miR-7 mediates the signaling pathway of NE affecting FSH and LH synthesis in pig pituitary

Abstract

MicroRNA-7 (miR-7) is an important modulator of a plenty of gene expressions and the interrelated biological processes, highly expressed in porcine pituitary. Norepinephrine (NE), acting as an important neurotransmitter or/and a hormone secreted excessively under stress, affects the synthesis and secretion of various hormones, including pituitary follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are the key hormones which regulate sexual maturation and reproductive functions. However, the relationship among NE, miR-7 and gonadotropin needs to be elucidated. The aim of this study was to identify whether miR-7 involved in the NE-adrenoceptor signaling pathway affects the synthesis and secretion of FSH and LH in porcine pituitary. Our results showed that the NE intracerebroventricular injection increased pituitary miR-7 level and the synthesis and secretion of FSH and LH in porcine, whereas the inhibition of either endogenous miR-7 or β-adrenergic receptors hindered the rise of FSH and LH synthesis induced by NE in cultured primary porcine anterior pituitary cells. Further, we identified the molecular type of β-adrenergic receptors and the signaling pathway in porcine pituitary, and we found that NE played its roles relying on adrenoreceptor beta 2 (β2AR) and the RAF/MEK/ERK1/2 signaling pathway. The phosphorylation of ERK1/2 upregulated miR-7 level which subsequently enhanced FSH and LH synthesis by targeting to Golgi glycoprotein 1 (GLG1). These suggest that miR-7 mediates NE’s effect on promoting FSH and LH synthesis in porcine pituitary.

Introduction

hormone (LH) and follicle-stimulating hormone (FSH) and regulates mammalian reproductive process through hypothalamus-pituitary-gonadal (HPG) axis (Christensen et al. 2012, Davis et al. 2013, Ramaswamy & Weinbauer 2014). There are reports that the stress, including the environment and serious threat or psychological pressure to human, profoundly affects the activities of the HPG axis (Lennartsson et al. 2012, Kageyama 2013, Oyola & Handa 2017, Heck & Handa 2019, Mohammadi et al. 2019), including the abnormalities of the synthesis and secretion of FSH and LH, in which the norepinephrine (NE) is involved (Lobo et al. 1983, Moberg 1991, Johnson et al. 1992, Toufexis et al. 2014, Gu et al. 2018). But up to now, the effects of NE on the synthesis and secretion of pituitary FSH and LH and the related mechanisms remain to be elucidated.

NE, one of the earliest identified neurotransmitters (Von Euler 1946, Schwarz & Luo 2015), functions as a hormone which regulates the physiological functions of the brain and other organs or systems of the body, such as nervous system (Agarwal et al. 1993). It has been well documented that NE exerts its effects by binding to and activating its receptors located on the surface of cells, which have been identified as two broad families known as alpha (α) and beta (β) adrenergic receptors (Denef & Baes 1982, Ahles & Engelhardt 2014). Previous studies have confirmed that β-adrenergic receptors are the predominant adrenergic receptors in the anterior pituitary gland (Denef & Baes 1982, De Souza 1985, 1987, Bedran-de-Castro et al. 1990); yet, it has not been elucidated whether the effects of the NE on the synthesis and secretion of FSH and LH rely on its binding to β-adrenergic receptors.

miRNAs are small noncoding RNAs, the expressions and functions of which have been extensively studied (Mohr & Mott 2015, Wierinckx et al. 2017). MicroRNA-7 (miR-7) is evolutionarily conserved and considered to be a prototypical neuroendocrine miRNA (Li et al. 2016), which is highly expressed in neurons and neuroendocrine organs, including pituitary gland (Yuan et al. 2015, Ahmed et al. 2017, He et al. 2018b). Functionally, miR-7 critically regulates sexual maturation and reproductive functions by regulating FSH and LH synthesis and secretion (Ahmed et al. 2017). However, little is known about the interaction between miR-7 and the NE. The present study was designed to investigate whether miR-7 mediated the effects of NE on pituitary FSH and LH synthesis in pigs.

### Materials and methods

#### Animal and tissue collection

Male Bama pigs (n=8), age 6–8 months with a weight of 25–30 kg, were purchased from Beijing Farm Animal Research Center. The pigs were housed in a room with natural length of photoperiod and had ad libitum access to the commercial pig diet and water during the entire experimental period. The pigs were intracerebroventricular (ICV) injected 20 μg/kg NE in artificial cerebrospinal fluid (ACSF) (n=4) or ACSF alone (n=4) at 10:00 h, then the pigs were set free in the room. The dosage of NE was chosen according to the report (Zhang et al. 2018). One hour later, at 11:00 h, the blood was collected by puncture through the jugular vein and then centrifuged to separate the serum and stored at −80°C before FSH and LH assay. The pigs were killed by electric shocks, after collecting the blood immediately at 11:00 h, and then slaughtered.

The pituitaries were collected after slaughter, cleaned and frozen in liquid nitrogen and then stored at −80°C for RNA isolation.

The additional pig pituitaries (n=45) were collected from the slaughter house for in vitro culture, in situ hybridization (ISH) and immunofluorescence studies.

All the animal experiments were approved by the institution’s ethics committee and were carried out in accordance with the principles and procedures of the Chinese Association for Laboratory Animal Sciences and the Helsinki Declaration. The protocol was approved by the State Key Laboratory of Agrobiotechnology, and all the procedures were in accordance with ethical standards.

#### Intracerebroventricular injection

The procedure of ICV injection was performed as previously described (van Eerdenburg & Dierx 2002, Zhu et al. 2010). The pig’s head was fixed in a stereotaxic apparatus and the orientation was adjusted for cannula placement as described by previous reports (Poceta et al. 1981). Assembled the interface-device and fixed the stainless steel cannula to the lateral ventricle and glued with a perforated steel collar by dental cement, then connected out a flexible PVC tube for later injections (Zhang et al. 2018). Sutured the scalp and left the pigs to recover for 1 week. All the injections started at 10:00 h and the injected reagents were adjusted to a volume of 1 mL.
Cell culture

For each in vitro experiment, anterior pituitary was taken apart from the whole pituitary gland and digested with collagenase II (Sigma). After being filtered and washed, the digested cells were resuspended and cultured in 12-well plates with Dulbecco’s modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; Gibco-Invitrogen) containing 10% (v/v) fetal bovine serum (FBS; Gibco-Invitrogen) and 1% (w/v) penicillin-streptomycin at 37°C in a highly humidified atmosphere of 95% (v/v) air and 5% (v/v) CO₂. The cells were cultured for 72 h in 10% FBS-DMEM/F12 and the culture medium was refreshed each 24 h, and the cells were cultured in DMEM/F12 for 12 h starvation, followed by transfection and drug treatments. Cells and the culture media were collected and stored at −80°C before assay. All the inhibitors were purchased from MCE (MedChemExpress, Shanghai, China).

To assay the effects of NE on miR-7 level and the synthesis and secretion of FSH and LH in pituitary, the primary cultured porcine anterior pituitary cells were treated with 10⁻⁷ mol/L NE for 0 h, 1 h, 3 h and 6 h. The NE concentration was selected and ameliorated according to our recent report (Qiu et al. 2019).

In order to over-express and knockdown miR-7 in primary cultured porcine anterior pituitary cells, 2 × 10⁻⁷ mol/L of each reagent in a miR-7 kit (RiboBio, Guangzhou, China) containing negative control of miR-7-mimics (nc-mi), miR-7-mimics (miR-7-mi), negative control of miR-7-inhibitors (nc-in) and miR-7-inhibitors (miR-7-in) were transfected for 12 h. The concentration and incubation time of miR-7 kit was selected according to the reference (Qiu et al. 2019). Lipofectamine 2000 agent (Invitrogen Life Technologies) was used for transfection according to the manufacturer’s instructions.

To find the relationship between miR-7 and gonadotropin, the porcine anterior pituitary cells were transfected with 2 × 10⁻⁷ mol/L nc-mi, miR-7-mi, nc-in or miR-7-in for 12 h.

To verify the relationship between miR-7, NE and gonadotropin, the porcine anterior pituitary cells were firstly transfected with 2 × 10⁻⁷ mol/L nc-in or miR-7-in for 12 h and then treated with or without 10⁻⁷ mol/L NE for another 1 h.

To study which type of adrenergic receptors were bound to NE, the primary cultured porcine anterior pituitary cells were treated with α-adrenergic receptor inhibitors (αAR-inhibitor, HY-B0362A) (Giussani et al. 1995) or β-adrenergic receptor inhibitor (βAR-inhibitor, HY-B0573) (Munabi et al. 2016) for 2 h in concentration of 2 × 10⁻⁵ mol/L according to the manufacturer’s procedures and followed treatments with or without 10⁻⁷ mol/L NE for another 1 h.

To investigate the signaling pathway of NE inducing FSH and LH synthesis and secretion mediated by miR-7, the primary cultured porcine anterior pituitary cells were treated with 2 × 10⁻⁸ mol/L GW5074 (Lei et al. 2008), 10⁻⁵ mol/L PD98059 (Rojewska et al. 2015), 2 × 10⁻⁶ mol/L LY294002 (Jiang et al. 2010) and 4 × 10⁻⁸ mol/L CYT387 (Monaghan et al. 2011) for 2 h, respectively, followed by the treatment with 10⁻⁷ mol/L NE or not for 1 h. The inhibitors concentrations and incubation time were selected and ameliorated according to the reference (Qiu et al. 2019).

To examine whether the ERK1/2 signaling pathway was activated by NE, the primary cultured porcine anterior pituitary cells were treated with 10⁻⁷ mol/L NE for 0 min (min), 5 min, 10 min, 20 min and 30 min. Further, in order to find which type of adrenergic receptors were involved in this pathway, the primary cultured porcine anterior pituitary cells were treated with 2 × 10⁻⁵ mol/L αAR-inhibitor or βAR-inhibitor for 2 h, then incubated with 10⁻⁷ mol/L NE or not for 10 min.

For the aim of finding out the target gene of miR-7, the primary cultured porcine anterior pituitary cells were transfected with 2 × 10⁻⁷ mol/L nc-mi or miR-7-mi for 12 h. In order to knockdown miR-7 target gene GLG1 in primary cultured porcine anterior pituitary cells, 2 × 10⁻⁷ mol/L negative-siRNA (nc-siRNA), GLG1-Sus-1566 (GLG1-siRNA1) or GLG1-Sus-1068 (GLG1-siRNA2) (GenePharma, Suzhou, China) were transfected for 6 h according to the manufacturer’s procedures. Further, to explore the relationship between GLG1 and gonadotropin, the primary cultured porcine anterior pituitary cells were transfected with 2 × 10⁻⁷ mol/L nc-siRNA or GLG1-siRNA1 for 6 h followed by a 1 h of 10⁻⁷ mol/L NE incubation.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from pigs’ pituitaries or the primary cultured porcine anterior pituitary cells using TRIzol reagent (TaKaRa) as per the manufacturer’s instructions. RT was conducted using the M-MLV reverse transcriptase kit (Promega) to synthesize complementary DNAs. RT-miR-7stem-loop primer: 5’-CCTCAACTGGTGCGGCTTGAGTCCCTCTTTTGTCTGCTTGC-3’.

RT-U6 primer: 5’-AACCCTTCACGAATTTGCGT-3’. Mixed transcription reactions were incubated in the DNA T100™ Thermal Cycler (Bio-Rad Laboratories). U6 and GAPDH were respectively used for normalization of miR-7.
or others' transcripts expression. Gene expression levels were measured by SYBR Green master mix (TaKaRa) in the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Primer sequences were showed in Table 1.

### In situ hybridization

The ISH of miR-7 applied using digoxigenin (DIG)-labeled locked nucleic acid probe (38181-05; Exiqon, Woburn, MA, USA) was performed as described by previous reports (Javelle & Timmermans 2012). Pigs' pituitaries were collected and fixed in 4% (w/v) paraformaldehyde. Cryosections (10 μm thick) were prepared. The samples were fixed for 10 min in 4% paraformaldehyde and treated with 5 μg/mL proteinase K for 10 min at room temperature, followed by acetylation for 10 min. The sections were then pre-hybridized at room temperature for 6 h and then hybridized with miR-7 probes at 48°C overnight. The signal was detected by an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics). The sections were incubated in nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in darkness at room temperature until the prospective intensity of staining was reached. The sections were photographed under a DMRB light microscope (Leica Microsystems).

### Immunofluorescence

For immunofluorescence, the porcine pituitaries were fixed into 5 μm and then dewaxed, rehydrated. For ISH of miR-7 and immunofluorescence double staining, we used the cryosections after ISH of miR-7. The antigen retrieval was carried out by high pressure in 121°C 0.01 mol/L sodium citrate buffer (pH 6.0) for 15 min. The sections and the cryosections were then treated with 10% (v/v) normal donkey serum or 10% normal goat serum in phosphate buffer saline (PBS) at room temperature for 1 h to block nonspecific binding sites, followed by the incubation with anti-ADRA1B antibody (1:50; Shanghai Shifeng biotechnology co., LTD, Shanghai, China), anti-β2AR antibody (1:50; sc-570; Santa Cruz Biotechnology), anti-LH antibody (1:50; ZM-0312; Zhongshan, Beijing, China) or anti-FSH antibody (1:50; ZM-0311; Zhongshan), and we used nonimmunized 10% (v/v) rabbit serum or 10% (v/v) mouse serum in PBS as controls, overnight at 4°C. After washing, the sections of cryosections were subsequently incubated with the secondary antibodies, which were cy2-conjugated donkey anti-rabbit IgG (1:200; 711-225-152; Jackson ImmunoResearch) or cy3-conjugated goat anti-mouse IgG (1:200; 115-165-003; Jackson ImmunoResearch) for 3 h and kept in darkness at room temperature. The nuclei were stained by DAPI (10236276001; Roche Applied Science) for 20 min. Finally, sections and cryosections were mounted with Vectashield (H-1000; Vector Laboratories, Burlingame, CA, USA) and photographed under a fluorescence microscope photograph system (Leica Microsystems).

### Western blot

Proteins were extracted from cells by using radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology). The protein concentrations were determined by the BCA assay kit (Vigorous Biotechnology, Beijing, China) according to the manufacturer's instructions. Western blots were performed as mentioned in our previous report (Zhang et al. 2018). Anti-ERK1/2 antibody (1:1000; 4695; Cell Signaling Technology) and anti-phosphorylated ERK1/2 (p-ERK1/2) antibody (1:2000; 4370; Cell Signaling Technology) were used.

### Dual-luciferase reporter assay

The psiCHECK™-2 vector (C8021; Promega) was used for constructing the dual-luciferase reporter genes. Wild-type and mutant 3′ UTR of SP1 and GLG1 mRNA containing XhoI and NotI sites were cloned by overlap PCR. Then, the sequences were inserted in the vector to construct

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**Table 1** Primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5′-3′)</th>
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<tbody>
<tr>
<td>miR-7</td>
<td>Sense: AGTTGTCGTGGAAGACTTAGT G</td>
</tr>
<tr>
<td>U6</td>
<td>Sense: CTCGCCGAGCA</td>
</tr>
<tr>
<td>FSH</td>
<td>Sense: CCATCTCCCAATCTGTCTC</td>
</tr>
<tr>
<td>LH</td>
<td>Sense: AGAGCTGACCTTGTACCCATC</td>
</tr>
<tr>
<td>FOS</td>
<td>Sense: TTCATCCCAACGGTGACTG</td>
</tr>
<tr>
<td>SP1</td>
<td>Sense: GACAGTGGGGCAGGTT</td>
</tr>
<tr>
<td>GLG1</td>
<td>Sense: CCCCGTCCCGGGAAG</td>
</tr>
<tr>
<td>CD38</td>
<td>Sense: ATGAAGCTGGGGCATGACGTC</td>
</tr>
<tr>
<td>PTGFRN</td>
<td>Sense: CCGAGGCCGCTGCTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense: TTTAACCTGGAACATGGAC</td>
</tr>
</tbody>
</table>
dual-luciferase reporter plasmids. The 293FT cells were cultured in 10% FBS-DMEM/F12 in 24-well plates. The cells were transfected using the Lipofectamine 2000 agent with a mixture containing the 200 ng/mL dual-luciferase reporter plasmid and 2×10⁻⁷ mol/L nc-mi or miR-7-mi for 24 h according to the reports (He et al. 2018b, Qiu et al. 2019), and luciferase activity was analyzed by using dual-luciferase reporter assay kit (E1910; Promega) on a Modulus II Microplate Multimode Reader (Turner Biosystems, Sunnyvale, CA, USA) in accordance with the manufacturer’s recommendations. For data analysis, the ratios of Renilla luciferase activity and firefly luciferase activity in mutant plasmids were used as controls.

**Radioimmunoassay (RIA)**

Samples of porcine serum and the cell culture media were collected at indicated times. FSH and LH concentrations were measured using RIA reagents (Beijing North Institute Biological Technology, Beijing, China) according to the manufacturer's procedures. Briefly, samples were labeled with ¹²⁵I-FSH and FSH or ¹²⁵I-LH and LH antiserum and counted in a gamma counter to obtain the results. Minimum detectable concentrations of FSH and LH were 2.5 mIU/mL and 5.0 mIU/mL. Interassay and intra-assay coefficients of variation were <10 and <5%.

**Cell counting**

Sections were scanned using a 20× magnification objective of a Leica microscope. Three fields of three different sections have been chosen, and the number of miR-7-, β2AR-, FSH- or LH-positive cells compared with total cell number was counted using the manual counter function of Adobe Photoshop CC 2018 software. Cell counting was performed by two investigators, and the results are expressed as the percentages of miR-7, β2AR, FSH or LH immune positive cells.

**Statistical analysis**

Data were presented as means±s.e.m. of at least three independent experiments using GraphPad Prism 7.0 software. Single comparisons were performed by Student’s t test, and multiple comparisons were conducted with one-way ANOVA. A value of P<0.05 was considered to be statistically significant, and P<0.01 was considered to be extremely significant.

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**Results**

**NE enhances miR-7's expression and promotes the synthesis and secretion of FSH and LH in porcine pituitary**

NE, acting as a neurotransmitter evoked and increased by various types of stressors, could affect the synthesis and secretion of FSH and LH in porcine pituitary. Our recent study has proved that miR-7 is highly expressed in porcine pituitary gonadotroph (He et al. 2018b). In order to confirm our hypothesis that miR-7 involves in the NE signaling pathway affecting the FSH and LH synthesis and secretion in porcine pituitary, 20 μg/kg NE and ACSF were respectively supplied through the ICV injection in male porcine, and 1 h later the pituitary and the serum were harvested. The pituitary miR-7, FSH and LH mRNA levels were detected by RT-PCR, and serum FSH and LH concentrations were assayed by RIA. The results showed that the miR-7, FSH and LH mRNA expression levels were stimulated by NE by 2.61, 1.82 and 2.09 times, respectively (Fig. 1A, B and C), while serum FSH and LH concentrations were increased by about 1.88 and 1.51 times (Fig. 1D and E).

In order to confirm the in vivo results, the cultured porcine primary anterior pituitary cells were treated with 10⁻⁷ mol/L NE for 0 h, 1 h, 3 h and 6 h respectively, then the miR-7, FSH and LH mRNA levels, medium FSH and LH concentrations were detected. The results demonstrated that 10⁻⁷ mol/L NE treatment for 1 h elevated the anterior pituitary cells miR-7 level by 2.50-fold (Fig. 1F) and that the anterior pituitary cells FSH and LH mRNA levels were increased by 4.81-fold and 3.57-fold (Fig. 1G and H), while the culture medium FSH and LH concentrations were elevated by 1.85-fold and 2.08-fold (Fig. 1I and J). Meanwhile, the effects of 3 h and 6 h NE treatments were not as significant as 1 h treatment.

**miR-7 mediates the NE signaling pathway affecting FSH and LH synthesis**

In order to verify whether miR-7 was involved in regulating the effects of NE on the pituitary FSH and LH synthesis, the cultured porcine anterior pituitary cells were respectively transfected with 2×10⁻⁷ mol/L nc-mi, miR-7-mi, nc-in or miR-7-in. After 12 h of incubation, the transfection efficacies were detected and the results showed that miR-7-mi increased miR-7 level over 82 times higher than the control nc-mi and miR-7-in...
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Exogenous NE stimulation induces a large increase of the miR-7 level and the secretion of FSH and LH in porcine pituitary. (A, B and C) Relative miR-7 (A), FSH (B) and LH (C) mRNA levels in porcine pituitary after 1 h 20 μg/kg NE or ACSF ICV injection were assayed by RT-PCR and normalized to U6 and GAPDH. n = 4. (D and E) FSH (D) and LH (E) concentration in serum after 1 h NE or ACSF ICV injected were assayed by RIA. n = 4. (F, G and H) Cultured primary porcine anterior pituitary cells were treated with 10⁻⁷ mol/L NE. Cells and cell culture media were collected at different time points. Relative miR-7 (F), FSH (G) and LH (H) mRNA levels in cells were assayed by RT-PCR and normalized to U6 and GAPDH. n = 3. (I and J) FSH (I) and LH (J) concentration in culture medium were assayed by RIA after treated with NE. n = 3. Results are means ± s.e.m. *P < 0.05, **P < 0.01 and ***P < 0.0001.

downregulated the endogenous miR-7 level by 87% compared to the control nc-in (Fig. 2A). Further analysis showed that miR-7 increased the FSH and LH synthesis and secretion in porcine anterior pituitary cells (Fig. 2B, C, D and E).

The primary porcine anterior pituitary cells after transfecting with 2 × 10⁻⁷ mol/L nc-in or miR-7-in were subsequently treated with 10⁻⁷ mol/L NE for another 1 h, and the FSH and LH mRNA levels and the concentration of FSH and LH were detected. The results showed that

miR-7 mediates the NE upregulating FSH and LH synthesis and secretion in primary cultured porcine anterior pituitary cells. (A) Primary cultured porcine anterior pituitary cells were transfected with 2 × 10⁻⁷ mol/L nc-mi, miR-7-mi, nc-in or miR-7-in for 12 h. Relative miR-7 level in cells were assayed by RT-PCR and normalized to U6. n = 3. (B and C) Relative FSH (B) and LH (C) mRNA levels in cultured primary porcine anterior pituitary cells were assayed by RT-PCR after nc-mi, miR-7-mi, nc-in or miR-7-in transfection and normalized to GAPDH. n = 3. (D and E) FSH (D) and LH (E) concentration in the culture medium after cells were transfected with nc-mi, miR-7-mi, nc-in or miR-7-in assayed by RIA. n = 3. (F and G) Primary cultured porcine anterior pituitary cells were transfected with 2 × 10⁻⁷ mol/L nc-in or miR-7-in for 12 h and then 10⁻⁷ mol/L NE was added for 1 h. Relative FSH (F) and LH (G) mRNA levels were assayed by RT-PCR normalized to GAPDH. n = 3. (H and I) FSH (H) and LH (I) concentration in culture medium were assayed by RIA after cells were transfected with nc-in or miR-7-in and treated with NE. n = 3. Results are means ± s.e.m. *P < 0.05 and **P < 0.01. NS, not significant.
miR-7 knockdown significantly inhibited the enhancing effects of NE on FSH and LH mRNA expressions and the synthesis of FSH and LH (Fig. 2F, G, H and I).

**NE affects miR-7 expression and gonadotropin synthesis and secretion via β2AR**

As the NE plays functions through its combining adrenergic receptors, we thus identify the molecular types of adrenergic receptors through which the NE affects miR-7 expression and gonadotropins synthesis in porcine pituitary gland. First, we guessed it was adrenoceptor alpha 1B (ADRA1B) or adrenoceptor beta 2 (β2AR) combined by NE (Fig. 3A and B). Second, we treated the primary cultured anterior pituitary cells with $2 \times 10^{-5}$ mol/L αAR-inhibitor or βAR-inhibitor for 2 h and followed the treatments with or without $10^{-7}$ mol/L NE for another 1 h. The miR-7, FSH and LH mRNA in the cells were detected by RT-PCR, and FSH and LH concentrations in the media were assayed by RIA. The results showed that αAR-inhibitor did not influence the upregulating effects of NE on miR-7, FSH and LH mRNA expressions and FSH and LH concentrations in the medium, but all of these enhancing effects of NE were blocked by βAR inhibitor (Fig. 3C, E, F, G and H).

Further, to confirm whether the effects of NE on miR-7 relied on β2AR, triple staining experiments for miR-7, β2AR and LH or FSH were performed and the results...
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NE-β2AR activates the RAF/MEK/ERK1/2 signaling pathway elevating miR-7 to up-regulate FSH and LH synthesis

In order to identify the signaling pathway of NE-β2AR affecting miR-7 and the synthesis and secretion of FSH and LH, the cultured primary porcine anterior pituitary cells were cultured in vitro, incubated with 2 × 10⁻⁸ mol/L RAF inhibitor GWS5074, 10⁻⁵ mol/L MEK inhibitor PD98059, 2 × 10⁻⁶ mol/L PI3K inhibitor LY294002 or 4 × 10⁻⁸ mol/L JAK inhibitor CYT387 for 2 h, followed by the treatment of 10⁻⁷ mol/L NE for 1 h. The cells miR-7, FSH and LH mRNA levels were then detected by RT-PCR, and the media concentrations of FSH and LH were assayed by RIA. The results showed that the additions of CYT387 and LY294002 had no significant effect on miR-7, FSH and LH induced by NE, whereas GWS5074 and PD98059 blocked the enhancing effect of NE on miR-7, FSH and LH expressions (Fig. 4A, B, C, D and E).

As the RAF/MEK lead to ERK activation (O’Hayre et al. 2017), we assayed the effects of NE on p-ERK1/2 after 0 min, 5 min, 10 min, 20 min and 30 min treatments...
miR-7 affects pituitary FSH and LH synthesis

Figure 5

GLG1 is the target gene of miR-7. (A) Porcine primary anterior pituitary cells were transfected with 2 × 10⁻⁷ mol/L nc-mi or miR-7-mi for 12 h. Relative mRNA levels of FOS, SP1, GLG1, CD38 and PTGFRN were detected by RT-PCR and normalized to GAPDH. n = 3. (B) Relative SP1 and GLG1 mRNA levels in porcine pituitary after 1 h 20 μg/kg NE or ACSF ICV injected were assayed by RT-PCR and normalized to GAPDH. n = 3. (C) Cultured primary porcine anterior pituitary cells were treated with 10⁻⁷ mol/L NE. Cells and cell culture media were collected at different time points. Relative SP1 and GLG1 mRNA levels in cells were assayed by RT-PCR and normalized to GAPDH. n = 3. (D) The predicted binding sites of miR-7 and 3'-UTRs of SP1 and GLG1. (E) Schematic representations of the hybridization between miR-7 and 3'-UTRs of SP1 and GLG1. (F) Schematic of inserted SP1 3'-UTR and GLG1 3'-UTR sequences into the psiCHECK™-2 vector. (G and H) Relative luminescence intensity detected by the Modulus™ II microplate multimode reader after miR-7-mi or nc-mi and dual-luciferase vectors cotransfected into 293FT cells. n = 3. (I) Primary cultured porcine anterior pituitary cells were transfected with 2 × 10⁻⁷ mol/L nc-in, miR-7-in, nc-mi or miR-7-mi for 12 h. Relative GLG1 mRNA level in cells was assayed by RT-PCR and normalized to GAPDH. n = 3. (J) Primary cultured porcine anterior pituitary cells were transfected with 2 × 10⁻⁷ mol/L nc-siRNA, GLG1-siRNA1 or GLG1-siRNA2 for 6 h. Relative GLG1 mRNA level in cells was assayed by RT-PCR and normalized to GAPDH. n = 3. (K and L) Primary cultured porcine anterior pituitary cells were transfected with 2 × 10⁻⁷ mol/L nc-siRNA or GLG1-siRNA1 for 6 h, followed by another 1 h incubation with 10⁻⁷ mol/L NE. Relative mRNA level of FSH (K) and LH (L) in cells were assayed by RT-PCR and normalized to GAPDH. n = 3. (M and N) FSH (M) and LH (N) concentrations in culture medium were assayed by RIA. n = 3. Results are means ± s.e.m. *P < 0.05, **P < 0.01 and ***P < 0.0001. NS, not significant. A full colour version of this figure is available at https://doi.org/10.1530/JOE-19-0331.
by 10\(^{-7}\) mol/L. NE in the cultured primary porcine anterior pituitary cells. The Western blot results showed that NE treatment increased p-ERK1/2 levels about 3.05-fold at 10 min (Fig. 4F). And further analysis showed that NE activated the p-ERK1/2 through β2AR in the cultured primary porcine anterior pituitary cells (Fig. 4G).

**GLG1 is a direct target of miR-7**

In order to find out the target genes of miR-7, we searched Sanger miRNA target database and TargetScan database, selected the relative factors in the signaling network regulating FSH and LH and used bioinformatics approaches to detect whether these genes have complementary sequences in 3’-UTRs with seed sequence of miR-7. Five genes-PTGFRN (prostaglandin F2 receptor negative regulator) (Ahmed et al. 2017), GLG1 (Golgi glycoprotein 1) (Ahmed et al. 2017), CD38 (CD38 molecule) (Chobanian et al. 1994), FOS (FJ murine osteosarcoma viral oncogene homolog) (He et al. 2018b) and SP1 (Sp1 transcription factor) (Thackray et al. 2009) were potential miR-7 target genes. The RNAHybrid database provided miR-7-binding site predictions by offering minimum free energy of hybridization between the 3′-UTRs of target genes and seed sequence of miR-7. Corresponding minimum free energy of the predicted five genes are –20.4 kcal/mol (PTGFRN), –24.2 kcal/mol (GLG1), –22.2 kcal/mol (CD38), –21.4 kcal/mol (FOS) and –24.4 kcal/mol (SP1). Furthermore, we detected mRNA levels of the five target genes after 12 h transfection of 2 × 10\(^{-7}\) mol/L miR-7-mi or nc-mi. The results showed that miR-7 upregulation did not have significant effects on PTGFRN, FOS and CD38 mRNA levels, whereas miR-7 upregulation sharply decreased SP1 and GLG1 mRNA by 92.13% and 95.32% (Fig. 5A). And NE increased the SP1 and GLG1 mRNA levels in porcine pituitary both in vivo and in vitro (Fig. 5B and C).

Further, we predicted binding sites of miR-7 to possible target genes (SP1 and GLG1) (Fig. 5D) and used the RNAHybrid to predict the minimum free energy of hybridization between miR-7 and predicted target genes (SP1 and GLG1) (Fig. 5E). Finally, we used the pSICHECK™-2 vector to clone the putative 3′UTR target site downstream of a luciferase reporter gene (Fig. 5F). We cotransfected 200 ng/mL pSICHECK™-2 vector (wild-type or mutant) and 2 × 10\(^{-7}\) mol/L miR-7-mi (or nc-mi) into 293FT cells. The results show that the luciferase activity of p-Luc-3′ UTR SP1 cotransfected with miR-7-mi had no evident changes compared with p-Luc-3′ UTR mutant SP1 (Fig. 5G), but the luciferase activity of the transfected cells with miR-7-mi and p-Luc-3′ UTR GLG1 decreased by 53.69% compared with the cells cotransfected with miR-7-mi and p-Luc-3′ UTR mutant GLG1 (Fig. 5H). Meanwhile, more analysis showed that GLG1 was inhibited by miR-7 (Fig. 5I).

For the further exploration of GLG1 function on gonadotropin, 2 × 10\(^{-7}\) mol/L nc-siRNA, GLG1-siRNA1 or GLG1-siRNA2 were transfected for 6 h in the primary cultured porcine anterior pituitary cells to knockdown the GLG1. The result showed that GLG1-siRNA1 could knockdown the 58.15% GLG1 in cells (Fig. 5J). Secondly, 2 × 10\(^{-7}\) mol/L nc-siRNA or GLG1-siRNA1 were transfected in cells for 6 h followed by another 1 h incubation with 10\(^{-7}\) mol/L NE, and the results indicated that GLG1 inhibited the synthesis and secretion of FSH and LH (Fig. 5K, L, M and N).

**Discussion**

The results present here prove that NE has functions to upregulate FSH and LH synthesis in porcine pituitary via β2AR. And this study, at first, demonstrates that miR-7 exerts its regulatory effect by mediating the NE-β2AR signaling pathway. In support, our in vitro and in vivo results show that NE effects on the pituitary to upregulate the expressions of miR-7, FSH and LH and all of FSH- and LH-positive cells expressed β2AR and miR-7 in porcine pituitary. Whereas these effects are omitted after miR-7 knockdown by miR-7-in and NE-β2AR is blocked by β2AR inhibitor.

The functions of miR-7 in different endocrine organs have been reported (Moberg 1991, Kredo-Russo et al. 2012, Glover et al. 2015, Degrefe et al. 2018). However, at first, our research indicates that miR-7 acts as a regulatory factor which involves in the process of NE-mediated FSH and LH synthesis. The first evidence is that miR-7 mRNA level is positively correlated with FSH and LH mRNA levels in porcine pituitary and FSH and LH concentrations in porcine serum under NE stimulation. Secondly, the overexpression of miR-7 in cultured porcine anterior pituitary cells increases FSH and LH synthesis, and the knockdown of miR-7 reduces cells FSH and LH synthesis. In addition, miR-7 knockdown blocked the enhancing effect of NE on FSH and LH synthesis. These data demonstrate that miR-7 enhanced by NE is a positive factor of FSH and LH synthesis in porcine pituitary.

Here, our results demonstrate that NE promotes porcine pituitary FSH and LH synthesis, which is in agreement with the reports in rats (Drouva & Gallo 1976, 1987).
miR-7 affects pituitary FSH and LH synthesis

Figure 6
Illustrative model of miR-7 mediates the up-regulating effects of NE on FSH and LH synthesis in porcine pituitary. NE up-regulates miR-7 expression through the RAF/MEK/ERK1/2 signaling pathway via β2AR. miR-7 inhibits GLG1 and GLG1 inhibits the synthesis and secretion of FSH and LH resulting in the enhancement of FSH and LH synthesis. A full colour version of this figure is available at https://doi.org/10.1530/JOE-19-0331.

Miyake et al. 1983, He et al. 1993, Gu et al. 2018), but there are few reports about the signaling pathway and the related mechanisms of NE affecting FSH and LH synthesis. The results of the present study confirm that the NE signal is conducted through β2AR/RAF/MEK/ERK1/2, and our new finding is that the ERK1/2 acts as a upstream molecule to elevate miR-7 expression through ERK1/2 phosphorylation, while the down-stream molecule is GLG1, which inhibits the synthesis of FSH and LH as a target gene of miR-7. This can be thought as a new pathway of NE affecting the synthesis of FSH and LH in porcine pituitary gland (Fig. 6).

Our results present here demonstrate that the target gene of miR-7 is GLG1, but our previous work has proved that miR-7 mediates the zearalenone signaling pathway by negatively regulating FSH synthesis and secretion by targeting FOS (He et al. 2018b). These infer that miR-7 may involve in different signaling pathways of zearalenone and NE. In support, NE and zearalenone have different effects on pituitary gonadotrophin, which are dependent on different physiological stages and the related hormone levels (Gu et al. 2018, He et al. 2018a, Zheng et al. 2019). However, the detailed molecular mechanisms of miR-7 affecting pituitary gonadotrophin need to be elucidated in the future studies.

The data presented here demonstrate that NE, acting as a neurotransmitter evoked by stressors, increases the miR-7 level via β2AR/RAF/MEK/ERK1/2, which subsequently promotes FSH and LH synthesis and secretion by targeting GLG1 in porcine pituitary. In light of these findings, the further studies about the interactions between miRNAs and neurotransmitters and their relevance are important for our understanding of the functions and related mechanisms of miRNAs.

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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Author contribution statement
Sheng Cui conceived and designed the research; Xin Li, Hongjiao Li, Jinglin Zhang, Di Zhang and Guojin Xu performed research; Xin Li and Hongjiao Li contributed equally; Di Zhang and Guojin Xu collected the data; Xin Li and Hongjiao Li analyzed the data; Sheng Cui and Xin Li wrote the manuscript.

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