Original Paper

Enhanced at Puberty-1 (Eap1) Expression Critically Regulates the Onset of Puberty Independent of Hypothalamic Kiss1 Expression

Chenxi Li Pin Li

Department of Endocrinology, Shanghai Children's Hospital, Shanghai Jiao Tong University, Shanghai, China

Key Words

Eap1 • Kiss1 • Lentiviral-mediated RNAi • Puberty

Abstract

Background/Aims: Enhance at puberty-1 (*Eap1*) is an intronless gene that regulates the onset of puberty through a network of hypothalamic genes. However, precise mechanistic events essential for *Eap1*-regulation of puberty have not been fully elucidated. *Eap1* is thought to promote the initiation of puberty through regulation of the hypothalamic metastasis-suppressor *Kiss1*. We aim to investigate this hypothesis by genetically perturbing *Eap1* through RNA interference *in vivo*. **Methods:** We first engineered and optimized four sets of shRNAs that target rat *Eap1* mRNA as well as one negative control shRNA. After generating lentiviral (LV) particles, we examined the suppression of *Eap1* in NRK-54E cell line to select the most efficient one. Sequentially, LV-*Eap1*-shRNA or controls including LV-eGFP and saline were intraventricular microinjected into 21-day-old rats. Rats were raised in appropriate conditions and we examined the time of vaginal opening, ovary physiology as well as hypothalamic puberty-regulatory genes at three developmental stages: juvenile (postnatal day PND25), early puberty (PND35), adult (PND42). **Results:** Hypothalamic suppression of *Eap1* delayed the onset of rat vaginal opening. Hematoxylin and eosin (H&E) staining revealed a significant reduction of corpus luteum (CL) at PND35, but at PND42 CL levels were normal relative to control. In conjunction with differences in phenotype and ovary morphology, *GnRH* expression and transcripts were also reduced at PND25 and PND35, while their levels were similar to control at PND42. *Kiss1* mRNA and protein levels were not significantly different at all three developmental stages. **Conclusion:** *Eap1* expression critically regulates puberty as well as *GnRH* expression. However, *Eap1*-regulation of puberty may not necessitate *Kiss1*/GPR54 signaling.

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Published by S. Karger AG, Basel

Pin Li

Department of Endocrinology, Shanghai Children's Hospital
Shanghai Jiao Tong University, No.355 Luding road, Shanghai (China)
E-Mail lipin21@126.com

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Introduction

The onset of puberty is regulated by the hypothalamic-pituitary-gonadal (HPG) axis, which is inactive during childhood. Activation of gonadotropin-releasing hormone (*GnRH*)-expressing neurons in the hypothalamus triggers the onset of puberty [1]. However, the exact molecular mechanism underlying sexual maturation in the brain remains poorly understood. In the past decades, several comprehensive studies have together revealed that hypothalamic control of puberty is not determined by a single gene. Instead, a sophisticated gene network in the hypothalamus controls this event hierarchically [2, 3]. In 2007, Heger et al. [4] first identified patterns of increased *C4ORF4* mRNA in the hypothalamus of nonhuman primates during puberty. Furthermore, they found that *C4ORF4* played an important role in regulating the onset of puberty as well as maintenance of reproductive function. They subsequently renamed it enhanced at puberty-1 (*Eap1*) and numerous studies have since examined this gene. However despite such efforts, the precise molecular mechanism of *Eap1*-regulation of puberty remains unclear. Matagneet et al. [5] reported that *Eap1* must be centrally regulated as they detect enhanced expression during puberty occurs even in the absence of ovaries. Ojeda et al. [6] reported that *Eap1* plays a dual role in transcriptional activities (it trans-activates secretion promoting genes while suppresses inhibitory genes of *GnRH* neuronal function). More recently, a mechanistic study demonstrated that *Eap1* is recruited to the *Kiss1* promoter, and paired immunofluorescence assays showed *Eap1* and kisspeptin are co-expressed in neurons in the medial basal hypothalamus [7]. This suggests that *Eap1* may transcriptionally regulate *Kiss1* gene expression. Several studies demonstrate that Kisspeptin robustly stimulates the secretion of *GnRH* through the *Kiss1*/GPR54 signal pathway and plays a pivotal role in the initiation of puberty. Furthermore, *Kiss1* neurons integrate several factors that influence *Gnrh*-neurons activation, these include nutritional status, environmental cues, and socioeconomic factors [8].

In agreement with other studies, we previously demonstrated that the number of *Eap1* immuno-positive cells in the hypothalamus peaked in early puberty but declined in adulthood [9]. Moreover, the *Eap1*-expressing neurons were proximally distributed around *kiss1* and *GnRH* expressing neurons. These data suggest that *Eap1* may regulate the HPG-axis through stimulation of the kisspeptin/GPR54 signaling pathway. To test this hypothesis, we utilized RNAi to experimentally examine the consequences of rat hypothalamic *Eap1* suppression in conjunction with observing key events associated with the onset of puberty, paralleled by specific detection of key regulators *Kiss1* and *GnRH*.

Materials and Methods

Animals

Sprague-Dawley rats at 21 days of age were purchased from Shanghai SLAC Laboratory Animal Co. (Shanghai, China) and randomly divided into three groups: saline, lentivirus (LV)-enhanced green fluorescent protein (eGFP), and LV-*Eap1*-shRNA. Upon arrival, they were subjected to intracerebroventricular microinjections between hours 13:00 and 17:00. The rats were then housed under controlled temperature (21±2°C) and humidity (55±10%) conditions, with a 12h light/12h dark cycle (lights on at hours 7:00–19:00). Food and water were provided ad libitum. The animals were inspected for vaginal opening every afternoon between hours 16:00 and 17:00. Finally, the rats were euthanized at the juvenile, early puberty, and adult stages (PND25, PND35, and PND42, respectively) between hours 16:00 and 17:00. All experiments were approved by the Institutional Animal Care and Use Committee of Shanghai, China.

Cell culture

NRK-52E, a kidney epithelial cell line (Cell Bank of the Chinese Academy of Sciences) which can express the *EAP1* gene, was cultured in a humidified atmosphere containing 5% CO₂ and 37°C. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS, Gibco), 1.5 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.

293T cells (Cell Bank of the Chinese Academy of Sciences) were cultured in DMEM containing 10% FBS, 4.0 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, and were cultured in a humidified atmosphere containing 5% CO₂ at 37°C.

Construction of lentiviral-Eap1-shRNA and efficacy test of LV-Eap1-shRNA

The Designer3.0 (Gene-Pharma) software was used to design four pairs of oligonucleotide sequences targeting the *Eap1* gene as well as a negative sequence,

as shown in Table 1. Each *Eap1*-shRNA sequence contained a 21-nt sense strand, a TTCAAGAGA-loop, and a 21-nt antisense strand. For the purpose of cloning, *Bam*HI (GATCC) and *Eco*RI (AATTC) sequences were respectively added to the 5' and 3' flanking regions. These oligonucleotide sequences were cloned into the LV3 vector (Gene-Pharma) through restriction enzyme-mediated digestion and ligation with T4 DNA ligase. The resulting constructs were then transfected into 293T cells after precipitation in calcium phosphate method [10]. This procedure was utilized to identify the *Eap1* shRNA that led to optimal suppression.

Lentiviral (LV) particle production

To engineer lentiviral (LV) particles, we adapted the RNAi-Mate (Gene-Pharma) protocol to co-transfect 293T cells with three packaging plasmids (pGag/Pol, pRev, and pVSV-G) with the described LV-shRNA vector. After 72h, the LV particles were collected, and the viral titer was determined after infecting separate 293T cells with serial dilutions of the viral stock, subsequent selection and determination of multiplicity of infection. The resulting virus was subsequently stored at -80°C.

NRK-52E cells were seeded in a six-well plate at a density of 4×10⁴ cells/well in antibiotic-free DMEM for 24h. In parallel, they were next infected with tittered LV mixtures containing 1 of 4 shRNA against *Eap1* (LV-Eap1-shRNA1-4) or control (LV-eGFP) as well as polybrene (5 µg/mL). Total RNA and protein were separately extracted 72 and 96h post infection to determine the magnitude of *Eap1* suppression.

Intracerebroventricular injections of LV particles

For intracerebroventricular injections, we adapted procedures from our previous study [11]. Briefly, 21-day-old rats were positioned in a stereotaxic instrument after they were deeply anesthetized with 1% sodium pentobarbital (0.5 mL/100 g body weight). The skin and periosteum were incised to expose the bregma point, and a microsyringe with 40 µL LV(-), LV-Eap1-shRNA1-4, or saline was inserted into the skull at a 90° angle (parameters: 0.8mm rostral from bregma, 1.5mm lateral from midline, and 3.5mm ventral from the surface of the skull). This was held for 5–10 min and then slowly injected at a speed of 4 µL/min, and the syringe was retained for another 5–10 min before it was removed.

Tissue preparation

Rats were anesthetized with 1% sodium pentobarbital (0.5mL/100 g body weight), decapitated to collect the whole brain and subsequently frozen on dry ice. Brains were serially sectioned using a cryostat. Two hypothalamic regions (preoptic area/anteroventral periventricular nucleus (POA/AVPV) and (arcuate nucleus ARC)) were microdissected according to the anatomical landmarks by a stereotaxic coordinate atlas. The POA/AVPV (POA which contains GnRH neurons while AVPV consists of one of the two populations of *Kiss1*-expressing neurons) was cut into 100-µm section and dissected from six consecutive sections. The ARC (the other population of *Kiss1*-expressing neurons) was cut into 300-µm section and dissected from eight consecutive sections. The tissues were also used to assay for RNA or protein expression. Finally, the ovaries were also collected and weighed from the euthanized rats.

Hematoxylin and eosin (H&E) staining and quantification of corpus luteum (CL)

Ovaries were fixed in 4% paraformaldehyde at 4°C for 8 h. Subsequently they were dehydrated in a series of ethanol concentrations, cleared in xylene, embedded in paraffin, and serially sectioned at 6 µm. Six representative sections were selected from each ovary; these were deparaffinized in xylene, hydrated

Table 1. Targeting sequences of Eap1 shRNAs and negative control

LV	Target	Sequence
LV-shRNA1	EAP1-rat-109	GGTTGCGTCAATTACGAAGGC
LV-shRNA2	EAP1-rat-1209	GCTGAAGCTGTTTCATTGAGTA
LV-shRNA3	EAP1-rat-1328	GCTTCAAATACCTGGAGTACG
LV-shRNA4	EAP1-rat-1839	GCTCATGTCAGTAGCAGACAC
LV(-)	Negative control	TTCTCCGAACGTGTACGCT

through a series of ethanol concentrations, and stained with H&E. Only the nascent CL defined as formed after ovulation, was counted. Nascent CL was distinguished from the large existing follicles through examination of angiogenesis between the granulosa cell layer and the theca layer. Although

existing and nascent CL are similar in size, degenerative processes such as fibrosis are more advanced in existing CL and was used as an additional marker for distinction.

Table 2. Primers used for q-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product length (bp)
Kiss1	AGCTGCTGCTTCTCCTCTGT	AGGCTTGCTCTCTGCATACC	152
GnRH	CCGCTGTTGTTCTGTTGACTGTG	GGGTTCTGCCATTGTATCCTC	150
Eap1	CTTGCTACCTGTGCG AC	GTGCTGTCTCGATCA CG	126
β-Actin	TGCCGCATCCTCTTCCT	GGTCTTTACGGATGTCAACG	191

Reverse transcription and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. After the RNA was concentrated and purified 1 µg of each sample was reverse-transcribed into cDNA, and qPCR was performed in a final mixture of 20 µL. The PCR conditions were as follows: initial denaturation at 95°C for 1 min. 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. And a final extension step was included at 72°C for 7 min. Each qPCR reaction was performed in triplicate. The primers (Table 2) were synthesized by Shanghai Sangon Biotech Co., Ltd., and β-actin primers were used as the internal control.

Western blotting

Cell or hypothalamus tissue samples were homogenized in lysis buffer (Thermo Fisher, USA) containing a protease inhibitor cocktail. The supernatant was collected after centrifugation at 14,000 rpm for 15 min at 4°C, and the protein concentration was determined using the bicinchoninic acid (BCA) assay kit. Protein samples were separated using 10% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (for *Eap1* and kisspeptin, respectively), and then transferred onto nitrocellulose (NC) membranes. The membranes were blocked with 5% nonfat milk for 1 h at 4°C, incubated with anti-*Eap1* (1:500, Sigma-Aldrich), or anti-kisspeptin (1:500, Cell Signaling Technologies) antibodies at 4°C overnight, or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000, Cell Signaling Technologies) at 4°C for 2 h. Subsequently, the membranes were rinsed three times with Tris-buffered saline with Tween (TBS-T) every 5–10 min, and probed with secondary antibody (1:10000, HRP labeled, Jackson, U.S.A.) at 24°C for 1 h. After three additional rinses with TBS, the membranes were developed using an enhanced chemiluminescence (ECL) reagent.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 13.0 statistical software was used for the analysis. The data were expressed as mean ± standard deviation (SD) and the significance of the differences between groups was analyzed using one-way analysis of variance (ANOVA). The level of significance was $p < 0.05$.

Results

Engineering and optimization of *Eap1* suppression in cells

We first sought to engineer a model that permits clear detection of *Eap1* suppression in tissue. LV3 backbones containing eGFP driven by a separate CMV promoter were used to engineer shRNA constructs as indicated in Fig. 1A, and the sequencing results authenticated each cloned construct (Fig. 1B, LV-shRNA4 is shown as an example). We also optimized lentivirus (LV) titer. Specifically, the viral stock solution was serially diluted to obtain four concentrations 10-fold apart, and these were subsequently used to infect 293T cells. Based on the population of GFP-fluorescent cells, the titer of the LV-shRNA was calculated at 3.8×10^8 TU/mL (Fig. 1C).

Fig. 1. A. Construction of shRNA. B. Validation of shRNA sequences. shRNA4 is shown as an example. C. To determine the titer of LV particles, four serial diluents of the virus stock solution were used to infect 293T cells.

Fig. 2. Determination of Eap1 suppression efficacy for respective LV-EAP1-shRNAs in Nk-52E cells. A, B. mRNA levels were analyzed after transfection with different shRNAs relative to controls LV-eGFP or saline. We observed reduction of Eap1 expression after 72 and 96 hours post transfection of the respective shRNAs. C. Eap1 protein concentrations were determined by western blots.

To determine the relative efficacy of *Eap1* suppression, NRK-52E cells were in parallel transfected with LV-*Eap1*-shRNAs or LV-eGFP. *Eap1* mRNA and protein levels were measured 72 and 96 hours post transfection. As shown in Fig. 2A and B, infection with LV-*Eap1*-shRNAs reduced *EAP1* transcripts, and relative to LV-eGFP, shRNA1-4 suppressed expression at 99.4, 95.7, 93.8, and 99.7% at 72h while at 82.5, 97.5, 17.6, and 98.7% at 96h, (Fig. 2 A and B). We further confirm relative suppression of *Eap1* protein expression through western blots (Fig. 2C and D). In summary, we engineered a GFP co-expressing lentiviral construct (LV-shRNA4) that effectively suppresses *Eap1* for the use in subsequent experiments.

In vivo suppression of Eap1 in rat hypothalamus via intracerebroventricular injection

After intracerebroventricular injection of the respective LV, we next confirmed LV particles penetrated the lateral ventricle to the third ventricle and infected proximal cells.

Fig. 3. GFP-positive cells were detected around the third ventricle after intracerebroventricular injection with LV particles. A,B. Images of confocal microscopy using 200-fold magnification are shown. C. Section from A with a higher level of magnification. Arrows represent infected GFP-positive cells.

To do so, we used confocal microscopy to detect GFP fluorescence. We observed that cells around the third ventricle (*Kiss1*-expressing neurons are mainly distributed in AVPV and ARC which is a thin cell layer around third ventricle) were GFP positive (Fig. 3).

We next examined the efficacy *Eap1* suppression in two hypothalamic regions. Rats microinjected with either LV-*Eap1*-shRNA, LV-eGFP or saline groups were euthanized at the three sexual developmental stages. As expected, RNAi of *Eap1* reduced mRNA levels in the LV-*Eap1*-shRNA injected mice relative to the saline or LV-eGFP injected groups at each of the three developmental stages in the POA/AVPV (Fig. 4 A). We did not observe differences in *EAP1* expression amongst the saline and LV-eGFP group as their differences were not significant ($P > 0.05$). Additionally, the *Eap1* protein expression was consistent with mRNA expression. Specifically, we observed reduced *Eap1* protein expression upon LV-*Eap1*-shRNA microinjection relative to saline or LV-eGFP injected groups at all three developmental ages in the AVPV (Fig. 4C). *Eap1* protein expression was not perturbed in each control microinjection group. In the ARC, we did not observe relative suppression of *Eap1* protein or mRNA expression between any of different ages groups (Fig. 4B,D). These results indicate we successfully suppressed *Eap1* in rat POA/AVPV, allowing subsequent examination of *Eap1* functions in the POA/AVPV.

Eap1 suppression delays vaginal opening and perturbs ovary development.

Eap1 regulates the onset of puberty and the reproductive cycle. We next examined vaginal opening every afternoon from hours 16:00 to 17:00h after the intracerebroventricular injection of LV-*Eap1*-shRNA. As shown in Fig. 5A, the average time of vaginal opening in the LV-*Eap1*-shRNA group was significantly delayed compared with that of the saline or LV-eGFP groups. Confirming *Eap1* POA/AVPV expression as a key component for sexual development. Furthermore, we used H&E staining to observe the ovarian morphology and function (Fig. 5C). At PND35, CL numbers were lower in the LV-*Eap1*-shRNA group relative to other developmental stages. However, there appeared to be no difference among groups at PND42 (Fig. 5D). Additionally, we also observe that the ovarian index (ovary weight/body weight) at PND35 was lower in *EAP1* suppressed group relative to other groups, and no changes were observed at other developmental stages (Table 3).

Hypothalamic GnRH and Kiss1 expression are differentially regulated by Eap1 suppression

GnRH, predominantly found in rat POA, integrates critical neuroendocrine signals for hypothalamic regulation of puberty and its expression reflects the activation of the HPG axis. To determine its regulation by *Eap1*, upon collecting total mRNA from POA/AVPV, we exam-

Fig. 4. Hypothalamic expression of *Eap1* gene post intracerebroventricular injection. A.B. qPCR analysis of *Eap1* mRNA obtained from POA/AVPV and ARC in the three groups from different age stages. *Eap1* mRNA in the POA/AVPV is reduced in the LV-*Eap1*-shRNA group (* $p < 0.05$, $n = 8$) relative to the saline and LV-eGFP injected groups in all age stages. However that of the ARC displayed no difference. C.D. Western blot analysis of *Eap1*. Equal amounts of protein lysates from different age groups were subjected to electrophoresis by SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies. A representative immunoblot is provided.

Fig. 5. Sexual development phenotypes associated with *Eap1* suppression. A. The onset of vaginal opening is significantly delayed in the LV-*Eap1*-shRNA group relative to the saline and LV-eGFP groups. B. Hypothalamic GnRH mRNA is reduced in LV-*Eap1*-shRNA groups in PND25 and PND35, but no differences were observed in the PND42 group (* $P < 0.05$). C. Pathological assessment of the follicle by H&E staining. The number of CL in the LV-*Eap1*-shRNA group was less at PND35 relative to control, whereas no differences were seen at PND42.

Table 3. Ovarian index (ovary weight/body weight) ($\bar{x} \pm s$) $\times 10^{-4}$. In the early puberty group, the ovarian index of the LV-shRNA group was significantly lower compared to the indices of the saline and LV (-) groups. *, $P < 0.05$; (n=8)

group	Juvenile	Early puberty	Adult
control	2.35 $\pm$ 0.16	3.24 $\pm$ 0.15	3.63 $\pm$ 0.23
LV(-)	2.38 $\pm$ 0.34	3.33 $\pm$ 0.24	3.76 $\pm$ 0.12
LV-shRNA	2.17 $\pm$ 0.16	2.68 $\pm$ 0.22*	3.72 $\pm$ 0.13

Fig. 6. KiSS1 expression after *in vivo* Eap1 suppression in the hypothalamus. KiSS1 expression in AVPV and ARC was assessed by qPCR (A, B) and (C, D) western blots normalized to GAPDH signals at each of the three labeled age stages. No significant differences were seen ($P > 0.05$).

ined relative *GnRH* transcript levels with qPCR, We noticed that *GnRH* was reduced in LV-*Eap1*-shRNA injected relative to control groups at the juvenile (PND25) and early puberty stages (PND35). However, we observed no difference amongst treatment groups at the adult stage (PND42) (Fig. 5B). Our results that associate *GnRH* regulation to *Eap1* coincided with phenotypic observations regarding vaginal opening and ovarian index.

Studies have demonstrated that *KiSS1* regulates secretion of *GnRH*. As a key focus, we aimed to determine whether the control of puberty by *Eap1* is mediated through *KiSS1*. In *Eap1* suppressed samples, we determined the overall expression of hypothalamic *KiSS1* with qPCR and western blots. Despite changes in *GnRH*, which is a perceived *KiSS1* transcripts were not significantly different amongst groups at each developmental stage (Fig. 6A, B, and C). We also did not observe differences in *KiSS1* protein expression (Fig. 6E, F, and G). Overall, our results indicate that *Eap1* regulated puberty through *GnRH* without affecting *KiSS1*.

Discussion

The loss of *Eap1* expression in the medial basal hypothalamus (MBH) of the nonhuman primate hypothalamus abrogates the menstrual cycle [12]. Additionally, Lomniczi et al. [13]. report a single nucleotide polymorphism of the *Eap1* gene was associated with amenorrhea or oligomenorrhea in *rhesus macaques*. These observations demonstrate that *Eap1* acts as a key developmental regulator of the female reproduction system in both rodents and nonhuman primates. In this study, we successfully engineered a lentiviral construct and

further optimized conditions to specifically suppress *Eap1* in POA/AVPV via intraventricular microinjection [11, 14]. *Eap1* suppression delayed onset of puberty. This observation was accompanied by a decline in ovarian function, as reflected by CL formation, during early-stage puberty. Our findings are consistent with previous studies [4]. Furthermore, we also note that *Eap1* suppression in the POA/AVPV led to a decrease in hypothalamic *GnRH* content, which reflected the observed phenotypic defects. Surprisingly, both mRNA and protein expression of the *Kiss1* gene in the AVPV were unchanged by *Eap1* suppression, which differs from our preceding hypothesis in which *Eap1* regulates the secretion of *GnRH* through *Kiss1*/GPR54 signaling.

Eap1 is an intronless gene located on chromosome 14q24.3 [15]. Reports agree that it acts upstream of the hypothalamic network that regulates reproductive development and functions. *Eap1* contains an N-terminal C4 zinc finger and a C-terminal RING finger domain [16]. The RING finger-containing domain is predicted to participate in protein-protein interaction and ubiquitination, while the zinc finger domain was hypothesized to be involved in the transcriptional modification of DNA or RNA [17, 18]. Previous studies have demonstrated that *Eap1* transactivates the *GnRH* promoter but represses the preproenkephalin promoter, inferring that it has a dual transcriptional activity [4]. In 2012, Mueller et al. [19] discovered that *Eap1* was regulated by *ttf1*, *cux1*, and *yy1*. These are upstream components of the Tumor-related gene (TRG) network that also regulate puberty. In HeLa cells, Muller et al. [7] utilized ChIP assays to demonstrate interactions between *Eap1* and *Kiss1* promoter; this in turn led to repression of *Kiss1* expression in Grip-Tite™ 293 MSR cells and GT1-7 neurons. These observations contradict our findings, and may result from differences in cell lineage or signaling context specific to each cell lines.

In our study, upon *in vivo* suppression *Eap1*, we observed no differences in *Kiss1* expression, whereas *GnRH* expression was significantly reduced. Our findings indicate *Eap1* signals through the HPG axis by direct regulation of *GnRH* neurons, which are primarily located in the POA [20]; and this appears to involve pathways other than *Kiss1*/GPR54. A previous study identifies that *Eap1* and *GnRH* are co-expressed in some neurons and *Eap1* directly promotes *GnRH* expression in GT1-7 cells. This depicts a model in which *Eap1* acts on *GnRH* neurons directly [4]. Nevertheless, our results also demonstrate that even upon *Eap1* suppression, *GnRH* and ovarian function are normal in adults. By visualizing eGFP-positive cells around the third ventricle, we estimate 60% of the AVPV/POA neurons were infected by the LV particles [21] and this sufficiently perturbed the onset of puberty. However, effects on puberty were temporal; as the *GnRH* expression levels eventually recovered. This implies that while *Eap1* is important, other factors may also regulate female puberty development, and these genes ultimately amount to a compensatory response. Additionally, despite our findings, we still cannot entirely exclude the role of *Kiss1*/GPR54 signaling in *Eap1*-driven puberty. *Kiss1* neurons consist of two populations (AVPV and ARC) and *Kiss1* neurons may exert distinct biology in each context [22]. As an example, AVPV *Kiss1* neurons display a positive feedback to estrogen signals while those of the ARC pertain a negative feedback effect. While we did not observe *Eap1* regulation of *Kiss1* expression in AVPV, it remains a possibility that it regulates *Kiss1* in ARC, in which additional studies are required are necessary to examine this potential regulation.

In temporally controlled fashion, puberty inhibitory genes are predominantly expressed, while these are reduced during the onset of puberty. On the other hand, expression of excitatory genes such as *Kiss1*, *Tac* are elevated and initiate sexual maturation [23]. Abreu et al. [24-26] reported that a loss-of-function mutation of makorin ring finger protein 3 (*MKRN3*) is a common cause of familial central precocious puberty; this implicates that *MKRN3* as a key repressor of puberty control as well as a new pathway of puberty regulation. The discovery of *MKRN3* emphasizes the importance of inhibitory signals in the reproductive signaling axis, and that suppression of *MKRN3* may also be a required for the initiation of puberty. More recently, *Eap1* was shown to be a component of the transcription-repressing complex, also known as interferon regulating factor-2 binding proteins; this inhibits the expression of downstream genes [27]. In conjunction with previous findings in which *Eap1*

represses other inhibitory genes. Including preproenkephalin, we speculate *Eap1* may broadly function as a transcriptional repressor of several inhibitory factors involved as well. Future studies are required to elucidate the effect of *Eap1* suppression of additional inhibitory genes such as *MKRN3*.

Currently, epidemiological studies indicate that the incidence of precocious puberty in girls has rapidly increased in recent years [28]. Precocious puberty is associated with short adult height, increased breast cancer risk, polycystic ovarian syndrome, reproductive system neoplasms and metabolic syndrome [29]. Improving our understanding of underlying mechanisms that regulate maturation of reproductive systems will contribute to effective strategies in the prevention or treatment of precocious puberty.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Grant No. 81370686).

Disclosure Statement

Nothing to declare.

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