Abstract. RFamide-related peptide-3 (RFRP-3) may be involved in the inhibition of kisspeptin, but there is no direct evidence that RFRP-3 can directly act on kisspeptin neurons. The present study aimed to investigate the role and mechanism of RFRP-3 and kisspeptin in the hypothalamic-pituitary reproductive axis. In order to detect the expression and localization of RFRP-3 and kisspeptin in dorsomedial hypothalamic nucleus, double immunofluorescence method combined with confocal microscopy were performed. RFRP-3 was injected into the lateral ventricle of ovariectomized estrogen primed rats. Blood and brain tissues were collected at 60-, 120-, 240- and 360-min. Serum levels of gonadotropin-releasing hormone, luteinizing hormone and follicle-stimulating hormone were detected by ELISA. Kisspeptin expression in hypothalamus was detected by western blotting. Finally, surface plasmon resonance was used to verify whether RFRP-3 can directly interact with kisspeptin. Confocal images indicated that RFRP-3 and kisspeptin were co-expressed in the same neurons in the hypothalamus of ovariectomized estrogen-primed rats. Serum concentrations of gonadotropin-releasing hormone, luteinizing hormone and follicle-stimulating hormone were demonstrated to be significantly reduced following microinjection of RFRP-3 into the lateral ventricle for 60, 120, 240 and 360 min compared with the corresponding saline groups. The expression levels of kisspeptin in hypothalamus were gradually decreased following microinjection of RFRP-3 into the lateral ventricle. In addition, the affinity constant (K_D) of RFRP-3 binding to kisspeptin was 6.005x10^{-5} M, indicating that RFRP-3 bound directly to kisspeptin in the range of protein-protein binding strength (K_D, 10^{-3}-10^{-6} M). In conclusion, RFRP-3 may regulate the hypothalamic-pituitary reproductive axis by inhibiting the expression of hypothalamic kisspeptin and direct binding.

Introduction

The hypothalamic-pituitary-gonadal axis is one of the main systems regulating mammalian reproductive function, including in humans (1). Hypothalamic secretion of gonadotropin-releasing hormone (GnRH) can stimulate the anterior pituitary gland to secrete gonadotropin, which can act on the gonads to stimulate the synthesis of steroid hormones, and promote sperm and egg production (2,3). In the early 21st century, researchers identified two new neuropeptides that could directly regulate the synthesis and secretion of GnRH; gonadotropin-inhibitory hormone (GnIH) was identified in 2000, and was revealed to inhibit the synthesis and secretion of GnRH in the hypothalamus of mammals and birds by acting on its receptor, neuropeptide FF receptor (NPFFR)1 (4). Mammalian RFRP-1 and RFRP-3 are homologs of poultry GnIH, and are key neuropeptides that regulate the reproductive function in vertebrates (5). Whereas kisspeptin is a neuropeptide encoded by the Kiss1 gene that was revealed to stimulate the synthesis and secretion of GnRH; gonadotropin-inhibitory hormone (GnIH) was identified in 2000, and was revealed to inhibit the synthesis and secretion of GnRH in the hypothalamus of mammals and birds by acting on its receptor, neuropeptide FF receptor (NPFFR)1 (4). Mammalian RFRP-1 and RFRP-3 are homologs of poultry GnIH, and are key neuropeptides that regulate the reproductive function in vertebrates (5). Whereas kisspeptin is a neuropeptide encoded by the Kiss1 gene that was revealed to stimulate the synthesis and secretion of GnRH by acting directly on the KISS1 receptor (KISS1R) of GnRH neurons (6,7). Previous studies have shown that these two neuropeptides may act on the reproductive axis via unilateral or bilateral linkage (8,9); however, to the best of our knowledge, the specific mechanism has not been clarified. It has been shown that RFRP-3 (mammalian homolog of GnIH) may be involved in the inhibition of kisspeptin via the calcium or protein kinase C-related signaling pathways (10), but there is currently no direct evidence that RFRP-3 can directly act on kisspeptin neurons.
The association between RFRP-3 and kisspeptin, as well as their role and mechanism in the hypothalamic-pituitary reproductive axis, were investigated in the present study. An ovariectomized estrogen-primed (OEP) rat model was first established and RFRP-3 was microinjected into the lateral ventricle. Co-localization of RFRP-3 and kisspeptin was assessed using laser confocal microscopy. Direct binding between RFRP-3 and kisspeptin was evaluated using surface plasmon resonance (SPR). The results indicated that there may be a direct interaction between RFRP-3 and kisspeptin neuropeptides in the hypothalamus of OEP rats, providing experimental evidence for the further study of the regulatory mechanism of the neuroendocrine reproductive axis.

Materials and methods

Animals. A total of 53 specific pathogen-free grade female Sprague Dawley (SD) rats (age, 6-7 weeks; weight, 200±20 g) were purchased from Beijing Huafukang Biotechnology Co., Ltd. (certificate no. 114013000674466). The rats were fed in a well-ventilated environment, at a constant temperature (22-25°C), humidity (50-54%) and a natural 12/12-h light cycle and free access to food and water. All animal experiments were conducted according to the ethical guidelines of Chengde Medical University and were approved by the ethical review board of Chengde Medical University (Chengde, China).

Animal treatment and grouping. The SD rats were fed adaptively for 1 week, then bilateral oophorectomy was performed under anesthesia with 0.01% PBST, incubated with the Alexa Fluor 568-labeled donkey anti-goat (cat. no. A11057) and Alexa Fluor 488-labeled donkey anti-rabbit (1:50; cat. no. A21206) secondary antibodies for RFRP-3 and kisspeptin, respectively (both from Thermo Fisher Scientific, Inc.) for 2 h at 4°C in the dark, then washed with 0.01% PBS-Tween-20% (PBST) for 5 min, and then incubated with goat anti-rat RFRP3 (1:25; cat. no. sc-32380) and rabbit anti-rat kisspeptin (1:50; cat. no. sc-15400; subtypes of kisspeptin not available; both from Santa Cruz Biotechnology, Inc.) polyclonal antibodies for 48 h at 4°C. The sections were washed with 0.01% PBST, incubated with the Alexa Fluor® 568-labeled donkey anti-goat (cat. no. A11057) and Alexa Fluor 488-labeled donkey anti-rabbit (1:500; cat. no. A21206) secondary antibodies for RFRP-3 and kisspeptin, respectively (both from Thermo Fisher Scientific, Inc.) for 2 h at 4°C in the dark, then washed with 0.01% PBST again. DAPI (cat. no. BS130A; Biosharp Life Sciences) was added and the samples were incubated for 5 min at room temperature in the dark. After washing, the sections were mounted and observed with a laser confocal microscope (magnification, x400; TCS SP8X; Leica AG).

ELISA. At 60, 120, 240 and 360 min after injection with RFRP-3, venous blood was collected from the rats of each group. After centrifugation at (3,000 x g) for 15 min at 4°C, the serum was isolated. The concentrations of GnRH, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the rat serum were determined with rat GnRH (cat. no. ml003038; Shanghai Enzyme-linked Biotechnology Co., Ltd.), rat FSH (cat. no. E-EL-R0391C; Elabscience Biotechnology, Inc.) and rat LH ELISA kits (cat. no. E-EL-R0026C; Elabscience Biotechnology, Inc.).

Western blot analysis. The fresh hypothalamic tissues were separated and the total proteins were extracted using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). The BCA method was used for protein quantification (Beijing Solarbio Science and Technology Co., Ltd.). Proteins were separated via 12% SDS-PAGE (20 µg per lane) and transferred onto a PVDF membrane (MilliporeSigma). After blocking with 5% skimmed milk for 2 h at room temperature, rabbit anti-rat kisspeptin polyclonal (1:50; cat. no. sc-15400; subtype of kisspeptin not available; Santa Cruz Biotechnology, Inc.) and anti-GAPDH antibodies (1:8,000; cat. no. AP0063; Bioworld Technology, Inc.) were added and the membranes were incubated overnight at 4°C. After washing with 2.5% TBS-Tween 20 three times, the goat anti-rabbit IgG HRP-conjugated secondary antibody (1:80,000; cat. no. E030120; Beijing Merida Technology Co., Ltd.) was added. After incubation.
for 1 h at room temperature, the membrane was washed three times and then analyzed using the Chemiluminescence image analysis kit (Tanon Science & Technology Co., Ltd.). Image J analysis software (version 1.46r; National Institutes of Health) was used to analyze the average gray value.

**SPR.** The interaction between kisspeptin and RFRP3 was analyzed using a Biacore T200 SPR system (Cytiva). Kisspeptin (cat. no. CFB170220017; Shanghai Qiang Yao Biotechnology Co., Ltd.) was diluted to 20 µg/ml with sodium acetate buffer (pH 5.5) and coupled to a CM5 sensor chip with a coupling amount of 5,720 response units (RU). PBS-P (10 mM phosphate buffer with 2.7 mM KCl, 137 mM NaCl and 0.05% surfactant P20, pH 4.5) was used as the running buffer and RFRP3 (cat. no. H-5846; Bachem AG) was diluted to five concentrations (3.125, 6.25, 12.5, 25 and 50 µM). Different concentrations of RFRP-3 were injected into the detection channel and the reference channel to perform the binding reaction at 4°C for 3 min. The experiment was performed by selecting the ‘Kinetics’ program. The samples were analyzed with an injection time of 60 sec, a flow rate of 15 µl/min and a dissociation time of 15 min. After each concentration cycle, the chip was regenerated with glycine-HCl (pH 2.0) at a concentration of 10 mM for 30 sec. After the end of the cycle, the obtained curve was subjected to data processing using Biacore Evaluation Software (T200 version 2.0; Cytiva) to obtain the kinetic parameters.

**Statistical analysis.** Data analysis was performed using SPSS v19.0 software (IBM Corp.). The results are presented as the mean ± standard deviation. Independent-samples t-tests were used to analyze differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Kisspeptin and RFRP3 are co-expressed in the nerve cells of the hypothalamus. To observe the localization of kisspeptin and RFRP-3 in the nerve cells of the hypothalamus, thionine staining and immunofluorescence labeling was performed. After thionine staining, the structures of the brain tissues in the rats were observed to be clear and intact with a light blue color. The nerve cells were densely distributed and neatly arranged with clear nucleoli; regions such as the medial dorsomedial nucleus of the hypothalamus (DMH), ventromedial nucleus of the hypothalamus (VMH) and arcuate nucleus (ARC) were clearly visible (Fig. 1A and B).
Double-labeling immunofluorescence was used to determine the expression and localization of RFRP3 and kisspeptin. It was observed that RFRP3 and kisspeptin were co-expressed in the neurons of the hypothalamus, as determined using laser confocal microscopy (Fig. 1C). RFRP3 showed red fluorescence, which was mainly expressed in the cytoplasm of the nerve cells, whereas kisspeptin was labeled green and mainly expressed in the nucleus. In addition, the nuclei were stained with DAPI and showed blue fluorescence. These results indicated that kisspeptin and RFRP-3 were co-expressed in the nerve cells of the hypothalamus.

Reduction of GnRH, LH and FSH in the serum by RFRP3. To determine the concentrations of GnRH, LH and FSH in the serum, ELISA was performed (Fig. 2). The concentration of serum GnRH was 10.36±1.13, 9.39±1.15, 14.82±1.22 and 7.99±1.24 ng/l at 60, 120, 240 and 360 min after the microinjection of RFRP3 into the lateral ventricle, while the corresponding GnRH concentrations in the control groups were 18.98±0.92, 18.55±0.91, 18.31±0.82 and 19.45±1.07 ng/l, respectively. The concentrations of GnRH in the RFRP3 groups were significantly lower compared with in the corresponding control groups (P<0.05; Fig. 2A).

The LH concentrations of the RFRP3 groups at the four time points were 12.79±1.27, 16.67±1.18, 9.68±0.82 and 14.06±1.17 ng/l, while those in the saline control subgroups were 22.53±0.99, 22.79±0.76, 22.41±0.99 and 22.47±0.94 ng/l, respectively. The concentrations of LH in the RFRP3 groups were significantly lower compared with in the corresponding control groups (P<0.05; Fig. 2B).

The FSH concentrations of the RFRP3 groups at the four time points were 16.72±0.67, 15.31±1.12, 17.82±0.74 and 16.42±1.04 ng/l, while those in the saline control subgroups were 20.49±0.68, 20.69±0.75, 20.32±0.95 and 19.58±0.85 ng/l, respectively. The concentrations of FSH in the RFRP3 groups were significantly lower compared with in the corresponding control groups (P<0.05; Fig. 2C). These results indicated that microinjection of RFRP-3 into the lateral ventricle inhibited the secretion of GnRH, LH and FSH in the serum of OEP rats, and these three hormones exhibited similar trends at the different time points.

Inhibition of hypothalamic kisspeptin by RFRP3. To demonstrate whether RFRP-3 had a regulatory effect on kisspeptin in the hypothalamus, western blot analysis was performed. The protein expression levels of kisspeptin were presented as the gray value ratio of kisspeptin to GAPDH. At 60, 120, 240 and 360 min after the microinjection of RFRP3, the protein expression levels of kisspeptin in the hypothalamus of the OEP rats were 115.93±1.77, 103.17±1.85, 113.24±1.92, and 72.79±1.92, respectively (Fig. 3). The expression levels of kisspeptin in the hypothalamus of OEP rats after the RFRP3 microinjection were significantly different compared with in the control groups (P<0.05; Fig. 3), showing a time-dependent decrease.
GnRH is recognized as the only hypothalamic neuropeptide that regulates gonadotropin secretion in vertebrates, which in turn stimulates the secretion of LH and FSH from the anterior pituitary (2,3). The present study showed that RFRP-3 could be co-expressed with kisspeptin in the nerve cells of the hypothalamus and that RFRP-3 could directly bind to kisspeptin. Microinjection of RFRP-3 into the lateral ventricle inhibited the concentrations of GnRH, LH and FSH in the serum, and decreased the protein expression levels of kisspeptin in the hypothalamus. In 2000, Tsutsui et al (4) identified a new hypothalamic neuropeptide that could inhibit the secretion of LH and FSH in the brain of Japanese quail. The sequence of this natural peptide contains 12 amino acids (SISKPSAYLPLRFa), with a RF amide peptide at the carboxy terminus, which was called GnIH (4). Since then, several studies (5,9-18) have found that GnIH is expressed in poultry, fish, amphibians and vertebrate mammals, including humans, and that the structure is highly conserved. Mammalian RFRP-1 and RFRP-3 are homologs of poultry GnIH, and are key neuropeptides that regulate the reproductive function in vertebrates (5). In previous years, numerous studies (14,19,20) have found that GnIH could regulate the feeding behavior of vertebrates, in addition to regulating reproduction. GnIH nerve fibers in sheep could be projected to neuropeptide Y, pro-opiomelanocortin, orexin and melanin-concentrating hormone neurons, which all play important roles in regulating feeding. Anjum et al (21) reported that the administration of GnIH in mice increased food intake, increased the expression levels of glucose transporter 4 and increased the synthesis of triglycerides in adipose tissue; in addition, GnIH reduced glucose uptake by down-regulating the expression levels of glucose transporter 8 and reduced testosterone synthesis, suggesting that GnIH plays a role in fat accumulation, in addition to negative regulation of testosterone synthesis. Mammalian GnIH neurons are mainly found in the periventricular nucleus (PeN) of the hypothalamus, the DMH, and the area between the DMH and the VMH (22). GnIH directly inhibits the secretion and release of GnRH via the GnIH receptor, NPFRR1, on the GnRH neurons, thereby inhibiting the synthesis and secretion of gonadotropin (5,14). Since only some GnRH neurons express NPFRR1, GnIH may regulate the GnRH axis via other interneurons, such as kisspeptin neurons. Studies have shown that RFRP-3 nerve fibers in female mice could be projected onto the anteroventral periventricular nucleus (AVPV) and the PeN of the kisspeptin neurons (23), and GnIH could also bind to NPFRR1 expressed by interneurons, such as the kisspeptin neurons, to inhibit the activity of these neurons, thereby inhibiting the function of the GnRH neurons (5).

Kisspeptin and its receptor, KISS1R, have a stimulating effect on reproductive function (6). Adolescent hypogonadism occurs in humans and rodents lacking functional kisspeptin or the kisspeptin receptor gene, which is characterized by low levels of gonadotropins and sex hormones, hypogonadism and infertility (24-27). Administration of exogenous kisspeptin can effectively promote the secretion of LH and FSH via a GnRH-dependent mechanism (26,28-31). Studies have shown that most GnRH neurons overexpress kisspeptin, and kisspeptin nerve fibers can be projected to GnRH neurons, thus directly activating GnRH neurons (26,29,32). Kisspeptin is a potent stimulator of GnRH release; however, little is known regarding the upstream pathway that regulates the synthesis and secretion of kisspeptin. Kisspeptin is mainly expressed in AVPV, PeN and ARC in the hypothalamus of

**Interaction between RFRP3 and kisspeptin.** To demonstrate whether RFRP-3 binds directly to kisspeptin, SPR was performed. The binding dissociation curve corresponding to each concentration was recorded and processed using Biacore T200 evaluation software. RFRP-3, RFamide-related peptide-3; RU, response units.
Effects cannot be excluded. To verify this hypothesis, SPR was used to determine reproductive function.

As previously described (35), the serum GnRH concentrations after 60, 120, 240, and 360 minutes following RFRP3 microinjection into the lateral ventricle of OEP rats were significantly decreased compared with the control group. These results showed that there was kisspeptin expression in the cytoplasm. The SPR results showed direct interaction between RFRP3 and kisspeptin; however, further experiments will be performed to indicate how they interact.

In conclusion, the present study demonstrated that RFRP3 could co-express with kisspeptin in nerve cells in the hypothalamus and RFRP3 could directly bind to kisspeptin. RFRP3 may regulate the hypothalamic-pituitary reproductive axis by inhibiting the expression of the hypothalamic kisspeptin protein. However, the factors affecting neuronal secretion of RFRP3 or kisspeptin require further research. The present study provides evidence for further understanding the regulatory mechanism of RFRP3 and kisspeptin in reproductive function and provides potential targets for the treatment of reproductive dysfunction, which could aid in exploring regulation of reproduction.

Acknowledgements

The authors would like to thank Ms Lei Chen (Graduate School, Chengde Medical University, Chengde, China) and Ms Xiaochao Liu (Graduate School, Chengde Medical University, Chengde, China) for their assistance with performing animal treatments.

Funding

The present study was supported by the open project of Key Laboratory of Family Planning and Eugenics of National Health and Family Planning Commission (grant no. 201502), the Natural Science Foundation of Hebei Province (grant no. H2013406115), the Plan Project of Hebei Provincial Science and Technology Department (grant no. 08276101D-20) and the Hebei Higher Education Research Project (grant no. QN2015121).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Authors' contributions

YQ and SW conceived and designed the study, and funded and supervised the study. LC, SY and LS performed the experiments, collected and analyzed data, and drafted the manuscript. MW, SG, and ZC participated performing experiments/acquiring data and manuscript revision. LC, SY and LS performed to detect the binding between the recombinant rat RFRP3 and the kisspeptin proteins (37,38). RFRP3 showed a binding and dissociation process with kisspeptin, which could be analyzed by kinetic methods. A small affinity value, indicating strong binding between these two proteins, was observed with binding rate, dissociation rate and affinity constants of 5.775x10^3 M^-1 s^-1, 3.467x10^-2 s^-1 and 6.005x10^-5 M, respectively. The affinity of RFRP3 to kisspeptin was in the range of protein-protein binding strength (K_D, 10^-7-10^-6 M), indicating that there may be a specific direct binding between the two proteins. Kisspeptin expression was higher in the nucleus, whereas RFRP3 expression was higher in the cytoplasm. In the preliminary experiments, single staining for kisspeptin was performed and the results showed that there was kisspeptin expression in the cytoplasm. The SPR results showed direct interaction between RFRP3 and kisspeptin; however, further experiments will be performed to indicate how they interact.

In conclusion, the present study demonstrated that RFRP3 could co-express with kisspeptin in nerve cells in the hypothalamus and RFRP3 could directly bind to kisspeptin. RFRP3 may regulate the hypothalamic-pituitary reproductive axis by inhibiting the expression of the hypothalamic kisspeptin protein. However, the factors affecting neuronal secretion of RFRP3 or kisspeptin require further research. The present study provides evidence for further understanding the regulatory mechanism of RFRP3 and kisspeptin in reproductive function and provides potential targets for the treatment of reproductive dysfunction, which could aid in exploring regulation of reproduction.

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The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

YQ and SW conceived and designed the study, and funded and supervised the study. LC, SY and LS performed the experiments, collected and analyzed data, and drafted the manuscript. MW, SG, and ZC participated performing experiments/acquiring data and manuscript revision. LC, SY and LS...
confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Review Board of Chengde Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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