Prenatal androgen excess enhances stimulation of the GNRH pulse in pubertal female rats

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Abstract

In adolescent girls with polycystic ovary syndrome (PCOS), neuroendocrine derangements manifest after the onset of puberty, characterized by rapid LH pulse frequency. The early mechanism underlying the pubertal regulation of the GNRH/LH pulsatile release in adolescents with PCOS remains uncertain. To determine the effects of prenatal androgen exposure on the activation of GNRH neurons and generation of LH pulse at puberty, we administrated 5α-dihydrotestosterone to pregnant rats and observed serum LH levels and expression of hypothalamic genes in female offspring from postnatal 4 to 8 weeks. The 6-week-old prenatally androgenized (PNA) female rats exhibited an increase in LH pulse frequency. The hypothalamic expression of neurokinin B (*Nkb* (*Tac2*)) and *Lepr* mRNA levels in PNA rats increased remarkably before puberty and remained high during puberty, whereas elevated *Kiss1* mRNA levels were detected only after the onset of puberty. Exogenous kisspeptin, NK3R agonist, and leptin triggered tonic stimulation of GNRH neurons and increased LH secretion in 6-week-old PNA rats. Leptin upregulated *Kiss1* mRNA levels in the hypothalamus of pubertal PNA rats; however, pretreatment with a kisspeptin antagonist failed to suppress the elevated serum LH stimulated by leptin, indicating that the stimulatory effects of leptin may be conveyed indirectly to GNRH neurons via other neural components within the GNRH neuronal network, rather than through the kisspeptin–GPR54 pathway. These findings validate the hypotheses that NKB and leptin play an essential role in the activation of GNRH neurons and initiation of increased LH pulse frequency in PNA female rats at puberty and that kisspeptin may coordinate their stimulatory effects on LH release.

Key Words
- androgen
- puberty
- gonadotropin-releasing hormone
- neurokinin B
- kisspeptin
- leptin
- polycystic ovary syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is a complex multifactorial disorder characterized by oligo-ovulation or anovulation, hyperandrogenism, polycystic ovaries, luteinizing hormone (LH) hypersecretion, and insulin resistance. Clinical, experimental, and genetic evidence supports the hypothesis that prenatal exposure to excessive maternal androgens induces the PCOS-related phenotype in female offspring (Xita & Tsatsoulis 2006, Forsdike *et al.* 2007, Abbott *et al.* 2009, Roland *et al.* 2010, Tyndall *et al.* 2012). Prenatal androgen excess resets the reproductive and metabolic trajectory of the growing fetus and reprograms target tissue differentiation and development, resulting in the development of PCOS in adolescents and adults (Franks 2012). Peripubertal reproductive and metabolic

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dysfunctions, including anovulation, hyperandrogenism, insulin resistance, and hyperinsulinemia, are present in adolescents with PCOS before the onset of puberty and persist during pubertal development (Huang *et al.* 2010). The significant overlap between these symptoms and the normal physiological changes associated with puberty mean that PCOS is possibly being under-evaluated in the adolescent population, raising serious concerns over the potential and extended public health consequences (Broder-Fingert *et al.* 2009). Therefore, a better understanding of the pathogenesis of PCOS in adolescents may be key for minimizing the development of symptoms and for preventing the onset of the long-term complications associated with this syndrome.

The activation of gonadotropin-releasing hormone (GNRH) neurons and the establishment of pulsatile GNRH secretion during late postnatal development are essential for initiating the process of puberty (Terasawa & Fernandez 2001). On the basis of the ability of mutations or deletion of the kisspeptin receptor (G protein-coupled receptor, GPR54 (KISS1R)) to cause hypogonadotropic hypogonadism and prevent normal pubertal maturation in humans and animal models, compelling evidence indicate that kisspeptin–GPR54 signaling activates the GNRH neurons and triggers the onset of puberty (Clarkson *et al.* 2010). Further studies demonstrated that exogenous kisspeptin potently stimulated GNRH-dependent LH secretion before puberty in both primates and rodents, supporting the hypothesis that kisspeptin has a critical role in GNRH/LH pulsatile release and normal pubertal maturation (Navarro *et al.* 2004, Plant *et al.* 2006). Recent studies have shown that mutations of the genes encoding either neurokinin B (*NKB*) or its cognate receptor (*NK3R*) resulted in hypogonadotropic hypogonadism, similar to mutations of the kisspeptin system (Guran *et al.* 2009, Topaloglu *et al.* 2009). Pulsatile administration of GNRH to adults with *NKB* or *NK3R* gene mutations restored serum LH and testosterone secretion in males and resulted in ovulation, pregnancy, and a normal birth in a female (Young *et al.* 2010). These data implicate NKB signaling as an essential component for the onset of puberty and the control of GNRH release. However, the effects of NKB on the regulation of GNRH/LH secretion remain controversial in humans and animal models. In the ewe, activation of NK3R with senktide, a potent and selective NK3R agonist, consistently stimulates LH secretion (Billings *et al.* 2010). In contrast to humans and sheep, senktide suppressed LH secretion in rats (Sandoval-Guzman & Rance 2004) and mice (Castellano *et al.* 2009).

In 2007, both NKB and kisspeptin, along with dynorphin (DYN), were shown to be colocalized in a single subpopulation in the arcuate nucleus (ARC) of the sheep (Goodman *et al.* 2007). Further studies demonstrate that a subset of neurons colocalizing kisspeptin, NKB, and DYN in the ARC, abbreviated as the KNDy subpopulation, are strongly conserved across multiple mammalian species, from rodents to humans, and are part of the neural network modulating pulsatile GNRH secretion. On the basis of further observations, researchers have speculated that kisspeptin stimulates and DYN inhibits GNRH pulses; however, the actions of NKB remain controversial. These data raise the possibility that dysfunctional coordination of the KNDy network may underlie neuroendocrine defects in clinical reproductive disorders, including PCOS (Lehman *et al.* 2010). Adolescents with PCOS show disruption in the regulation of the GNRH pulse generator, characterized by rapid LH pulse frequency (Burt Solorzano *et al.* 2010). To date, the limited investigations have focused on the early neurobiological mechanisms underlying pubertal regulation of the GNRH/LH pulsatile release by kisspeptin and NKB in adolescents with PCOS.

The aim of this study was to determine the effect of intrauterine androgen exposure on LH secretion and the role of kisspeptin and NKB neurons in the alteration of GNRH/LH pulsatile secretion in female offspring of androgen-exposed rats at peripubertal stage. The levels of serum LH and expression of hypothalamic genes in prenatally androgenized (PNA) female rats were observed at postnatal weeks 4, 6, and 8. Through i.c.v. administration, we investigated whether the resulting neuroendocrine derangements could be attributed to the modulation of kisspeptin and NKB neurons in the hypothalamic ARC of PNA rats. We also elucidated the interaction of kisspeptin, NKB, and leptin on the regulation of pulsatile GNRH/LH secretion at puberty.

Materials and methods

Animals

Both female and male Sprague–Dawley rats were purchased from the Lab Animal Center of Nanjing Medical University and animal care was conducted in accordance with the Animal Research Committee Guidelines of Nanjing Medical University. The rats were housed at 25 °C under a 12 h light:12 h darkness cycle, with a humidity of 65–70%. Water and food were available and animals were allowed to eat and drink *ad libitum*. The Institutional Ethics Committee of Nanjing Medical University approved all the experimental procedures.

Female rats were mated with males overnight and copulatory plugs were examined the next morning. The

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day on which copulatory plugs were verified was arbitrarily considered as day 1 of gestation. On days 16–19 of gestation, the rats received s.c. injections of either 5 mg/day 5α-dihydrotestosterone (DHT; Dr. Ehrenstorfer GmbH, Augsburg, Germany) dissolved in 500 μl sesame oil (Sigma)/benzyl benzoate (Sigma) (DHT, $n=30$) or a vehicle as a control (C, $n=24$). The dose of DHT was selected on the basis of previous experiments (Foecking *et al.* 2005, Yan *et al.* 2013). All litters were weaned and females were separated from males at 21 days of age. Female offspring were observed from 4 weeks (prepuberty) to 8 weeks (late puberty) (Fig. 1).

Experimental design

Experiment 1: measurement of serum LH in PNA rats At postnatal weeks 4, 6, and 8 blood samples were collected by orbital puncture after the rats were fasted overnight and anesthetized with pentobarbital sodium at 0800 h. In addition, to determine whether prenatal androgen excess induced the increased LH pulse frequency in female rats at puberty, 6-week-old PNA ($n=8$) and control rats ($n=7$) were anesthetized and blood samples were taken by jugular venipuncture every 10 min for 3 h (between 0800 and 1100 h). After each blood sample was drawn (100 μl), an equal volume of heparinized saline (25 U heparin sodium/ml normal saline) was infused. The sera were stored at −80 °C until LH measurement.

Experiment 2: effect of prenatal androgen excess on expression of hypothalamic genes in PNA rats To analyze whether prenatal androgen exposure influenced the expression of genes associated with the regulation of GNRH/LH secretion in the hypothalamus of female rats during peripubertal development, the mRNA levels of *Kiss1*, *Kiss1r*, *Nkb*, *Nk3r* (*Tacr3*), *Npy*, *Npyr* (*Npy1r*), *Lepr*, *Gnrh* (*Gnrh1*), *Agrp*, and *Pomc* were evaluated in hypothalamic samples from peripubertal PNA rats. At the ages of 4, 6, and 8 weeks the female rats were decapitated and their hypothalamuses were immediately dissected, as described previously (Quennell *et al.* 2011). The hypothalamic samples were frozen in liquid nitrogen and stored at −80 °C until RNA analysis.

Experiment 3: coexpression of kisspeptin/LEPR and NKB/LEPR in the hypothalamic ARC of PNA rats This experiment was intended to determine whether kisspeptin and NKB neurons in the hypothalamic ARC of pubertal female rats coexpress the leptin receptor. The 6-week-old PNA ($n=6$) and control rats ($n=6$) were anesthetized and perfused with ice-cold normal saline, followed by 4% paraformaldehyde for brain collection. Brains were removed from the skulls, postfixed overnight in 4% paraformaldehyde, and transferred gradually into 20 and 30% sucrose until settled. Coronal sections (40 μm thick) throughout the ARC were cut from each brain on a freezing microtome (Leica, Nussloch, Germany) for immunofluorescence staining.

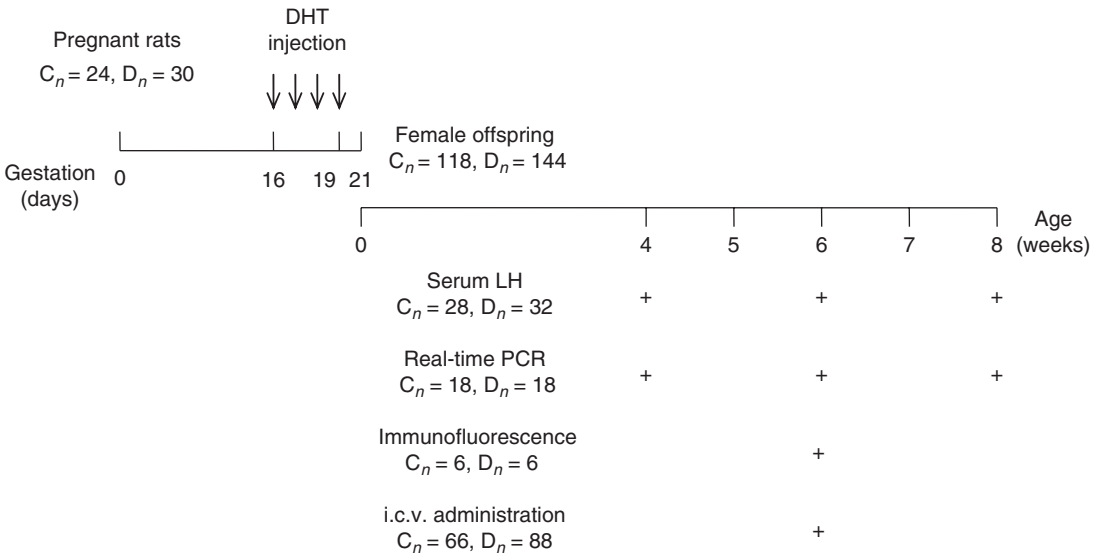

Figure 1
Study schema with the number of animals used in each experiment. C_n, number of control rats; D_n, number of rats treated prenatally with DHT; DHT, 5α-dihydrotestosterone.

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Experiment 4: effects of central administration of kisspeptin, senktide, and leptin on LH secretion in pubertal PNA rats This experiment was intended to assess whether i.c.v. administration of kisspeptin, senktide, or leptin affected serum LH secretion in pubertal PNA rats. The 5-week-old female rats were anesthetized and an i.c.v. infusion cannula (RWD Life Science, Shenzhen, China) was implanted in the lateral cerebral ventricle. The insertion point was 1.5 mm lateral and 1 mm posterior to bregma and 4.0 mm below the surface of the skull. The animals were allowed to recover for 7 days. The PNA ($n=16$) and control groups ($n=16$) were equally divided and received i.c.v. injections of kisspeptin (1 nmol/10 μl, Phoenix Pharmaceuticals, Belmont, CA, USA) or vehicle (10 μl 0.9% sodium chloride) respectively. The dose concentration of kisspeptin was selected on the basis of previous studies that reported that this dose potently elicited LH secretion in rats (Castellano *et al.* 2006, 2009). Blood samples were collected by jugular venipuncture before (0 min) and 15, 30, and 60 min after kisspeptin injection. The sera were stored at −80 °C until LH measurement. Central administration of senktide (600 pmol/10 μl, Sigma) and leptin (1 nmol/10 μl, Sigma) was conducted in the animals and sera were collected, following protocols similar to those for kisspeptin administration. The doses of senktide and leptin were selected on the basis of previous studies (Roa *et al.* 2008, Navarro *et al.* 2012). The integrated LH secretory responses were expressed as the area under the curve (AUC) over a 60 min period after the administration.

Experiment 5: effects of central administration of kisspeptin, senktide, and leptin on expression of hypothalamic genes in pubertal PNA rats This experiment was intended to explore whether the interaction of kisspeptin, NKB, and leptin was responsible for the impaired regulation of GNRH/LH secretion in PNA rats at puberty. The 5-week-old female rats had an i.c.v. infusion cannula implanted, as described for experiment 4. One week later, the PNA ($n=18$) and control rats ($n=18$) were decapitated 30 min after central administration of kisspeptin, senktide, or leptin. The hypothalamus was immediately dissected, frozen in liquid nitrogen, and stored at −80 °C until RNA analysis.

Experiment 6: effect of leptin on serum LH secretion in pubertal PNA rats pretreated with a kisspeptin antagonist To investigate whether leptin stimulates LH secretion indirectly by activating the ARC kisspeptin neurons at puberty, 6-week-old PNA rats were pretreated with a kisspeptin receptor antagonist (kisspeptin-234) and then serum LH levels were evaluated after administration of leptin. The 5-week-old PNA rats had an i.c.v. infusion cannula, implanted as described for experiment 4. One week later, the PNA rats ($n=8$) were treated with kisspeptin-234 (2 nmol/5 μl, Tocris Bioscience, Bristol, UK) over 5 min, followed by leptin (1 nmol/5 μl) 10 min later. Negative control rats ($n=7$) were pretreated with kisspeptin-234 followed by vehicle (5 μl 0.9% sodium chloride); positive control rats ($n=7$) received vehicle before leptin treatment.

Measurement of serum LH

The serum LH concentrations were measured using commercial rat LH ELISA Kits (Shibayagi Co. Ltd, Gunma, Japan), according to the manufacturer's recommendations. The sensitivity of the LH assay was 0.313 ng/ml. The intra- and inter-assay coefficients of variation were <5%.

RNA analysis by real-time PCR

The real-time PCR was conducted using a slight modification of a previously described method (Li *et al.* 2010). Total RNA was isolated from hypothalamic samples using TRIzol reagent (Invitrogen) and 1 μg RNA from each rat was reverse-transcribed into cDNA. The primers used for the assessment of hypothalamic genes are listed in Table 1. The nucleotide sequences for these genes were designed using Primer Premier 5.0 Software (Premier Biosoft International, Palo Alto, CA, USA) and based on NCBI rat reference sequences. The primers were synthesized commercially by Invitrogen. β-actin was used as the internal standard. The final reaction mixture contained 1 μl cDNA, 10 pmol each forward and reverse primer, 0.4 μl ROX reference dye, and 10 μl SYBR Premix Ex Taq (Takara Bio, Dalian, China) in a total reaction volume of 20 μl. The PCR conditions for the genes, except for *Nkb*, were 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The amplification conditions for *Nkb* were 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. Quantitative analysis of genes was carried out using the StepOnePlus Real-Time PCR System and StepOne Software v2.1 (Applied Biosystems) according to the recommendations of the manufacturer.

Double-label immunofluorescence

Free-floating sections were collected, rinsed with PBS, and preincubated with blocking solution (10% donkey serum) for 60 min at 37 °C. The sections were then incubated with a solution containing chicken anti-leptin receptor antibody (Abcam, Inc., Cambridge, MA, USA) and either

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Table 1 The primers used for assessment of hypothalamic genes by real-time PCR

Gene	Primer sequence (5′– 3′)	Product size (bp)	GenBank accession no.
Kiss1		152	NM_181692
Forward	AGCTGCTGCTTCTCCTCTGT		
Reverse	AGGCTTGCTCTCTGCATACC		
Kiss1r		222	NM_023992
Forward	GCAGACCGTCACCAATTTCT		
Reverse	GGGAACACAGTCACGTACCA		
Nkb		101	NM_019162
Forward	GAGGAACAGCCAACCAGACA		
Reverse	GAGTGGAGTGCTTTTCTGCAC		
Nk3r		111	NM_017053
Forward	AGCAGCTGAAGGCTAAACGA		
Reverse	GGTAGATCGCAGTGAGAATGAA		
Npy		98	NM_012614
Forward	AGAGATCCAGCCCTGAGACA		
Reverse	TCACCACATGGAAGGGTCTT		
Npyr		89	NM_001113357
Forward	ACGTTCGCTTGAAAAGGAGA		
Reverse	CATGACGTTGATTCGTTTGG		
Lepr		185	NM_012596
Forward	CCCCCACTGAAAGACAGCTT		
Reverse	GGCTTCACAACAAGCATGGG		
Gnrh		150	NM_012767
Forward	CCGCTGTTGTTCTGTTGACTGTG		
Reverse	GGGGTTCTGCCATTTGATCCTC		
Agrp		76	NM_033650
Forward	AAGCTTTGGCAGAGGTGCTA		
Reverse	GACTCGTGCAGCCTTACACA		
Pomc		76	NM_139326
Forward	TCCTCAGAGAGCTGCCTTTC		
Reverse	CCTGAGCGACTGTAGCAGAA		
β-actin		191	NM_031144
Forward	TGCCGCATCCTCTTCCTC		
Reverse	GGTCTTTACGGATGTCAACG		

rabbit anti-kisspeptin antibody (Merck Millipore, Billerica, MA, USA) or rabbit anti-NKB antibody (Phoenix Pharmaceuticals) for 24 h at 4 °C. Then, the sections were washed again with PBS and incubated with donkey anti-chicken antibody conjugated to Alexa Fluor 488 (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) and donkey anti-rabbit antibody conjugated to Alexa Fluor 555 (Invitrogen) for 30 min at 37 °C. After further washes, free-floating sections were mounted on slides and cover slips were applied. The images were taken using a Zeiss LSM 710 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany) and analyzed using ZEN 2011 Image Software (Carl Zeiss). The numbers of single- and double-labeled neurons were counted in four sections that contained the caudal portion of the ARC in each animal by two persons blinded to the origin of the sections.

Statistical analysis

The serum LH pulses were analyzed using the PULSAR Analysis Software (Mostari *et al*. 2013). The effects of DHT on serum LH and hypothalamic genes expression at the same development stage were analyzed by two-tailed Student's *t*-tests. In the 6-week-old PNA and control rats treated with kisspeptin, senktide, or leptin, serum LH values from the treatment groups and vehicle groups were compared at each time point using repeated measures ANOVA. The 0- to 60-min AUC for the integrated LH secretory responses after central administration was analyzed by two-way ANOVA. All results were expressed as the mean ± S.E.M., $P<0.05$ was considered significant.

Results

Effect of prenatal androgen excess on serum LH in peripubertal female rats

The morning serum LH levels did not significantly differ between the PNA groups and control groups at the ages of 4, 6, and 8 weeks ($P>0.05$; Fig. 2A). Nevertheless, the serum LH pulse frequency in 6-week-old PNA rats was significantly higher than that for the control rats ($P<0.05$;

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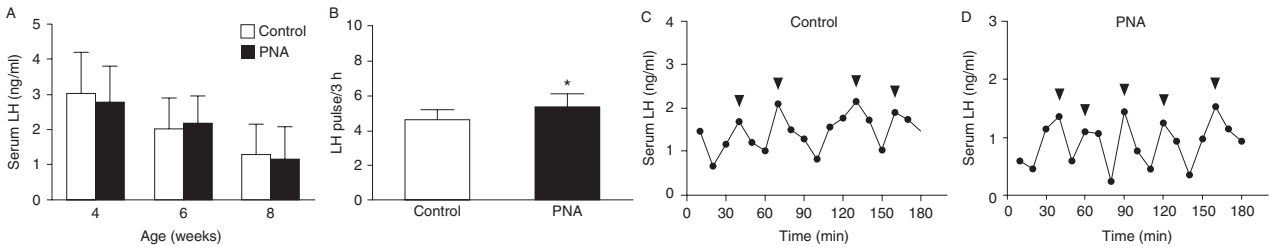

Figure 2
Effect of prenatal DHT treatment on serum LH release in peripubertal female rats. (A) The morning serum LH concentrations in control ($n=21$) and prenatally androgenized (PNA) rats ($n=24$) at postnatal weeks 4, 6, and 8. (B) At the age of 6 weeks, blood samples were taken by jugular venipuncture every 10 min from 0800 to 1100 h and the LH pulse frequency was measured in control ($n=7$) and PNA female rats ($n=8$). (C and D) Representative examples showing serum LH pulses from 0800 to 1100 h in 6-week-old control (C) and PNA female rats (D). Arrowheads indicate peaks of LH pulses determined by PULSAR Analysis Software. Values are expressed as the mean ± S.E.M. *$P<0.05$ vs corresponding control values.

Fig. 2B). The amplitude of LH pulses was not significantly different between the two groups (data not shown).

Effect of prenatal androgen excess on expression of hypothalamic genes in PNA rats

PNA treatment resulted in markedly elevated mRNA levels of *Kiss1* in the hypothalamus of 6- and 8-week-old PNA rats compared with the controls ($P<0.01$), whereas no differences were observed in the levels of *Kiss1* mRNA at the age of 4 weeks between two groups (Fig. 3A). At postnatal weeks 4, 6, and 8 the hypothalamic expression levels of *Nkb* mRNA were significantly increased in the PNA rats compared with the controls ($P<0.01$ or $P<0.05$), and were especially high in the 4-week-old PNA rats (Fig. 3C). Moreover, elevated *Nk3r* mRNA levels were

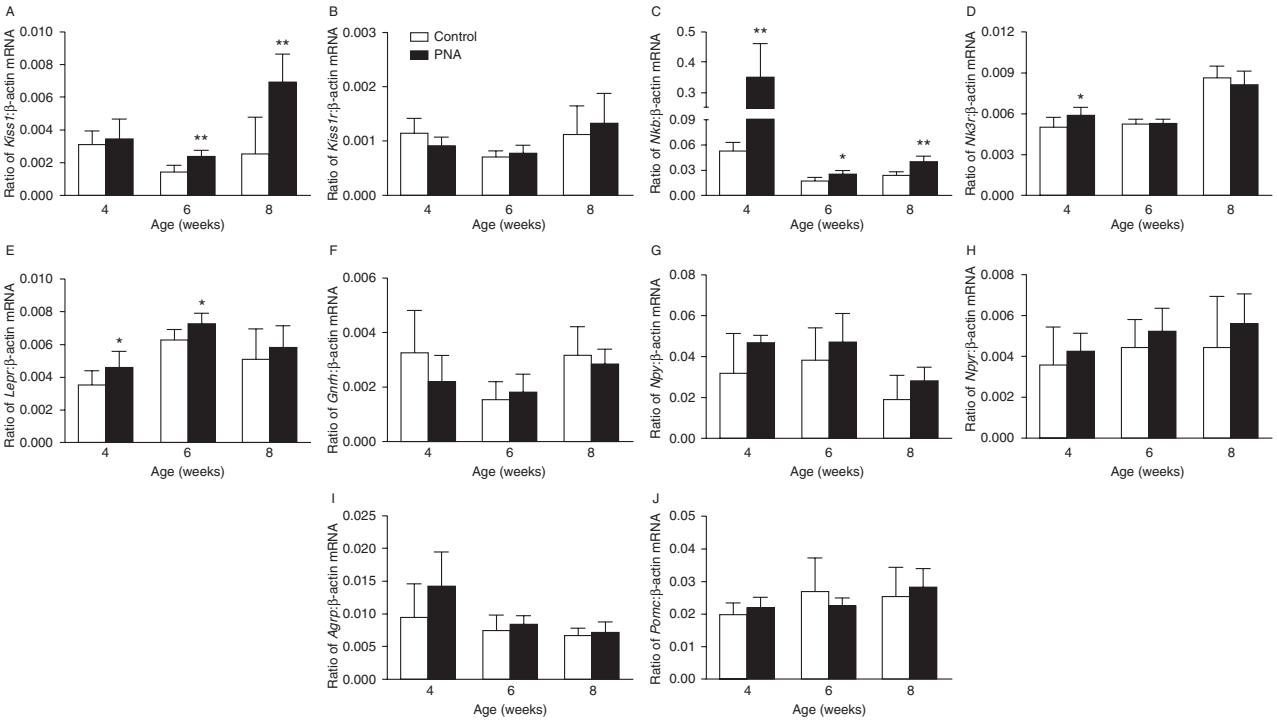

Figure 3
Effect of prenatal DHT treatment on the hypothalamic genes expression in peripubertal female rats. The values represent mRNA expression levels of (A) *Kiss1*, (B) *Kiss1r*, (C) *Nkb*, (D) *Nk3r*, (E) *Obrb*, (F) *Gnrh*, (G) *Npy*, (H) *Npyr*, (I) *Agrp*, and (J) *Pomc* in the hypothalamus of control ($n=18$) and prenatally androgenized (PNA) rats ($n=18$) at postnatal 4, 6, and 8 weeks. Values are expressed as the mean ± S.E.M. *$P<0.05$ and **$P<0.01$ vs corresponding control values.

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detected in the hypothalamus of PNA rats at postnatal 4 weeks ($P<0.05$; Fig. 3D). The 4- and 6-week-old PNA rats had significantly increased *Lepr* mRNA levels in the hypothalamus compared with the controls ($P<0.05$), whereas the 8-week-old PNA rats did not show altered *Lepr* mRNA levels (Fig. 3E). No differences were observed in the mRNA levels of *Kiss1r*, *Gnrh*, *Npy*, *Npyr*, *Agrp*, and *Pomc* between two groups at any development stage ($P>0.05$; Fig. 3B, F, G, H, I and J).

Coexpression of kisspeptin/LEPR and NKB/LEPR in the hypothalamic ARC of pubertal PNA rats

The colocalization of kisspeptin/LEPR and NKB/LEPR in the ARC of 6-week-old PNA rats was observed using double-label immunofluorescence. A cluster of cell bodies with kisspeptin immunoreactivity were detected in the ARC, with more kisspeptin-positive neurons per section in the caudal region than in the middle and rostral region of this nucleus. In the caudal ARC, nearly 90% of kisspeptin-positive neurons coexpressed LEPR (Fig. 4A, B and C). Moreover, numerous NKB-immunoreactive neurons were also observed in the caudal ARC of 6-week-old PNA rats. LEPR immunoreactivity was detected in some NKB-containing cells; the percentage of NKB neurons expressing LEPR was ~70% (Fig. 4D, E and F). Double labeling of kisspeptin/NKB and leptin receptor by immunofluorescence revealed that all three substances were concomitantly expressed in the ARC of pubertal PNA rats.

Actions of kisspeptin, senktide, and leptin upon LH secretion in pubertal PNA rats

After i.c.v. injection of kisspeptin, LH levels at 15, 30, and 60 min were significantly higher in both the 6-week-old PNA group and the control group compared with vehicle control group ($P<0.01$ or $P<0.05$; Fig. 5A and B). In particular, the PNA and control rats displayed five- and threefold increases in circulating LH levels at 60 min after administration of kisspeptin respectively. Senktide, the NK3R agonist, induced a robust increase in serum LH levels at 15, 30, and 60 min after central administration in 6-week-old PNA rats ($P<0.01$ or $P<0.05$; Fig. 5E). In the control group, LH levels at 15 and 30 min were markedly elevated after injection of senktide ($P<0.01$ or $P<0.05$), whereas LH levels at 60 min decreased and were not significantly different from those for animals that received the vehicle (Fig. 5D). The 6-week-old PNA rats treated with

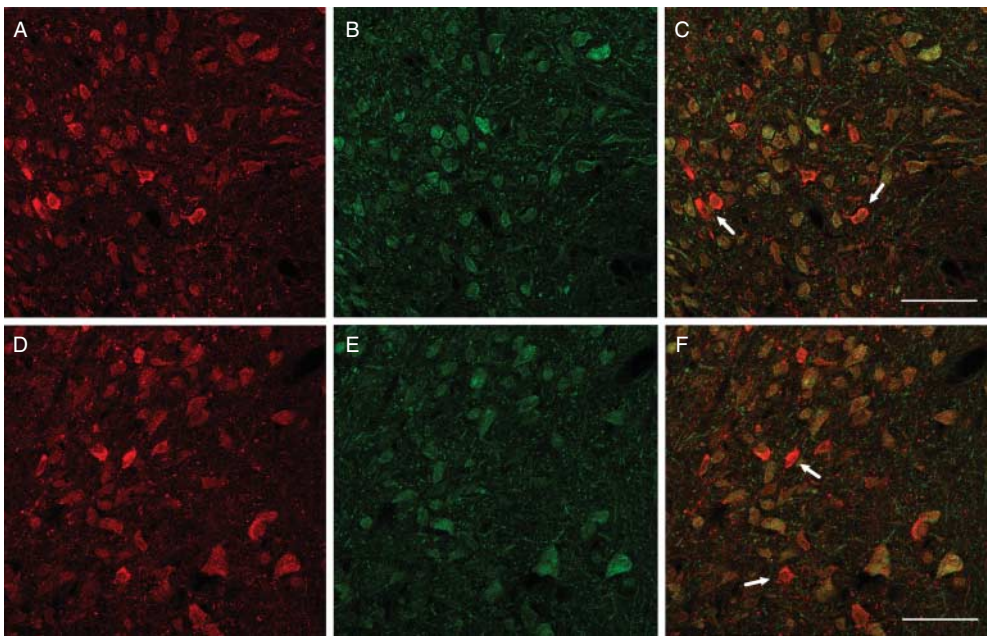

Figure 4
Coexpression of kisspeptin/LEPR and neurokinin B (NKB)/LEPR in the hypothalamic arcuate nucleus (ARC) of prenatally androgenized (PNA) rats. Confocal microscopic images of sections stained by double-label immunofluorescence for kisspeptin (A, red), NKB (D, red), and LEPR (B and E, green) in the caudal ARC of 6-week-old PNA rats. (C and F) Computer-aided merged images of A and B and of D and E respectively. The arrows in (C) indicate certain cell bodies containing kisspeptin immunoreactivity, but not LEPR. The arrows in (F) show exclusively NKB-immunoreactive cell bodies. Scale bars represent 50 μm.

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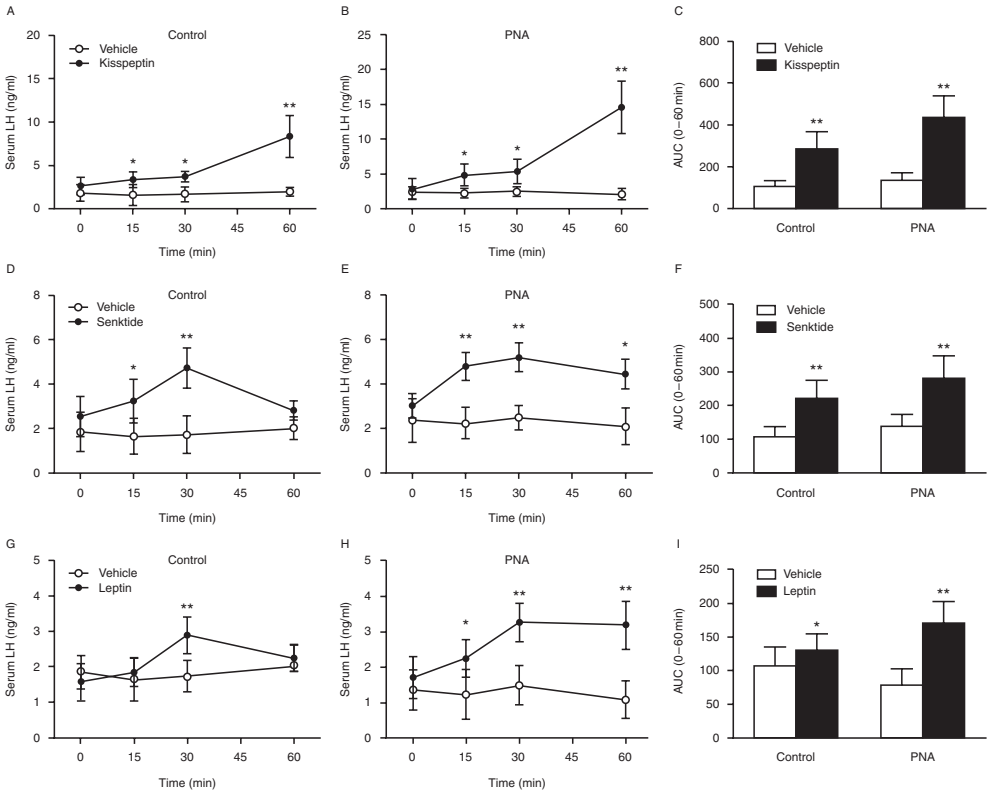

Figure 5
Effects of central administration of kisspeptin, senktide, and leptin on LH secretion in the 6-week-old female rats. Kisspeptin (1 nmol/10 μl), senktide (600 pmol/10 μl), leptin (1 nmol/10 μl), or saline (10 μl, vehicle) were acutely injected into the lateral cerebral ventricle of 6-week-old prenatally androgenized (PNA) and control rats, and blood samples were collected by jugular venipuncture before (0 min) and 15, 30, and 60 min after administration. (A and B) The serum LH levels after central injection of kisspeptin or vehicle in control (A; $n=16$) and PNA rats (B; $n=16$). (D and E) The serum LH levels after central injection of senktide or vehicle in control (D; $n=16$) and PNA rats (E; $n=16$). (G and H) The serum LH levels after central injection of leptin or vehicle in control (G; $n=16$) and PNA rats (H; $n=16$). (C, F, and I) Corresponding 0- to 60-min area under the curve (AUC) values for control and PNA rats. Values are expressed as the mean ± S.E.M. *$P<0.05$ and **$P<0.01$ vs corresponding control values.

leptin exhibited markedly higher LH levels at 15, 30, and 60 min ($P<0.01$ or $P<0.05$; Fig. 5H). In the control rats, the levels of serum LH significantly increased at 30 min after injection of leptin compared with the vehicle group ($P<0.01$), whereas no difference was detected in LH levels at 15 and 60 min between two groups (Fig. 5G). In addition, kisspeptin, senktide, and leptin treatment induced significantly increased 0- to 60-min AUC values in both the PNA rats and the control rats compared with the vehicle group ($P<0.01$ or $P<0.05$; Fig. 5C, F and I).

Effects of central administration of kisspeptin, senktide, and leptin on hypothalamic genes expression in pubertal PNA rats

After central injection of kisspeptin, the expression levels of *Nkb* and *Lepr* mRNAs were not altered in the hypothalamus of PNA and control rats ($P>0.05$; Fig. 6A and B). Likewise, the mRNA levels of *Kiss1* and *Lepr* were not significantly different between senktide treatment groups and vehicle groups ($P>0.05$; Fig. 6C and D). An increasing trend in hypothalamic *Kiss1* mRNA levels was observed in the animals treated with senktide, although it did not reach statistical significance (Fig. 6C). In both the 6-week-old PNA rats and the control rats, the hypothalamic *Kiss1* mRNA levels increased markedly after leptin administration ($P<0.01$; Fig. 6E), whereas the levels of *Nkb* mRNA did not differ between the leptin groups and the vehicle groups (Fig. 6F).

Effect of leptin on serum LH secretion following pretreatment with a kisspeptin antagonist in pubertal PNA rats

On the basis of the stimulatory effects of leptin on LH secretion and hypothalamic *Kiss1* mRNA levels in pubertal

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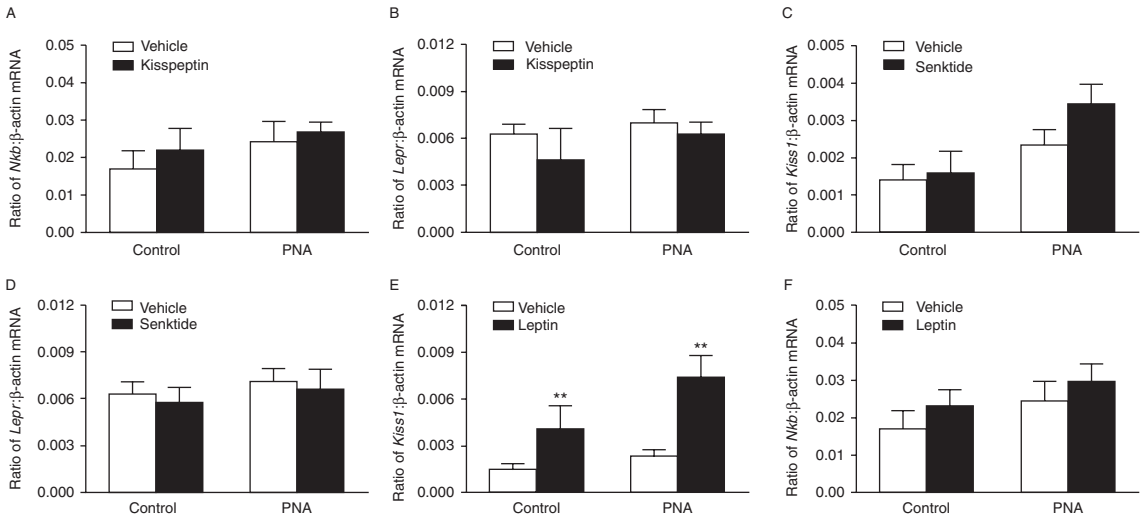

Figure 6

Effects of central administration of kisspeptin, senktide, and leptin on expression of hypothalamic genes in 6-week-old female rats. (A and B) The values represent expression levels of *Nkb* (A) and *Lepr* (B) mRNAs in the hypothalamus of control ($n=6$) and prenatally androgenized (PNA) rats ($n=6$) after treatment with kisspeptin or vehicle. (C and D) The values represent the expression levels of *Kiss1* (C) and *Lepr* (D) mRNAs in the hypothalamus of control ($n=6$) and PNA rats ($n=6$) after treatment with senktide or vehicle. (E and F) The values represent expression levels of *Kiss1* (E) and *Nkb* (F) mRNAs in the hypothalamus of control ($n=6$) and PNA rats ($n=6$) after treatment with leptin or vehicle. Values are expressed as the mean ± S.E.M. $^{**}P<0.01$ vs corresponding control values.

PNA rats, we next sought to determine whether leptin stimulates LH secretion indirectly by activating the ARC kisspeptin neurons. In 6-week-old PNA rats, i.c.v. administration of kisspeptin-234 resulted in a suppression of LH secretion (Fig. 7A). Nevertheless, pretreatment with kisspeptin-234 failed to suppress the elevated serum LH levels stimulated by leptin (Fig. 7C). In the group treated with kisspeptin-234 and leptin, the AUC values for the 60-min posttreatment period were significantly higher than those for the 60-min baseline control period ($P<0.01$; Fig. 7D). No difference was observed in the 60-min posttreatment AUC values between the treatment group and the positive control group ($P>0.05$).

Discussion

In this study, we investigated the effect of prenatal androgen exposure on LH secretion in female rats during the peripubertal period. The morning serum LH levels did not significantly differ between the PNA groups and control groups at postnatal weeks 4, 6, and 8. Moreover, we observed the serum LH concentrations every 10 min for 3 h and found that the 6-week-old PNA rats exhibited increased LH pulse frequency. In previous studies, hyperandrogenemic girls with PCOS exhibited elevated concentrations of serum LH, accompanied by an increase in pulse frequency and amplitude (van Hooff *et al.* 2000). Animal models have shown that prenatal androgen treatment induce accelerations of LH pulsatile release in primates (Abbott *et al.* 2009), sheep (Forsdike *et al.* 2007, Steckler *et al.* 2009), and rodents (Foecking *et al.* 2005), in accordance with the present results. Our observations confirm the concept that an early disturbance due to intrauterine androgen excess reprograms nervous tissue development of the fetus and irreversibly alters neuroendocrine function in female offspring.

On the basis of the functional role of kisspeptin and NKB in the mediation of the GNRH pulse generator in the ARC, we speculated that alterations in these neuropeptides may be associated with the higher LH pulse frequency in PNA female rats. In accordance with this prediction, we found that hypothalamic *Kiss1* mRNA levels were markedly elevated in the 6- and 8-week-old PNA rats compared with controls. Our data indicated that prenatal treatment with DHT induced increased *Kiss1* mRNA expression in the hypothalamus of PNA rats after the onset of puberty and that the hypothalamic kisspeptin population may be involved in higher LH pulse frequency at puberty. It has been reported that *Kiss1* and *Gpr54* knockout mice failed to go through puberty at the correct time; however, some studies showed evidence of gonadotropic activity after several months (Chan *et al.* 2009, Colledge 2009), raising the possibility that other neural components within the GNRH neuronal network may

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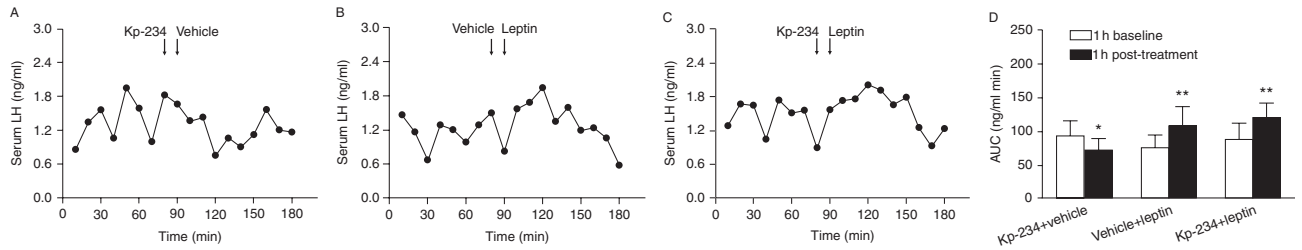

Figure 7

Effect of leptin on LH secretion following pretreatment with a kisspeptin receptor antagonist (Kp-234) in prenatally androgenized (PNA) female rats. Representative LH profiles indicating the effect of i.c.v. injection of (A) Kp-234+vehicle, (B) vehicle+leptin, or (C) Kp-234+leptin on LH secretion in the 6-week-old PNA rats. The arrows indicate central administration. (A) Central injection of Kp-234 resulted in suppression of LH secretion. (B) The levels of serum LH markedly increased after central injection of leptin. (C) Pretreatment with Kp-234 failed to suppress the elevated serum LH levels stimulated by leptin. (D) Area under the curve (AUC) values for the 60-min pretreatment period (baseline) and the 60-min posttreatment period are compared in the experiment summary. Kp-234, kisspeptin-234. Values are expressed as the mean±S.E.M., $n=22$. *$P<0.05$ and **$P<0.01$ vs 60-min baseline control period within the same treatment group.

partly replace kisspeptin–GPR54 signaling. Our study demonstrated that, at postnatal weeks 4, 6, and 8 the hypothalamic expression levels of *Nkb* mRNA in PNA rats were significantly elevated compared with the levels for controls and were especially high in the 4-week-old PNA rats. Moreover, the increased *Nk3r* mRNA levels were also detected in the hypothalamus of 4-week-old PNA rats. On the basis of these observations, we speculated that intrauterine androgen exposure resulted in the increased hypothalamic *Nkb* and *Nk3r* mRNA levels in PNA rats before puberty and NKB–NK3R signaling may be essential for the activation of GNRH neurons and increased LH pulse frequency at the peripubertal stage. These findings provide evidence that prenatal androgen treatment induces differential changes in the expression of several genes involved in the mediation of GNRH/LH pulsatile release in peripubertal PNA rats. Nevertheless, our observations differ from those reported by some studies in animal models. In another study of rats, DHT treatment had no effect on *Kiss1* mRNA in the hypothalamus; however, it significantly elevated *Kiss1* mRNA levels in pituitary tissue, ovary, and adipose tissue (Brown *et al.* 2009). A study on female sheep found that prenatal androgen treatment resulted in a reduction by half of the number of NKB cells, whereas the number of kisspeptin cells remained at levels similar to those in the KNDy subpopulation of control females (Cheng *et al.* 2010). The conflicting observations in these studies may result from differences between the action of DHT and testosterone, different neuron populations, species differences, and different age/sex groupings.

In this study, to investigate whether kisspeptin and NKB are involved in the activation of GNRH neurons and the increased LH pulse frequency in PNA rats, we evaluated the serum LH concentrations in 6-week-old female rats after central administration of kisspeptin and senktide. After injection of kisspeptin, LH levels at 15-, 30-, and 60-min were significantly higher in both the 6-week-old PNA group and the control group compared with levels for groups that received an injection of vehicle. In particular, the PNA and control rats displayed five- and threefold increases in circulating LH levels at 60 min after kisspeptin treatment respectively. These findings demonstrated that exogenous kisspeptin provided tonic drive to GNRH neurons and potently increased LH secretion in pubertal female rats. Our observations are consistent with the findings reported for other studies in animal models. In primates, repetitive administration of kisspeptin increased plasma LH levels before puberty (Shahab *et al.* 2005, Plant *et al.* 2006). Moreover, kisspeptin administration potently activated GNRH neurons in the rodent (Han *et al.* 2005, Dumalska *et al.* 2008, Pielecka-Fortuna *et al.* 2008) and induced GNRH secretion in the ewe (Messager *et al.* 2005).

We also found that senktide elicited a robust increase in serum LH levels at 15 and 30 min after central administration and LH levels at 60 min remained significantly elevated in 6-week-old PNA rats. In the control group, LH levels at 15 and 30 min were markedly elevated after injection of senktide, whereas at 60 min LH levels had decreased and were not significantly different from those for the vehicle group. Our data demonstrated that exogenous NK3R agonist exerted robust stimulation of GNRH neurons and a prolonged increase in LH concentrations in pubertal female rats, and senktide was more efficacious at stimulating LH release in the PNA group than in the control group. To date, the pharmacological actions

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of NKB on the regulation of pulsatile GNRH/LH secretion remain controversial. The central administration of senktide markedly decreased serum LH levels in ovariectomized rats treated with low levels of estradiol (Sandoval-Guzman & Rance 2004). Inhibitory effects on LH release were also observed after central injection of either NKB or senktide in ovariectomized goats (Wakabayashi *et al.* 2010) and mice (Navarro *et al.* 2009). In contrast, activation of NK3R with senktide consistently stimulated LH secretion in ewes during the follicular phase (Billings *et al.* 2010). In prepubertal rhesus monkeys, stimulation of LH secretion was observed after i.v. infusion of either NKB or senktide (Ramaswamy *et al.* 2010). These paradoxical observations may be explained by species differences in the efficacy of pharmacological agents and in the location of NK3R, as well as the gonadal status of animal models. The steroid environment may alter the basal activity of the neurons, the relative levels of gene expression, and the number or responsiveness of receptors (Rance *et al.* 2010).

Studies showed that i.c.v. coadministration of NKB and kisspeptin amplified kisspeptin's stimulatory effects in male rodents, whereas coadministration of NKB and kisspeptin to mouse hypothalamic explants inhibited kisspeptin's positive effect on GNRH release, indicating that NKB modulates the effect of kisspeptin on LH secretion, and that the pharmacological actions of NKB or senktide are complicated (Corander *et al.* 2010, Rance *et al.* 2010). On the basis of further observations, researchers have speculated that NKB is the trigger that initiates synchronous firing of KNDy neurons, and that kisspeptin in KNDy cells is the output that drives GNRH pulses (Lehman *et al.* 2010). In this study, after central injection of senktide, an increasing trend in hypothalamic *Kiss1* mRNA levels was observed in the 6-week-old PNA rats, although it did not reach statistical significance. We also found that the hypothalamic expression of *Nkb* mRNA in PNA rats increased markedly before puberty and remained high during the pubertal stage, whereas increased *Kiss1* mRNA levels in the hypothalamus were observed only after the onset of puberty. Therefore, it is reasonable to infer that NKB signaling has an integral role in the activation of GNRH neurons and initiation of increased LH pulse frequency during peripubertal development stage in PNA rats, and that the hypothalamic kisspeptin population may coordinate NKB's stimulatory effects on LH release.

Leptin, an important adipokine, has an indirect interaction with GNRH neurons (Quennell *et al.* 2009, Louis *et al.* 2011). Recent findings have indicated that leptin receptors were expressed in ARC kisspeptin neurons at a modest level (Cravo *et al.* 2011). Nevertheless, conditional transgenic deletion of leptin revealed that the direct action of leptin on kisspeptin neuron did not appear to be required for fertility or pubertal maturation (Donato *et al.* 2011). In this study, the 4- and 6-week-old PNA rats had significantly increased *Lepr* mRNA levels in the hypothalamus compared with control rats. We also found that kisspeptin- and NKB-positive cells coexpressed leptin receptors in the ARC of PNA rats. To determine whether the interaction of kisspeptin, NKB, and leptin was responsible for the increased LH pulse frequency in pubertal PNA rats, we quantified the levels of *Kiss1* and *Nkb* mRNAs in the hypothalamus of 6-week-old PNA rats after central administration of leptin. The results indicated that leptin upregulated *Kiss1* mRNA expression in PNA rats; however, pretreatment with a kisspeptin antagonist failed to suppress the elevated serum LH stimulated by leptin. On the basis of these observations, we speculated that the stimulatory effects of leptin may be conveyed indirectly to GNRH neurons via other neural components within the GNRH neuronal network, rather than through the kisspeptin–GPR54 pathway. It has been proposed that the effect of leptin on impaired regulation of the hypothalamic–pituitary–gonadal axis may be involved in the pathogenesis of PCOS (Blüher & Mantzoros 2007). In previous studies, we observed that pubertal PNA female rats exhibited hyperleptinemia (Yan *et al.* 2013). These findings indicated that hyperleptinemia at puberty may participate indirectly in the stimulation of the GNRH pulse generator and initiation of rapid LH pulsatile release in PNA rats.

In summary, prenatal androgen exposure induced elevated LH pulse frequency in pubertal female rats. The hypothalamic expression of *Nkb* and *Lepr* mRNAs in PNA rats were markedly increased before puberty and remained high during the pubertal stage, whereas increased *Kiss1* mRNA levels were detected only after the onset of puberty. Exogenous kisspeptin, NK3R agonist, and leptin exerted tonic stimulation of LH secretion in the 6-week-old PNA rats. Leptin upregulated *Kiss1* mRNA expression in pubertal PNA rats; however, pretreatment with a kisspeptin antagonist failed to suppress the elevated serum LH stimulated by leptin. On the basis of these results, we speculated that NKB and leptin play an essential role in the activation of GNRH neurons and initiation of increased LH pulse frequency in PNA female rats at puberty and kisspeptin may coordinate their stimulatory effects on LH release.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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