rats, the number of postpubertal GnRH neurons increased 5.5-fold compared with prepubertal neurons (**Figure 1A, C**). In the preoptic area of female rats, the number of postpubertal GnRH neurons increased 7.1-fold compared with prepubertal neurons (**Figure 1B, D**). The number of GnRH-EGFP neurons in the preoptic area between male and female rats was not significantly different (**Figure 1E**).

**The effect of prepuberty and postpuberty on the morphology of GnRH-EGFP neurons in the preoptic area**

Three morphological phenotypes of GnRH-EGFP neurons with medium-sized somata (of 10–16 μm) were detected in both the prepubertal and postpubertal preoptic area. Although fewer (approximately 0.82%) multipolar neurons were detected, GnRH neurons were generally unipolar and bipolar (**Figure 2**). In the preoptic area frontal sections, the majority of unipolar GnRH neurons were oval-shaped with a long dendrite that emerged from the basal part of the soma and extended ventrally (**Figure 2**). In contrast, bipolar GnRH neurons were mostly fusiform-shaped with both dorsal and ventral dendritic extensions (**Figure 2**). Approximately one third of both unipolar and bipolar neurons exhibited oblique dendritic orientation (**Figure 2**). Some GnRH neurons displayed differing morphologies, such as a unidirectional extension of both dorsal and ventral dendrites (**Figure 2B**), bifurcation of dendrites (**Figure 2C**), and two dendrites of different thickness originating from the basal region of the soma (**Figure 2D**). These complex neuronal morphologies were mainly observed in preoptic area bipolar GnRH cells of prepubertal rats (**Figure 2**). In contrast, the postpubertal preoptic area principally exhibited typical bipolar and unipolar GnRH neuronal features (**Figure 2I–L**). The number of unipolar and bipolar GnRH neurons in the preoptic area at prepuberty was similar (**Figure 3A**). Postpubertal GnRH neurons were primarily bipolar compared ($P < 0.05$; **Figure 3B**). No sex differences were found in the number of unipolar and bipolar neurons (**Figure 3A, B**).

Because spine-like processes were detected in nearly all the preoptic GnRH neurons, both unipolar and bipolar GnRH neurons were subdivided into the smooth type with few small processes (the number of processes ≤ 5) and the irregular type with many spine-like processes in the soma and proximal dendrite (the number of processes > 5). In prepubertal and postpubertal rats of both sexes, unipolar GnRH neurons were primarily an irregular type ($P < 0.01$; **Figure 3C, D**), whereas the bipolar neurons were mostly a smooth type ($P < 0.05$ or $P < 0.01$; **Figure 3E, F**). In prepubertal rats, irregular GnRH neurons were similar to the smooth type (**Figure 3G**). The number of smooth type neurons in postpubertal rats was not significantly higher than the irregular type (**Figure 3H**). Both the smooth and irregular GnRH-EGFP neurons were significantly ($P < 0.05$) increased following the development of sexual maturation (**Figure 3A–H**). No significant differences were observed in the GnRH neuronal sub-types between males and females at both maturation periods (**Figure 3I, J**).

**The effect of prepuberty and postpuberty on the number of spine-like processes of GnRH-EGFP neurons in the preoptic area**

Spine-like processes were observed in all examined GnRH-EGFP neurons, including the somata and dendrites (**Figure 4A–D**). Because the distal extremities of most dendrites were cut off during processing of the sections, the length of every dendrite appeared to be largely different. Furthermore, the majority of processes were present within the first 50 μm of the dendrites, and counts of processes revealed the number of processes according to their length from the soma. We found a gradual reduction in the number of processes with increasing distance from the soma (**Figure 4A–C**). Therefore, in the present study, soma and the initial 50-μm length of dendrite were used for counting the number of spine-like processes and synaptophysin appositions in GnRH-EGFP neurons. In prepubertal rats, the average number of spines in smooth GnRH neurons was 3.5 ± 1.02 and the mean irregular type in unipolar bipolar GnRH neurons consisted of 16.9 ± 4.15 spine-like processes. The number of spine-like processes increased in postpubertal neurons (19.7 ± 5.83), but no significant differences were detected between prepubertal and postpubertal rats (**Figure 4E, F**). Sex differences in the number of spine-like processes

were not detected in prepubertal or postpubertal GnRH neurons (**Figure 4E**).

**The effect of prepuberty and postpuberty on the synaptic inputs to GnRH-EGFP neurons in the preoptic area**

To evaluate synaptic inputs to GnRH neurons, we examined the apposition of synaptophysin to the somata and proximal dendrites of GnRH neurons by the confocal analysis. Plain images showed that numerous synaptophysin-immunoreactive punctae were in contact with GnRH neuronal somata and proximal dendrites (**Figure 4A–C**). However, few synaptophysin punctae was found adjacent to GnRH neurons. Three-dimensional rotation images showed that the apposition of synaptophysin punctae to GnRH neurons was approximately one third of the plan images. Some punctate staining of synaptophysin in the plan images was observed to be in contact with GnRH neuronal dendrites and/or somata, whereas three-dimensional image rotation along the dorsoventral axis indicated that they had exited the neuronal dendrites/somata (**Figure 4A, B**). This punctate staining of synaptophysin adjacent to the GnRH neurons in the plan images did not make contact with the dendrites and/or somata of GnRH neurons. The close apposition of synaptophysin with GnRH neurons occurred mainly on the proximal dendrites of GnRH neurons and with fewer observations on the somata and distal dendrites (**Figure 4A–D**). In the irregular GnRH neurons of postpubertal rats, a significant ($P < 0.05$) increase of close appositions between synaptophysin and GnRH neurons was found in both sexes, and was not observed in the prepubertal rats (**Figure 4F**). However, no differences were detected between prepubertal and postpubertal male and female rats. No significant differences were found in the number of synaptophysin punctae in smooth GnRH neurons of both prepubertal and postpubertal rats (data not shown).

## Discussion

Puberty is regarded as a process of maturation of reproductive function, representing the transition from childhood to adulthood in mammals (Ebling, 2005; Clarkson and Herbison, 2006a; Monje et al., 2010). GnRH neurons in the preoptic area may be altered morphologically during the pubertal period when their activities are upregulated (Cottrell et al., 2006; Sharif et al., 2013). Identifying the morphological changes of GnRH neurons following reproductive maturation is helpful in understanding the regulatory mechanisms underlying pubertal onset. GnRH-EGFP transgenic animals exhibit stronger EGFP expression in GnRH neurons (Spergel et al., 1999; Cariboni et al., 2007; Rosati et al., 2011; Shapiro et al., 2011; Vollaard et al., 2011) and clearly show dendritic arborizations and spine-like processes of these cells (Clarkson and Herbison, 2006b; Cottrell et al., 2006; Rosati et al., 2011). Therefore, in the present study, GnRH-EGFP transgenic rats were used for the morphological investigation of GnRH neurons (including synaptic inputs) in the preoptic area.

GnRH-EGFP neurons at both prepubertal and postpubertal stages in animals reveal a typical distribution pattern of GnRH neurons. The majority of GnRH cell bodies are present in the preoptic area where these neurons are involved in the generation of GnRH/luteinizing hormone, which initiates ovulation in mammals (Kinoshita et al., 2005; Clarkson and Herbison, 2006b; Adachi et al., 2007). However, the number of GnRH neurons varies greatly. Our results showed a predominance of GnRH-EGFP neurons in postpubertal rats over prepubertal animals. However, a previous study has shown a constant distribution of GnRH neurons throughout postnatal development in rats (Gao et al., 2011). The discrepancy between the two studies may lie in the different animals and methods used. The topographical distribution of GnRH neurons in the forebrain is fully established at the earliest neonatal stages, which may be involved in sexual dimorphism of the preoptic area (Gao et al., 2011). Results from our study indicate that a decline in GnRH synthetic capacity or transcriptional activity at prepuberty may result in lower EGFP expression in GnRH neurons of the transgenic rats. Furthermore, although the activity of GnRH neurons at prepuberty is lower, these neurons may have the capacity to synthesize GnRH, and the total cell number may remain unchanged during postnatal development. Therefore, low expression of GnRH peptide in wild-type rats may be labeled by immunocytochemistry. Sex differences throughout development in GnRH neurons of the preoptic area that were detected by Gao et al. (2011) were not observed in the present study. The reason for this is unknown and should be clarified in future studies.

Bipolar and unipolar GnRH neurons have been previously classified as smooth type and irregular type with spine-like processes (Krisch, 1980; Sharova et al., 2013). Similarly to previously studies using immunocytochemistry, GnRH-EGFP neurons in the present model exhibited spine-like processes on bipolar neurons as well as on unipolar neurons (Saito et al., 2010; Gao et al., 2011; Sharif et al., 2013; Srivastava et al., 2014). These processes were observed on almost every GnRH-EGFP neuron. Because dendritic spines are typically involved in excitatory synapses (Clarkson and Herbison, 2006a; Cottrell et al., 2006), both unipolar and bipolar GnRH neurons in the present study were further subdivided into smooth type with few small processes and irregular type with numerous spine-like processes on somata and dendrites. Most unipolar neurons showed the irregular type whereas the majority of the bipolar neurons were the smooth type at all ages. Irregular GnRH neurons predominated at prepuberty, whereas no significant differences were detected between the two sub-types at postpuberty. Both sub-types of GnRH neurons significantly increased during the puberty period. However, a previous study using immunocytochemistry revealed a decrease in smooth neurons and an increase in irregular neurons with a constant total number of GnRH neurons during postnatal development (Sharif et al., 2013). The reason for the discrepancy between the two studies may be a technical variation and a different mode of

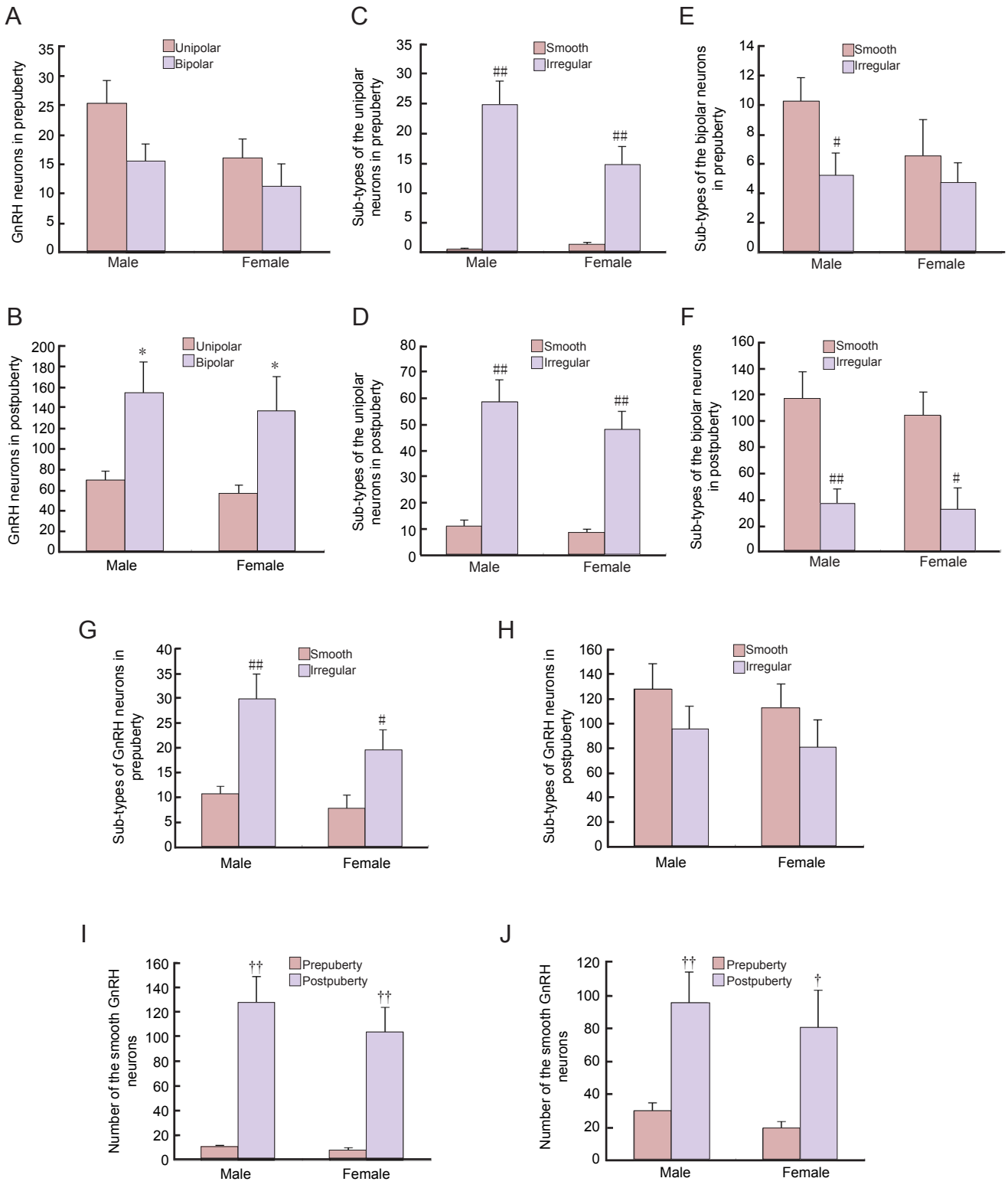


**Figure 3 Counts of GnRH-EGFP neurons in the preoptic area in both sexes.**
Counts of both unipolar and bipolar GnRH neurons in (A) prepubertal and (B) postpubertal rats. Counts of both smooth and irregular types in (C) prepubertal and (D) postpubertal unipolar GnRH neurons. Counts of the two sub-types in bipolar GnRH neurons at (E) prepuberty and (F) postpuberty. Counts of both smooth and irregular GnRH neurons in (G) prepubertal and (H) postpubertal rats. Counts of (I) smooth and (J) irregular GnRH neurons in rats at different times. (A–J) Data were expressed as mean ± SEM (/1,000-fold field) and there were four rats in each group. Statistical differences were analyzed using paired Student's *t*-tests. $*P < 0.05$, *vs.* unipolar; $\#P < 0.05$, $\#\#P < 0.01$, *vs.* smooth type; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, *vs.* prepuberty. GnRH: Gonadotropin-releasing hormone; EGFP: enhanced green fluorescent protein.

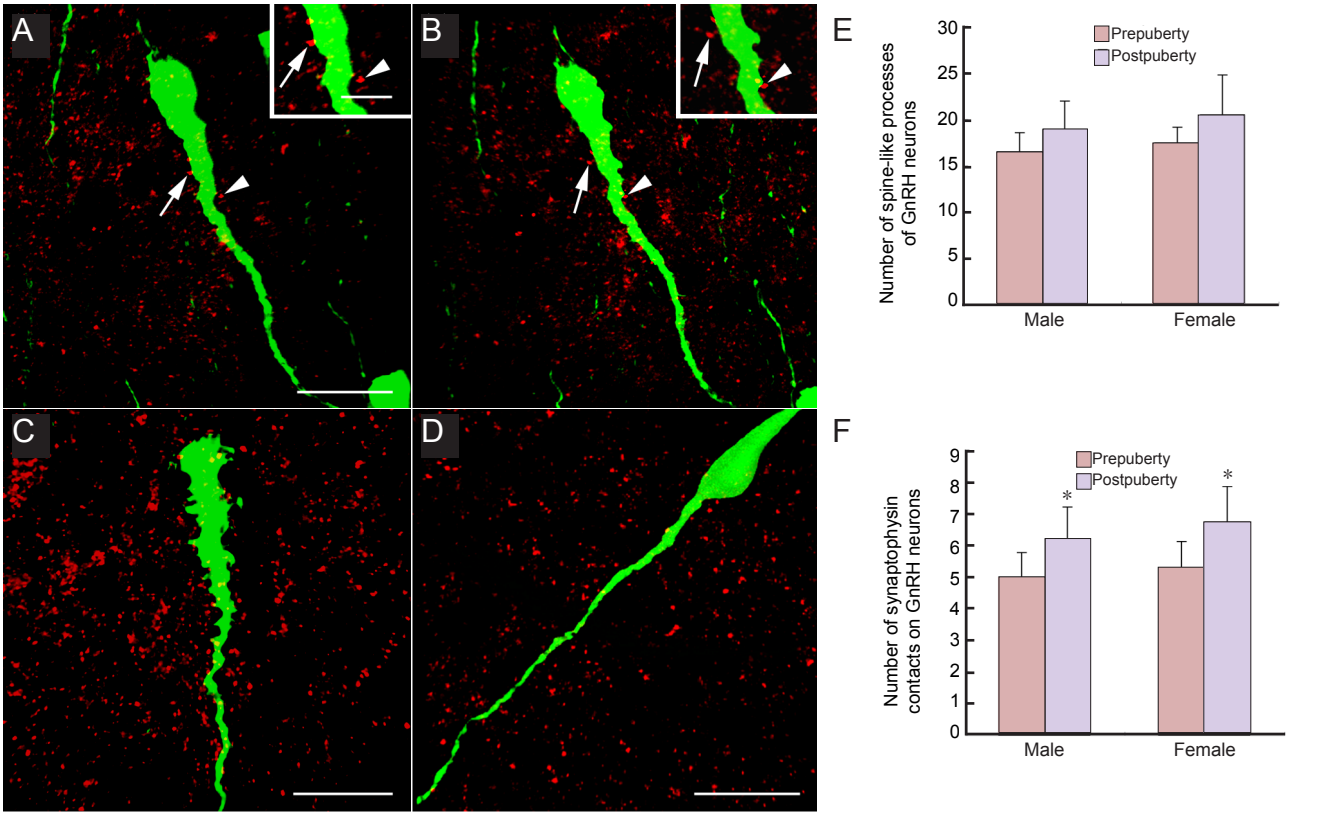


**Figure 4 The effect of prepuberty and postpuberty on the synaptic inputs to GnRH-EGFP neurons in the preoptic area.**
Confocal laser scan images of synaptophysin punctae (red) adjacent to GnRH neurons (green) in (A, B) prepubertal and (C, D) postpubertal rats. (A) Fluorescence staining showing that synaptophysin punctae contacts with the GnRH neuron and is mostly present on proximal dendrites (arrow) and scarcely on somata (arrowhead indicates synaptophysin punctae not in contact with the GnRH neuron). (B) Synaptophysin punctae adjacent to the GnRH neuron (arrow) (shown in A) exits the dendrite. Synaptophysin punctae not in contact with the (A) neuron in the fluorescence staining is in contact with the neuron in three-dimensional rotated images along the dorsoventral axis (A–D). Insets are higher-power views of synaptophysin punctae, indicated by an arrow and an arrowhead in (A) and (B), respectively. (C) Synaptophysin punctae in close appositions with GnRH neurons consisting of many spine-like processes. (D) Fewer synaptophysin punctae adjacent to GnRH neurons. (E) Counts of spine-like processes between prepubertal and postpubertal rats. (F) Counts of synaptophysin punctae in contact with GnRH neuron in prepubertal and postpubertal rats. $*P < 0.05$, *vs.* prepuberty. GnRH: Gonadotropin-releasing hormone; EGFP: enhanced green fluorescent protein. Scale bars: 25 μm (A–D), 10 μm in insets.

animal selectivity.

Immunofluorescence for GnRH neurons in mice has revealed that spines are principally present on somata and the first 30 μm of dendrites (Cottrell et al., 2006). However, the study did not show spines on dendrites beyond this length. GnRH-EGFP transgenic rats in the present study provided a clearer image than that of immunofluorescence. However, the majority of spine-like processes were detected on the proximal dendrites of irregular GnRH neurons. A trend was only observed for an increase in spine-like processes on GnRH neurons during puberty. In mice, spine numbers on GnRH-EGFP neurons increase two-fold between the end of the second week after birth and adulthood (Cottrell et al., 2006). Numerous spine-like processes were also seen on the cell bodies (Cottrell et al., 2006; Rosati et al., 2011). Biocytin filling of GnRH-EGFP neurons may provide a better identification of small processes on neurons compared with the present GnRH-EGFP neurons. In the present study, the somata exhibited fewer processes. The spines are known to represent the location of synaptic inputs to neurons (Krisch, 1980; Shepherd, 1996; Cariboni et al., 2007; Xu et al., 2011; Merriam et al., 2013; Sharova et al., 2013). Therefore, excitatory synaptic inputs to GnRH neurons may be predominantly transmitted *via* the spine-like structures of irregular GnRH neurons. An increase in spine density or number has been suggested to elevate direct excitatory inputs to GnRH neurons during puberty (Cottrell et al., 2006).

Ultrastructural studies in rats have revealed the presence of synapses on all GnRH neurons, including both smooth and irregular types (Xu et al., 2011; Sharova et al., 2013). However, irregular GnRH neurons possess more synaptic profiles compared with smooth neurons (Sharova et al., 2013). Nevertheless, the functional distinction of the two neuronal subtype is unknown. Smooth GnRH neurons are considered to be associated with sensory input emerging from the olfactory epithelium (Cheung et al., 2011; Sukhbaatar et al., 2014). Lesion of either the olfactory epithelium or olfactory bulb disturbs reproductive function and sexual behavior (Sotonyi et al., 2010; Navarro et al., 2011). Irregular GnRH neurons may receive multiple synaptic inputs or be integrated in a

complex formation (Sharova et al., 2013). Similarly to previous studies, the present immunofluorescence findings showed that most synaptic inputs to GnRH neurons consisted of spine-like processes (Clarkson and Herbison, 2006b; Cottrell et al., 2006). These neurons have been suggested to receive multiple synaptic inputs from GABAergic (Cottrell et al., 2006; McFarlane et al., 2011), glutamatergic (Lin et al., 2003; McFarlane et al., 2011), and kisspeptin fibers (Kinoshita et al., 2005; Clarkson and Herbison, 2006b). Gamma-aminobutyric acid (GABA) and glutamate are the principal inhibitory and excitatory neurotransmitters, regulating the activity of GnRH neurons *via* their respective receptors expressing GnRH neurons in the preoptic area (Lin et al., 2003; Kroll et al., 2010; Papanikolaou et al., 2011; Quintanar et al., 2011). GABA induces a switch from depolarization to hyperpolarization in GnRH neurons of pubertal female mice (Ottem et al., 2002), and rapid GABA activation excites GnRH neurons in adult mice (Han et al., 2002). The excitatory action of GABA on GnRH neurons has also been reported in rats (Kim et al., 2011). Under current-clamp conditions in the rat, the activation of GABA receptor subunits depolarizes GnRH neurons (Kim et al., 2011).

The present study revealed fewer synaptophysin appositions to GnRH neurons, which is consistent with electron microscopic observations showing that GnRH neurons receive relatively sparse synaptic input (Yin et al., 2008; Sharova et al., 2013). Synaptophysin punctae were mostly present on the proximal dendrites of irregular GnRH neurons and rarely on somata and smooth neurons. Synaptophysin is a 38 kDa glycoprotein present on the membrane of neuronal presynaptic vesicles, which may be involved in the formation of synaptic vesicles and exocytosis (Jahn et al., 1985; Arauz et al., 2010). Terminals of the preoptic area containing GABAergic and glutamatergic vesicles that colocalize with synaptophysin provide abundant synaptic inputs to GnRH neurons (McFarlane et al., 2011). Activation of GnRH neurons is a key event in the onset of puberty (Ebling, 2005; Clarkson and Herbison, 2006a; Monje et al., 2010). Kisspeptin is regarded as the most potent activator of GnRH neurons (Clarkson and Herbison, 2006b; Abbasi et al., 2013) and its input and subsequent response are increased during postnatal development in the mouse (Yu et al., 2013). Findings from the present study revealed an increase of synaptophysin punctae in contact with the GnRH neuron during puberty. This result corroborates with Clarkson and Herbison (2006b) who have shown synaptic inputs to GnRH neurons during reproductive maturation. Images from previous studies have shown numerous immunofluorescent punctae adjacent to GnRH neurons (Clarkson and Herbison, 2006b; Cottrell et al., 2006; Rosati et al., 2011). In a similar manner, the present study also showed numerous synaptophysin-immunoreactive punctae closely adjacent to the GnRH neuron in the plain photograph. However, in three-dimensional rotation images, few synaptophysin punctae were in contact with this neuron. Thus, GnRH neurons may receive fewer synaptic inputs, which has been suggested by ultrastructural studies in rats (Han et al., 2005; Yin et al., 2008; Karamizadeh et al., 2013; Rosenfield et al., 2013; Sharova et al., 2013; Thornton et al., 2014). The differences in the number of synaptic inputs to GnRH neurons may reflect the differences between rats and mice or the method of observation (Lee et al., 2012; Lem et al., 2012; Rosenfield et al., 2012). Further morphological studies and the inclusion of electrophysiological studies in both species will be important to effectively clarify this discrepancy.

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**Author contributions:** *Liu Q and Xue HG conceived and designed the experiments. Gai XD performed the experiments. Sun WQ analyzed the data. Li C provided experimental reagents/materials/analysis tools. All authors approved the final version of the manuscript.*

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