Grid1 Regulates the Onset of Puberty in Female Rats

Jing Ye
Anhui Agricultural University
Ping Qin
Anhui Agricultural University
Hailing Li
Anhui Agricultural University
Tiezhu Kang
Anhui Agricultural University
Wenyu Si
Anhui Agricultural University
Zhiqiu Yao
Anhui Agricultural University
Ya Liu
Anhui Agricultural University
Tong Yu
Anhui Agricultural University
Yunhai Zhang
Anhui Agricultural University
Yinghui Ling
Anhui Agricultural University
Hongguo Cao
Anhui Agricultural University
Juhua Wang
Anhui Agricultural University
Yunsheng Li
Anhui Agricultural University
Fugui Fang (fgfang@163.com)
Anhui Agricultural University https://orcid.org/0000-0002-3203-6687

Research

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Abstract

The present study aimed to investigate whether *Grid1*, encoding the glutamate ionotropic receptor delta type subunit 1(GluD1), influences the onset of puberty in female rats. First, we detected the expression of *Grid1* mRNA and its protein in the hypothalamus from infancy to puberty. Second, we evaluated the suppression of *Grid1* expression by Lentivirus-*Grid1* (LV-*Grid1*) in primary hypothalamus cells through measuring the expression level of *Grid1*. Finally, LV-*Grid1* was intracerebroventricular injected (ICV) into 21-day-old rats and to investigate the effect of *Grid1* suppression on puberty onset *in vivo*. Results showed that GluD1 immunoreactivity could be detected in the arcuate nucleus (ARC), paraventricular nucleus (PVN), and periventricular nucleus (PeN). *Grid1* mRNA levels were the lowest at prepuberty. Treatment of hypothalamic neurons with LV-*Grid1* decreased the mRNA expression levels of *Grid1* and *Rfrp-3* (encoding RFamide-related peptide 3, RFRP 3), but increased that of *Gnrh* (encoding gonadotropin-releasing hormone, GnRH). After 7 days of ICV LV-*Grid1* into rats, the *Grid1* mRNA was significantly reduced (by 46%), *Gnrh* mRNA *expression* was significantly increased, but *Rfrp-3* mRNA levels were decreased. The time of rat vaginal opening (VO) was earlier in the LV-*Grid1* group; the concentrations of luteinizing hormone (LH), estradiol (E_2), and progesterone (P_4) in serum were significantly increased; and the ovaries were significantly larger. Our study revealed that *Grid1* affects the onset of puberty by regulating the level of GnRH and RFRP3.

Background

Puberty plays an important role in the growth and development of animals, and its onset is closely related to the animal's sexual maturity and reproductive ability. The onset of puberty is a well-organized biological process that is controlled by the reproductive neuroendocrine systems [1, 2]. A traditional neuroendocrine study found that the hypothalamus-pituitary-gonadal axis (HPGA) plays a key role in the onset of puberty [3]. At the onset of puberty, the activity of GnRH secreted by neurons become active and GnRH neurons pulse release GnRH. GnRH neurons in the ARC of the hypothalamus synthesize and secrete GnRH, which is stored in the median eminence (ME). GnRH enters the pituitary portal system by pulsed secretion or enters the blood through the cerebrospinal fluid. GnRH stimulates the gonadotroph of the anterior pituitary gland to release LH and follicle stimulating hormone (FSH), which are necessary for regulating the secretion of peripheral target gonadal hormones and promoting the production of mature gametes through blood circulation[4]. This release of GnRH promotes the secretion of gonadotropins, LH, and FSH, which target the gonads to trigger puberty. The secretion of GnRH neurons affected by many factors, for example, GPR54 stimulates GnRH neurons directly[5], γ-aminobutyric acid(GABA) has an inhibiting effect on GnRH secretion[6], and Glutamate (Glu) promotes the secretion of GnRH[7]. Unlike primates, there may be no tonic central inhibition in rats, and the onset of puberty requires the establishment of a glutamatergic system and other auxiliary nervous systems[8]. Over the last few decades, comprehensive studies have demonstrated that hypothalamic control of puberty is not determined by a single gene, rather a complex gene network in the hypothalamus exerts hierarchical control of this event [9].

As the main excitatory neurotransmitters, glutamate is widely distributed in the brain and plays an important role in neurotransmission. It affects the growth and development, maturation, repair of neurons in the brain and affects the process of neurotransmission[10]. The glutamatergic system is the main excitatory neurotransmission system in the central nervous system (CNS) and 40% of synapses function via this system [11]. Glutamate is widely distributed in CNS, of which cerebellum, temporal lobe, frontal lobe, hypothalamus, caudate nucleus, lenticular nucleus and amygdala are most distributed. To date, several putative associated genes (DRD1, DTNBP1, MTHFR and TPH1) have been identified that are not only linked to glutamate conversion and regulation of glutamatergic neurotransmission, but also to structural proteins important in the development or maintenance of glutamatergic synapses [12]. The glutamate receptors in the CNS can be classified into metabotropic glutamate receptors and ionotropic glutamate receptors. The delta family of ionotropic glutamate receptors has two members, GluDl and glutamate ionotropic receptor delta type subunit 2 (GluD2) [13]. Genetic association studies have indicated that the *Grid1* gene, encoding GluD1, is a strong candidate gene for bipolar disorder, schizophrenia, and major depressive disorder [14–21]. Copy number variation studies have also suggested that *Grid1* is involved in autism spectrum disorders (ASD) [22–24]. Furthermore, the *Grid1* gene maps to the human 10q22-q23 genomic region at the site of recurrent deletion associated with cognitive and behavioral abnormalities [25, 26]. *Grid1* is widely distributed in the brain of adult mice, and its expression level is higher in cerebral cortex, striatum, cerebellar cortex and limbic system (hippocampus, nucleus accumbens, bed nucleus of stria terminalis). And it was highly expressed in the inner hair cells, spiral ganglion and satellite cells, vestibular ganglion and vestibular hair cells in cavies and rats, but relatively low in Corti's organ cells and vestibular Sertoli cells in the cochlea basement and low expression in other areas of CNS[27, 28].

Grid1 is involved in the glutamatergic signaling pathway, which directly regulates GnRH [29]. And previous studies in our laboratory showed that the *Grid1* mRNA and methylation pattern were different in prepubertal and pubertal goats [30]. Therefore, we speculated that *Grid1* may regulate the onset of puberty. In the present study, we first detected the expression of *Grid1* and its encoded protein in the hypothalamus of female rats at different developmental stages using quantitative real-time reverse transcription PCR (qRT-PCR) and immunofluorescence (IF). Then, we used RNA interference (RNAi) experiments to verify the inhibitory effect of *Grid1* on the hypothalamus in female rats by observing key genes and hormones that are related to the onset of puberty.

Methods

Animals

Adult Sprague-Dawley rats were purchased from Anhui Medical University laboratory animals Center [license number: SCKL (Anhui) 2017-001]. The rats were housed in the animal center of the Anhui Agricultural University and allocated into breeding pairs after feeding one week. The rats were reared in individual cages, and provided with a standard rodent diet and water, and were maintained at 22 °C ± 2 °C with a humidity of 55% ± 1.5%), and a 12/12-h light/dark cycle.

Experimental design

Experiment 1. Change of Grid1 mRNA *and protein level in the hypothalamus of rats from infant to puberty.* The animals were euthanized after anesthesia at infancy (postnatal day 10, PND10, n = 6), prepuberty (PND28, n = 6), and puberty (PND35–40, n = 6). After the rats were euthanized, the hypothalamus was surgically removed, immediately frozen in liquid nitrogen, and stored at − 80 °C until the qRT-PCR or preserved in 4% paraformaldehyde for IF analysis. The anatomical position of ARC, PVN and PeN are refer to *The Rat Brain* by George Paxinos and Charles Watson[31].

Experiment 2. Effects of Grid1 knockdown on the expression of genes associated with puberty in hypothalamic cells in vitro. Under aseptic conditions, hypothalamic neurons were isolated from PND1 female rats and incubated at 37 °C in an atmosphere of 5% CO_2. LV-*Grid1* was added on the third day of culture and total RNAs were extracted on the seventh day. Finally, the expression of *Grid1*, *Gnrh*, and *Rfrp-3* mRNA were detected using qRT-PCR.

Experiment 3. Effects of Grid1 knockdown on puberty onset, reproductive hormones of rats, litter size, and offspring weight. Eighteen female rats were randomly divided into LV-*Grid1* group, negative control (LV-(-))group, and control group, with six rats in each group. On PND21, all the rats treated with LV-*Grid1*, LV-(-), or saline by ICV injection. On the one hand, we collected hypothalamus to detect the expression level of *Grid1* mRNA and other puberty-related genes, and serum to detected the level of reproductive hormones after at 7 days after ICV injection. On the other hand, we observed the time at VO, the expression of genes related to reproduction and reproductive hormone levels in all rats. Finally, we mated the female rats with normal male rats and analyzed the litter size and offspring weight.

Primary hypothalamic cell culture

Primary hypothalamic cells were isolated from PND1 female rats. After rapid removal of the brain, the hypothalamus was dissected out. The tissues were cut into fragments and dispersed in 0.125% trypsin (Gibco, Grand Island, NY, USA) and DNase I (Biomiga, San Diego, SD, USA) for 20 min at 37 °C. Then, Dulbecco's modified Eagle's medium (DMEM) medium (Hyclone, South Logan, UT, USA) with 10% fetal bovine serum (Sijiqing, Huzhou, China) was added for trypsin inactivation and the tissues were disassociated gently by mechanical trituration. About 1 ml of cells were plated onto 6-well plates (coated with poly-D-lysine for 6 hr) for further culture. After 24 hr, DMEM medium was replaced with Neurobasal-A medium (Gibco, Grand Island, NY, USA) supplemented with 2% B-27 serum-free supplement (Gibco, Grand Island, NY, USA) at 37 °C in an atmosphere of 5% CO_2. Primary cells were cultured for 5 days before the experiments.

LV Construction

293T cells were co-transfected with Lentiviral expression particles and three kinds of packaging plasmids (pGag/Pol, pRev, and pVSV-G) using RNAi-Mate (Gene-Pharma, Shanghai, China), and were changed to complete medium at 6 hr after transfection. After 72 hr, the LV particles were collected and concentrated

to obtain a high titer lentivirus. Viral titers were subsequently determined in 293T cells. The resulting virus was then stored at -80 °C.

LV transfection

The experiment was performed on primary hypothalamic cells that had been cultured for three days. To 16 μl of LV-*Grid1*, LV-(-), or saline, we added Neurobasal-A medium supplemented with 2% B-27 serum-free supplement to 100 μl. The mixture was added to the 6-well plates containing the cells, which were then incubated at 37 °C in an atmosphere for 72 hr.

LV construction and selection of the optimal titer

Our primary objective was to design a model that can clearly detect the effect of *Grid1* suppression *in vitro*. As shown in Supplementary Fig. 1A, short hairpin RNA (shRNA) constructs targeting *Grid1* were constructed using the lentivirus (LV3) backbone, which contains an enhanced green fluorescent protein gene driven by a separate cytomegalovirus promoter, and the sequencing results verified the correctly cloned construct (Supplementary Fig. 1B). We then optimized the lentivirus titer. Specifically, virus stock solutions were 10-fold serially diluted to obtain four concentrations and were then used to infect 293T cells. Based on the population of GFP-fluorescent cells, the titer of the LV-shRNA was calculated as 1×10^9 TU/ml (Supplementary Fig. 1C).

ICV injection

For ICV injections, we adjusted our process according to a previous study [32]. Briefly, 21-day-old rats were deeply anesthetized using 2% sodium pentobarbital (0.2 ml/100 g body weight) and positioned in a stereotaxic apparatus. Under aseptic conditions, the head of the rat was shaved and the skin and periosteum were incised to expose the bregma point. Once the area had been prepared, a microsyringe with 1.5 μl LV-(-), LV-*Grid1*, or saline was inserted into the skull at a 90° angle in a position 2.5 mm posterior to the bregma, 0.5 mm lateral to the midline, and 8.6 mm inferior to the skull. This was held for 5 min, then slowly injected at a rate of 0.1 μl/min and retained for another 5 min before the syringe was removed.

Reverse transcription and qRT-PCR

The animals were euthanized by chloral hydrate, then collected blood, and whole tissues were excised and snap-frozen in liquid nitrogen. These operations are performed at nine o'clock every morning to ensure the stability of serum hormones. Total RNA was extracted using an OMEGA E.Z.N.A.™ Total RNA Kit II (Omega, Norcross, GA,USA) and reverse-transcribed into cDNA using an EasyScript One-Step gDNA removal and cDNA Synthesis SuperMix (TransScript, Beijing, China) according to the corresponding manufacturer's specifications. All the reactions were performed in triplicate in a 20 μl total reaction volume. The cDNA obtained after reverse transcription was diluted 10-fold before qPCR. The following qPCR amplification program was used: 95 °C for 10 min; then 40 cycles of 95 °C for 15 sec, and 60 °C for 1 min; with a terminal hold at 4 °C. We used the Primer Premier5 online software to design the primers and evaluated their specificity using BLAST at NCBI. The list of the primers is shown in Table 1 and

Gapdh was used as the control housekeeping gene. We collected the cycle threshold (Ct) from each reaction, and the expression level of each gene was evaluated using the $2^{-\Delta\Delta CT}$ method[33].

Table 1
Primer sequences for qRT-PCR

Gene	Forward primers	Reverse primers	Product length (bp)
Grid1	GGACTTCAGCAAGCGATAC	AACACGAATATGAGCACACC	104
Gnrh	GCCGCTGTTGTTCTGTTGAC	CTGGGGTTCTGCCATTTGA	133
Rfrp-3	CCAAAGGTTTGGGAGAACAA	GGGTCATGGCATAGAGCAAT	127
Glud1	GACGCATCTCCGCTACTG	CAAATCCCTGAACAACAAACG	129
Gapdh	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGGTGCA	134

Fluorescence immunolocalization of GluD1

The sections were deparaffinized with 100% xylene and rehydrated using a gradient ethanol series. The sections were then dipped in 10% Bull Serum Albumin (BSA, Amresco, Solon, OH, USA) and incubated at room temperature for 20 min to block non-specific antigens. Rat anti-GluD1 primary polyclonal antibodies (Abcam, Cambridge, MA, USA) were then added and incubated for 18 hr at room temperature. Thereafter, the sections were incubated with donkey anti-rat immunoglobulin G-fluorescent dye NL557 (R&D Systems, Minneapolis, MN, USA) secondary antibodies for 1 hr at room temperature. The sections were incubated with Vectashield medium containing 4,6-dimercapto-2-phenylindole (DAPI; blue nuclear dye; Vector Laboratories) for 20 min in the dark. Finally, the sections were blocked with 50% glycerol in the dark. Negative controls were not incubated with primary antibodies, but only treated with secondary antibodies. The sections were observed under the microscope (OLYMPUS IX71, Germany) and mean fluorescence intensities of sections were analyzed by Image-Pro Plus6.0.

Effect of Grid1 knockdown on GluD1, E_2, and P_4 concentrations in serum

Serum GluD1 (XQ-JS2680, Origin Biotech, Hong Kong, China), FSH (CK-E30597R, Origin Biotech, Hong Kong, China), LH (CK-E30623R, Origin Biotech, Hong Kong, China), E_2 (CK-E30581R, Origin Biotech, Hong Kong, China) and P_4 (CK-E30580R, Origin Biotech, Hong Kong, China) concentrations were measured using commercial enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. Briefly, the test sample were diluted five times with Sample Dilution buffer in 96well plates, and 100 µl of HRP-conjugate reagent was added to each well, which were covered with an adhesive strip and incubated for 60 min at 37 °C. The 50 µl chromogen solution was added to each well, gently mixed and incubated for 15 min at 37 °C. Finally, 50 µl of Stop Solution was added and the optical density was read at 450 nm using a microtiter plate reader for 15 min. The FSH assay had a sensitivity of 0.1 IU/l, for LH it was 1.0

mIU/ml, for E_2 it was 1.0 pg/ml, and for P_4 it was 0.1 ng/ml, with an intra-assay coefficient of variation of less than 15%. The coefficient of the standard curves was 0.9900.

Hematoxylin and eosin (H&E) staining

Ovaries were fixed in 4% paraformaldehyde at 4 °C for more than 8 hr. Subsequently, the ovaries were dehydrated through a series of ethanol concentrations, cleaned in xylene, and embedded in paraffin. Finally, the ovaries sectioned serially at 5 μm. The sections were then deparaffinized in xylene, hydrated through a series of ethanol concentration, and stained with H&E. The method to distinguish primordial follicles, primary follicles and secondary follicles is refer to Andrea S. K. etc.[34].

Statistical analysis

All values are analyzed as the mean ± SD using the SPSS 19.0 software package (IBM Corp., Armonk, NY, USA). Differences were considered to be significant at $P < 0.05$.

Results

The expression of the Grid1 mRNA and level of the GluD1 protein in hypothalamus from infant to pubertal rats

We used qRT-PCR and IF to detect the expression of Grid1 and GluD1 in the hypothalamus at different stage. qRT-PCR revealed that the *Grid1* mRNA level in the hypothalamus at infancy was significantly higher ($P < 0.05$) than that at prepuberty and puberty, and the *Grid1* mRNA level at prepuberty was significantly lower than that at puberty ($P < 0.05$) (Fig. 1A). GluD1 immunoreactivity (IR) was detected in the ARC, PVN, and PeN (Fig. 1B). The fluorescence intensity of GluD1 was similar in the ARC and PVN from infancy to puberty ($P > 0.05$); however, the GluD1 IR in the PeN of infant rats was significantly lower than that in prepubertal and pubertal animals ($P < 0.05$) (Fig. 1C).

Transfection of LV-Grid1 into primary hypothalamic cells

To determine the relative efficacy of *Grid1* suppression, hypothalamic cells were transfected with LV-*Grid1* or LV-(-) in parallel. We diluted the stock lentivirus concentrate 10-fold to obtain four lentivirus concentrations before transfection. Figure 2A shows the fluorescence intensity of the transfected cells, which for the undiluted stock solution reached the maximum at 72 hr (Supplementary Fig. 2). Therefore, we chose to use the stock solution for subsequent transfections of primary hypothalamic cells. *Grid1* mRNA levels were measured at 72 hr after transduction. As shown in Fig. 2B, transduction with LV-*Grid1* reduced the *Grid1* transcriptional level at 72hr relative to that in LV-(-)-transduced or control-transduced cells. Thus, LV-*Grid1* could effectively to suppress *Grid1* expression and was suitable for use in subsequent experiments. In addition, in LV-*Grid1*-transfected cells, the expression of *Rfrp-3* was also suppressed, while *Gnrh* expression increased, and the expression of *Glud1* (encoding glutamate dehydrogenase 1) was no different (Fig. 2B).

The suppressive efficiency of LV-Grid1, puberty-related gene expression, and serum reproductive hormone concentrations in rats

To verify the knockdown efficiency of the lentiviruses *in vivo*, we excised the hypothalamus of rats at 7d after ICV, extracted the RNA, and used qRT-PCR to verify the expression of *Grid1* mRNA. Compared with the control group and LV-(-) group, *Grid1* mRNA levels were reduced by 46% in the hypothalamus of the LV-*Grid1* group (Fig. 3A). The *Gnrh* mRNA increased significantly ($P < 0.05$) and *Rfrp-3* mRNA decreased ($P < 0.05$), while that of *Glud1* was no different compared with the control group after ICV ($P > 0.05$) (Fig. 3A). Meanwhile, the ovarian follicles were faster in the LV-*Grid1* group, and there were more tertiary follicles compared with those in the control and LV-(-) groups. In contrast, there were more primary and secondary follicles in the control and LV-(-) groups than in the LV-*Grid1* group (Fig. 3B). The concentrations of GluD1, E_2, P_4, FSH, and LH in serum obtained at 7 d after ICV injection were not significantly different among the groups ($P > 0.05$) (Fig. 3C-D). Meanwhile, there was no significant difference in ovarian weight and size among the three groups (Table 2, Supplementary Fig. 3a).

Table 2
Ovarian weight and size at 7 days after ICV injection

	Weight (mg)	Transverse diameter (cm)	Longitudinal diameter (cm)	Transverse perimeter (cm)	Longitudinal perimeter (cm)
Control	16.975 ± 0.357	0.416 ± 0.027	0.266 ± 0.012	1.074 ± 0.094	0.787 ± 0.077
LV-(-)	18.500 ± 1.323	0.455 ± 0.012	0.269 ± 0.007	1.185 ± 0.067	0.813 ± 0.034
LV-*Grid1*	16.00 ± 0.957	0.430 ± 0.020	0.270 ± 0.014	1.129 ± 0.036	0.816 ± 0.037
All data are shown as the mean ± SD.					

Effect of Grid1 knockdown on the time of VO, puberty-related genes, and serum reproductive hormone concentrations in rats

We examined the VO every morning from 8:00 to 9:00 after ICV injection of lentivirus in PND21 rats. As shown in Fig. 4A, on average, the time of rat VO occurred significantly earlier in the LV-*Grid1* group compared with that of the control group or LV-(-) group ($P < 0.05$). Then, female rats were mated with normal male rats to study the change of average litter size and weight of their progeny. Interestingly, no differences were found among the groups ($P > 0.05$) (Fig. 4B, Table 3).

Table 3
The weight of offspring

Groups	Sex (n = 6)	Weight (g)
Control	Male	6.719 ± 0.347
	Female	6.434 ± 0.380
LV-(-)	Male	6.578 ± 0.627
	Female	6.271 ± 0.583
LV-*Grid1*	Male	6.554 ± 0.296
	Female	6.301 ± 0.120
All data are shown as mean ± SD.		

We observed effect of *Grid1* knockdown on *Grid1*, *Gnrh*, *Rfrp-3*, and *Glud1* mRNAs levels in the hypothalamus, histology of the ovaries and the serum GluD1, E_2, and P_4 concentrations of rats in the LV-*Grid1* group showing VO and in the control and LV-(-) groups at matched ages. We found the expression of *Grid1* mRNA in the hypothalamus similar in the control and LV-(-) groups ($P > 0.05$) (Fig. 5A). Meanwhile, the level of *Gnrh*, *Rfrp-3*, and *Glud1* mRNA after ICV injection of LV-*Grid1* in 21 d rats also showed no difference compared with the control and LV-(-) groups ($P > 0.05$) (Fig. 5A). The number of corpora lutea (CLs) in LV-*Grid1* group was more than that in the control and LV-(-) group, and the number of primary and secondary follicles in LV-*Grid1* group also similar to the control group and LV-(-) group (Fig. 5B). By contrast, in the serum from rats in the LV-*Grid1* group rats with VO, the concentrations of E_2, P_4 and LH were significantly increased ($P < 0.05$), and the concentrations of GluD1 and FSH not different compared with those in the control group and LV-(-) group at the same age ($P > 0.05$) (Fig. 5C-D). Interestingly, the weight, transverse diameter, longitudinal diameter, and longitudinal perimeter of the ovaries obtained from the LV-*Grid1* group were significantly larger than those of the control and LV-(-) groups (Table 4, Supplementary Fig. 3b).

Table 4
Ovarian weight and size of rats which LV-*Grid1* group showing VO and the control group and LV-(-) group at matched age after ICV injection

	Weight (mg)	Transverse diameter (cm)	Longitudinal diameter (cm)	Transverse perimeter (cm)	Longitudinal perimeter (cm)
Control	42.550 ± 0.519	0.430 ± 0.035	0.3288 ± 0.022	1.211 ± 0.103	0.881 ± 0.085
LV-(-)	42.350 ± 0.777	0.493 ± 0.012	0.3672 ± 0.013	1.259 ± 0.031	0.885 ± 0.068
LV-*Grid1*	50.900 ± 0.528*	0.566 ± 0.018*	0.436 ± 0.014*	1.410 ± 0.063	1.205 ± 0.033*
All data are shown as mean ± SD.*$P < 0.05$					

Discussion

Glutamate is widely distributed in the brain and is a major excitatory neurotransmitter. It plays an important role in neurotransmission and affects the growth, maturation, repair and neurotransmission of neurons in the brain [10]. Glutamate participates in the regulation of various functions of the nervous system. The HPGA can be reactivated in the juvenile stage by treatment with neurotransmitters such as glutamate and kisspeptin [35]. The mechanism of the onset of the puberty is believed to involve initiation of the HPGA [36]. In this process, pulsatile GnRH secretions from specialized hypothalamic neurons stimulate hormone cascades and gonadal activation [37]. Combined with previous studies in our laboratory, the methylation level of *Grid1* changes during the onset of puberty in rats and goats [30, 38]. In the present study, we found that GluD1 IR localized to hypothalamic nuclei, especially in the PVN, PeN, and ARC, which are implicated in GnRH secretion [39–41]. The fluorescence intensity in PeN of infant rat is low than pubertal rat, it remind there are affinity between different nucleus of hypothalamus and puberty onset. In addition, the level of *Grid1* mRNA in the hypothalamus of prepubertal rats was significantly lower than that during puberty. These results indicated that *Grid1* might modulate hypothalamic and pituitary hormones, but how *Grid1* regulates the hormone secretion and affect onset of puberty require further research.

Lentiviral vectors have been developed as replication-defective retroviral vectors that can infect non-dividing and mitotic cells. Lentiviral vectors have two distinct properties: Long-term stability and the reduced likelihood of eliciting an immune response; therefore, they are ideal for i*n vivo* genetic experiments. Lentiviral vector-mediated RNA interference technology combines the advantages of a lentiviral vector with those of RNA interference when using a sequence that is specifically targeted to inhibit gene expression. These tools have been proven to be effective in a variety of mammalian cells and in many disease models [42–46].

In the present study, to investigate whether *Grid1* is involved in the regulation of GnRH, we constructed a lentiviral vector expressing an shRNA targeting *Grid1*, which was transduced into primary neurons of the

hypothalamus, resulting in significant suppression of *Grid1* expression. Suppression of *Grid1* caused an increase in the expression level of *Gnrh* mRNA in hypothalamic cells, whereas the expression level of *Rfrp-3* mRNA decreased. *Grid1*, as a glutamate receptor on neurons, acts as an excitatory neurotransmitter in many synapses in the CNS. When the expression of *Grid1* is decreased, it might affect the secretion of RFRP 3. Studies have shown that RFRP 3 inhibits the secretion of GnRH from nerve cells [47, 48], and reduce the secretion of LH[12]. Therefore, we speculated that when the *Grid1* expression is suppressed, the expression of *Rfrp-3* mRNA is decreased, thereby promoting GnRH secretion, resulting in an increase in the expression of *GnRH* mRNA. Therefore, the results indicated that *Grid1* knockdown might affect the key genes related to puberty in female rats.

One of the most important neuroendocrine events associated with reproduction, leading to an increase in LH, requires hypothalamic synthesis of progesterone [49]. In this study, we observed that the concentrations of E_2, P_4, GluD1, FSH and LH in serum obtained from the rats after ICV injection with LV-*Grid1* 7 days similar to those of the LV-(-) and control groups, we speculated that the ovaries are immature and insensitive to hormonal stimulation at PND28. Interestingly, in the serum from rats in LV-*Grid1* group with VO, the concentrations of E_2, P_4, and LH were significantly increased, while the concentrations of GluD1 and FSH were similar to those in the age-matched control and LV-(-) groups. At this stage, the LV-*Grid1* group showed higher concentrations of E_2 and P_4, which would cause the LH surge and estrous cyclicity. The change of LH secretion pattern is the result of pulsed release of GnRH with the development, because the pulsed GnRH release also increases in puberty[50]. Hypothalamus release GnRH, which promotes pituitary release LH and FSH, and they stimulate gonadal release gonadal hormone. At puberty, hypothalamus sensitivity to gonadal decreased, and then the inhibitory effect of inhibiting factors which inhibit release of GnRH was relieved, and the sexual center changed from inhibitory to excitatory stage.

In the present study, at 7 days after ICV injection of LV-*Grid1*, *Grid1* mRNA expression was reduced by 46% compared with that in the control group, whereas the *Gnrh* mRNA level was significantly increased, and the *Rfrp-3* mRNA expression was significantly reduced. These results implied that suppression of *Grid1* might cause changes of in the process of puberty in female rats. In rats, the effect of *Grid1* appears to continue into the onset of puberty. Our study indicate that Grid1 may affect HPGA by regulating GnRH neurons. However, there was no difference in the level of *Grid1*, *Glud1*, *Rfrp-3*, and *Gnrh* mRNA between in the LV-*Grid1* group showing VO and in the control and LV-(-) groups at matched ages. Despite this, ICV injection to suppress *Grid1* significantly advanced the time of puberty onset in rats. Combing the time of VO and the change of reproduction-related genes, we speculate that the effect of LV-Grid1 may be time-sensitive after puberty onset, which no effect on *Gnrh*, *Rfrp-3* and *Glud1* mRNA when puberty onset. This was accompanied by enhanced ovarian development, as reflected by CLs formation or the promotion of follicle development, during early-stage puberty.

Li et al. [32] reported that hypothalamic suppression of Enhance at puberty-1 (*Eap1*) delayed the onset of rat VO and *Eap1*-regulation of puberty may not necessitate KISS1/GPR54 signaling. This implied the *Eap1* plays an important role in the regulation of puberty, which, together with GnRH, forms a new

pathway of puberty regulation. Combined with the results our study, further studies are required to determine the effect of *Grid1* suppressed on additional genes such as *Eap1* or other signaling pathways during the onset of puberty.

Conclusion

In summary, *Grid1* and its associated proteins are altered during the development of puberty, and *Grid1* knockdown *in vivo* and *in vitro* affected the expression of genes related to puberty, as well as advancing the time of VO, changing the secretion of reproductive hormones, and increasing the weight and size of the ovaries. These results suggest that *Grid1* regulates the onset of puberty in female rats; however, the mechanism remains be further studied.

Abbreviations

GluD1: glutamate ionotropic receptor delta type subunit 1; LV-Grid1: Lentivirus-Grid1; ICV : intracerebroventricular injected; ARC: arcuate nucleus; PVN: paraventricular nucleu; PeN: periventricular nucleus; RFRP-3: RFamide-related peptide 3; GnRH: gonadotropin-releasing hormone; VO: vaginal opening; LH: luteinizing hormone; E2: estradiol; P4:progesterone; HPGA: hypothalamus-pituitary-gonadal axis; ME: median eminence; FSH: follicle stimulating hormone; GABA: γ-aminobutyric acid; Glu: Glutamate; CNS: central nervous system; GluD2: glutamate ionotropic receptor delta type subunit 2; qRT-PCR: quantitative real-time reverse transcription PCR; IF: immunofluorescence; RNAi: RNA interference; PND: postnatal day; H&E: Hematoxylin and eosin; IR: immunoreactivity; CLs: corpora lutea

Declarations

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Authors’ contributions

Conceived and designed the experiments: F.F.G., and Y.J.; Performed the experiments: Q.P., and L.H.L.; Analyzed the data: Y.J., and S.W.Y. and Y.Z.Q.; Contributed reagents/materials/analysis tools: L.Y., Y.T., Z.Y.H., L.Y.H., C.H.G., W.J.H., L.Y.S., F.G.F.; Wrote the manuscript: Y.J

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Availability of data and materials

All data generated through this study are included in this article.

Ethics approval and consent to participate

All procedures involving animals were approved by the Animal Care and Use Committee of Anhui Agricultural University.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Figures

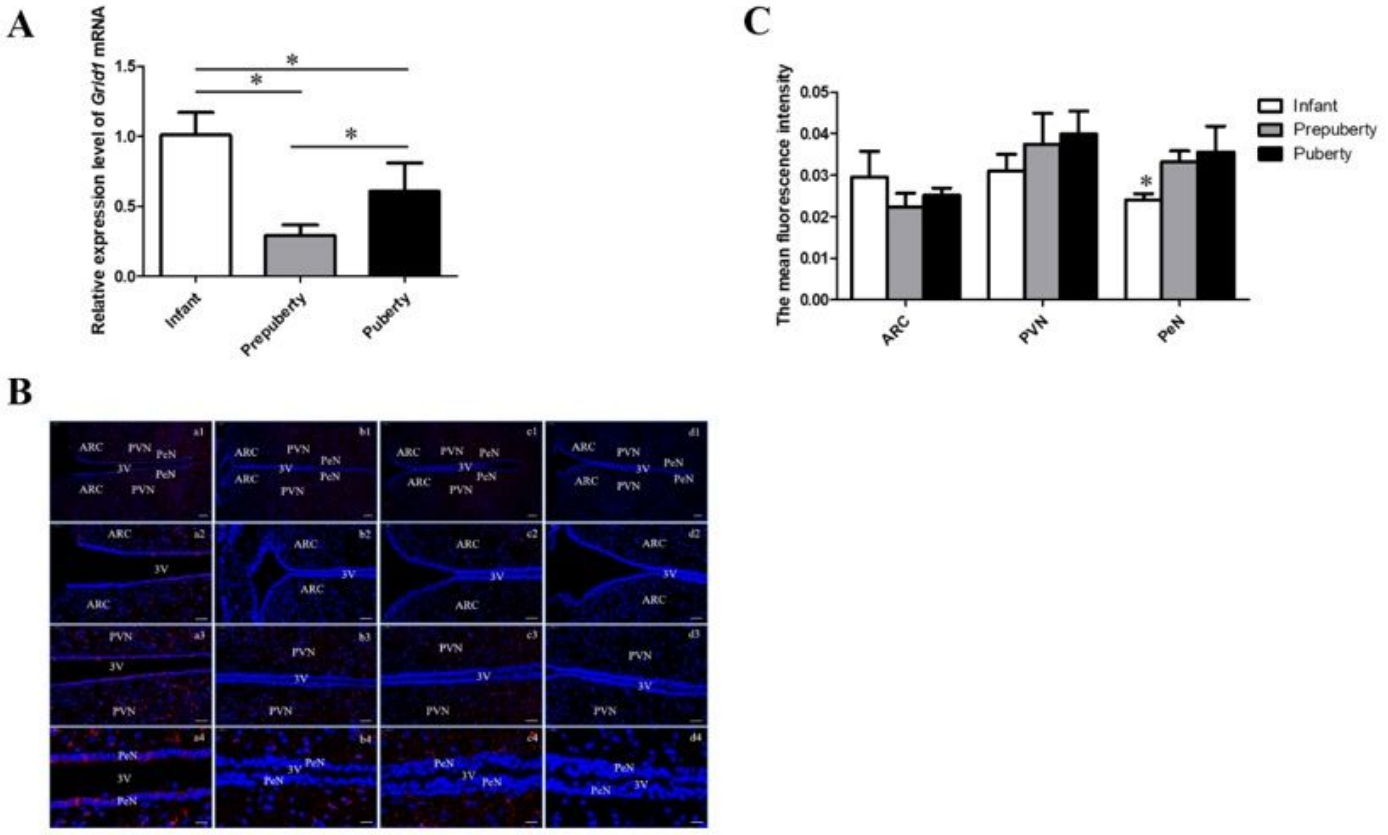

Figure 1

Level of Grid1 mRNA and distribution of the GluD1 protein in the hypothalamus of female rats from infancy to puberty. (A) Relative Grid1 mRNA expression levels in the hypothalamus from infancy to puberty. (B) Localization of Grid1-immunopositive cells in hypothalamic nuclei in female rats. (a1–a4), infant; (b1–b4), prepuberty; (c1–c4), puberty; (d1–d4), negative control. (C) The mean fluorescence intensity of the GluD1 protein in the ARC, PVN, or PeN from infancy to puberty. Error bars: SD. Scale bars: (a1-d1) 100 μm; (a2–d2, a3–d3 and a4-d4) 50 μm.

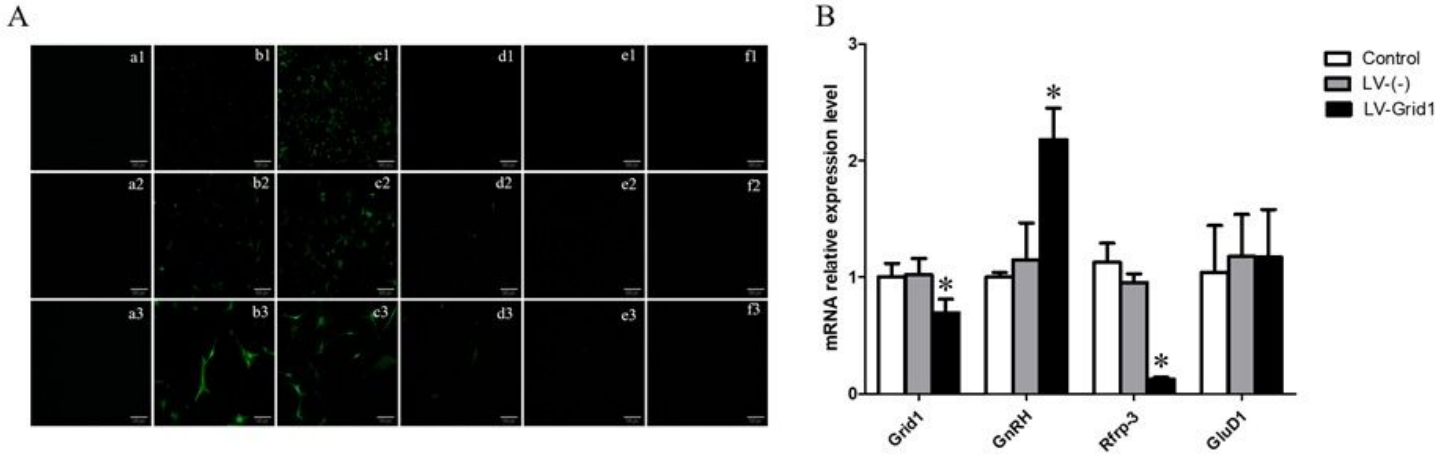

Figure 2

The effect of LV-Grid1 in hypothalamus cells on the expression level of Grid1 and puberty-related genes. (A) Four serial dilutions of the virus stock solution were used to infect hypothalamus cells. (a1-3) Control group; (b1-3) LV-(-) group; (c1-3)1 × 109 TU/ml; (d1-3) 1 × 108 TU/ml; (e1-3) 1 × 107 TU/ml; (f1-3) 1 × 107 TU/ml; (B) Levels of Grid1 mRNA and Gnrh, Rfrp-3, and Glud1 in hypothalamus cells infected by LV-Grid1 with at 1 × 109 TU/ml. Error bars: SD. Scale bars: (a1-f1) 500 μm; (a2–f2)200 μm; (a3-f3)100μm.

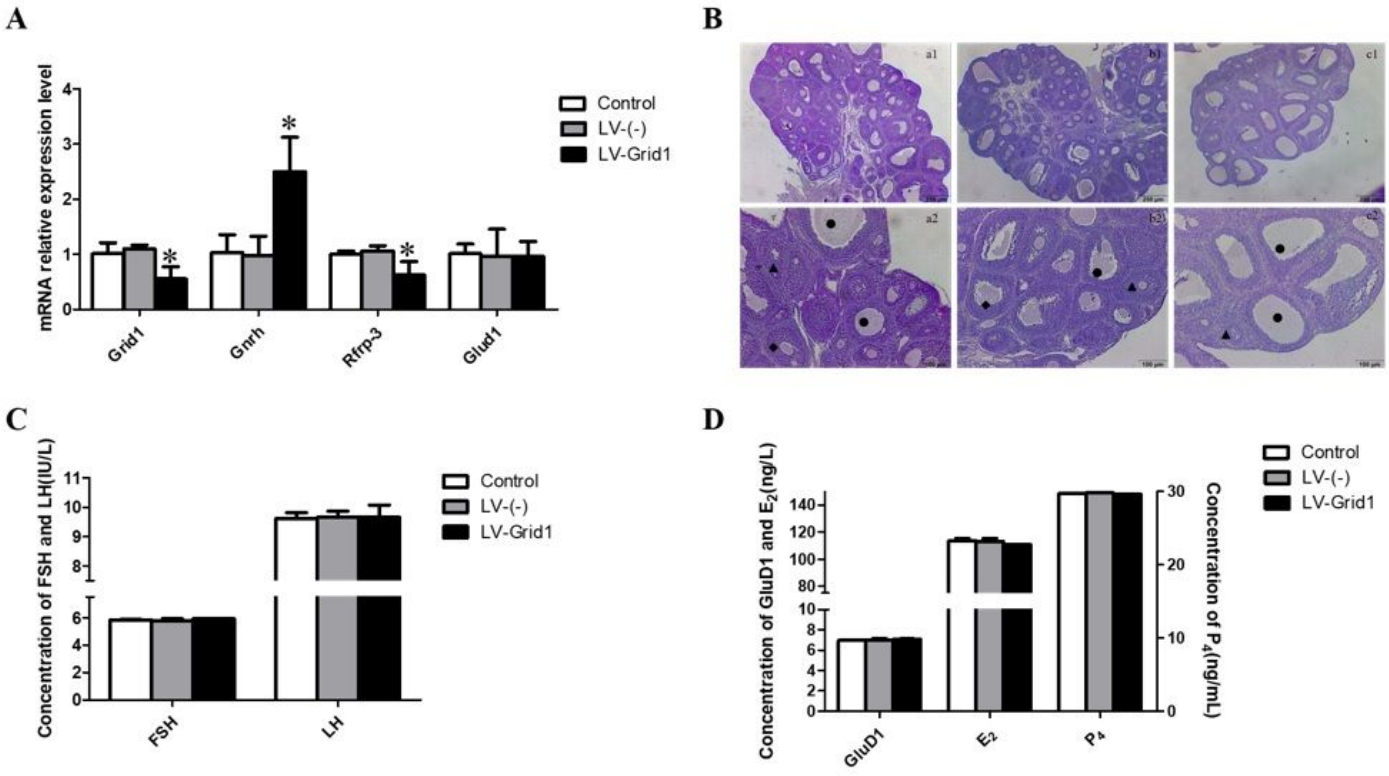

Figure 3

The effect of LV-Grid1 on female rats after 7 days of LV-Grid1 ICV injection. (A) The expression level of Grid1, Gnrh, Rfrp-3, and Glud1 mRNA in the hypothalamus (*P < 0.05, n=6). (B) Histological assessment of follicles using H&E staining (a: Control group; b: LV-(-) group; c: LV-Grid1 group; ▲primary follicle; ▯secondary follicles; ●Tertiary follicles; ▯corpus luteum). (C) Concentration of FSH and LH in rat serum. (D) Concentration of GluD1, E2, and P4 in rat serum. Error bars: SD.

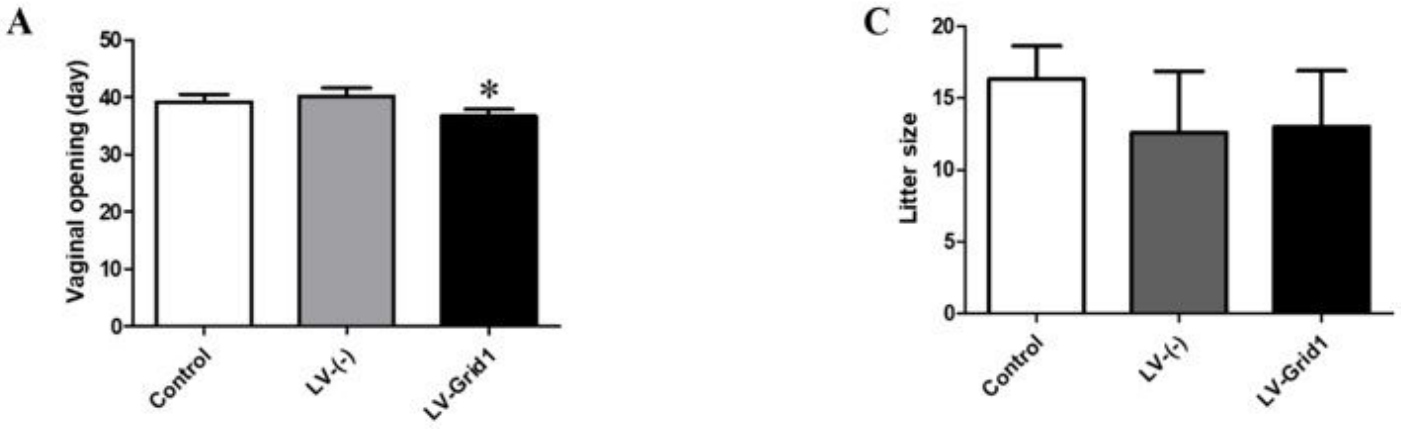

Figure 4

Sexual development phenotypes associated with Grid1 suppression. (A) VO occurred significantly earlier in the LV-Grid1 group compared with that in the control (saline) and LV-(-) groups (*P < 0.05). (B) There was no difference in litter size after LV-Grid1-treated female rats were mated with normal male rats. Error bars: SD.

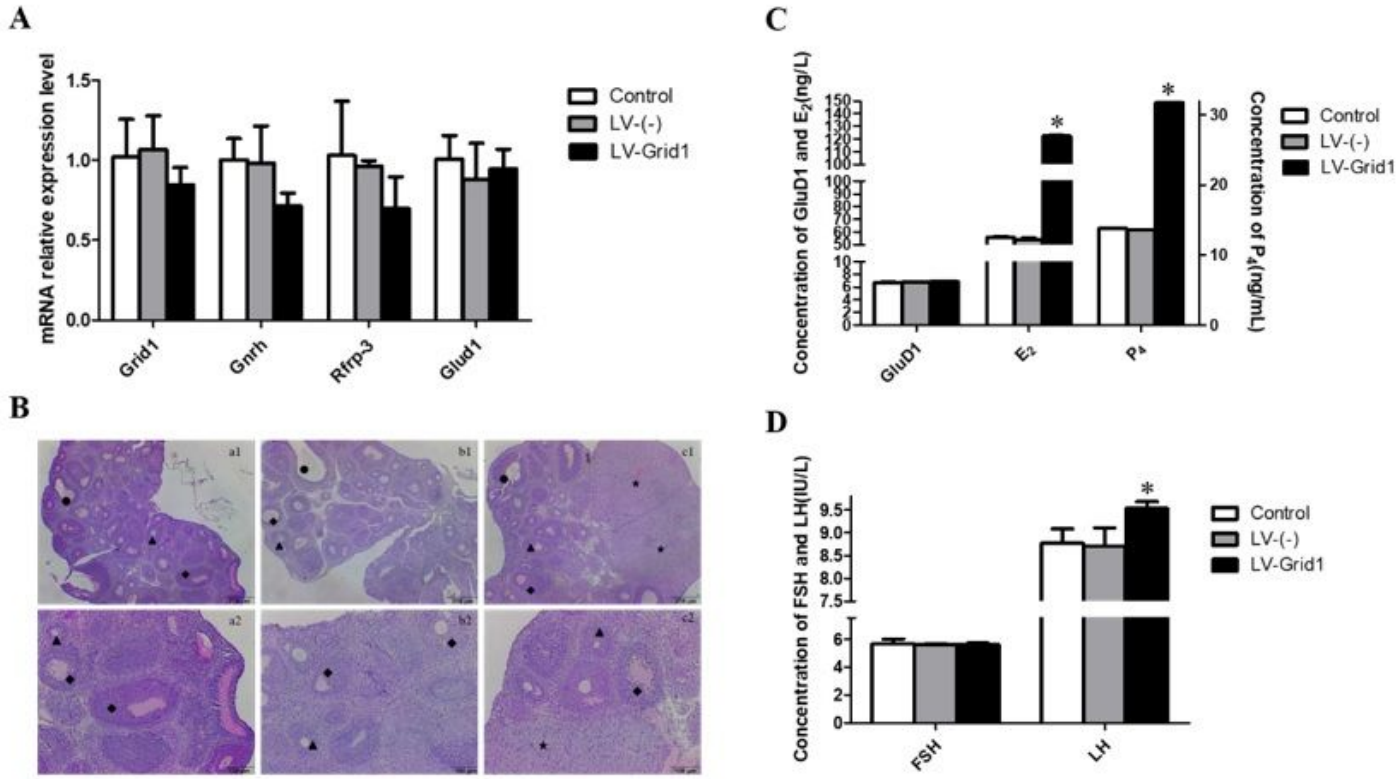

Figure 5

Reproductive-related genes, ovarian morphology and reproductive hormones associated with Grid1 suppression. (A) No differences were observed in the hypothalamic Grid1, Gnrh, Rfrp-3, and Glud1 mRNA

in LV-Grid1 groups during puberty. (B) Histological assessment of follicles using H&E staining (a: Control group, n=6; b: LV-(-) group, n=6; c: LV-Grid1 group, n=6; ▲primary follicle; ▯secondary follicles; ●Tertiary follicles; ▯corpus luteum). (C) The concentration of E2 and P4 in serum increased in LV-Grid1 group, but no difference was observed in the concentration of GluD1 in serum (*P < 0.05, n=6). (D) The concentration of LH in serum increased in LV-Grid1 group (*P <0.05, n= 6). Error bars: SD.

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