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Effect of RFRP-3 on reproduction is sex- and developmental status-dependent in the striped hamster (*Cricetulus barabensis*)☆

Lei Zhao [a,1], Min Zhong [a,b,1], Hui-Liang Xue [a,1], Ji-Shun Ding [a], Shuo Wang [a], Jin-Hui Xu [a], Lei Chen [a], Lai-Xiang Xu [a,*]

[a] College of Life Science, Qufu Normal University, 273165, China
[b] Department of Biological Sciences, Auburn University, AL 36849, USA

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ABSTRACT

RFamide-related peptides (RFRPs) are orthologous to gonadotropin-inhibitory hormone (GnIH) inhibiting gonadotropin release. There are only two RFRP sequences (RFRP-1 and RFRP-3) encoded in rodents. RFRP-3, which was considered as a hypothetical inhibitor on GnRH, shows a stimulatory effect on the male Syrian and male Siberian hamster in short days. As a dominant rodent pest in northern China farmland, the striped hamster (*Cricetulus barabensis*) has higher reproductive activities and could act as a model to study the mechanism of reproduction. However, the effect of RFRP-3 on the reproductive activity for the striped hamster is less understood. In the study, we cloned 643 bp *RFRP* cDNA from the striped hamster hypothalamus, which contained an ORF of 570 bp encoding two RFamide-related peptide (RFRP) sequences: SPAPANKVPHSAANLPLRF-NH_2 (*C. barabensis* RFRP-1) and TLSRVPSLPQRF-NH_2 (*C. barabensis* RFRP-3). We also investigated the expression variation of *RFRP* mRNA and *GnRH* mRNA in the hypothalamus from hamsters with different developmental statuses (7-week-, 13-week- and 1.5-year-olds) using FQ-PCR, in which the 13-week-old female individuals were in estrous. The striped hamsters that are 7 weeks and 1.5 years old are non-breeding individuals, and those that are 13-week hamsters have breeding phenomena. The highest hypothalamus *RFRP* mRNA level was found in breeding males as compared to non-breeding males. Conversely, the lowest *RFRP* mRNA level in the hypothalamus was observed in breeding females, with no significant level when the breeding females were compared to the 7-week-old individuals. Additionally, the investigation of *GnRH* expression level showed a declining expression trend across the developmental stages (7-week-, 13-week- and 1.5-year-olds) in both sexes. Significant negative and positive relationships were detected in the 13-week estrous female ($r = -0.997$, $P = 0.035$) and the 13-week male ($r = 0.998$, $P = 0.029$) striped hamsters respectively, which suggest that RFRP-3 has inhibitory and stimulatory effects on female and male adults respectively. Our results suggest that the effects of RFRP-3 on reproduction are sex- and developmental status-dependent in the striped hamster.

1. Introduction

Phe-Met-Arg-Phe-NH_2 (FMRFamide) was first isolated from the ganglia of the venus clam *Macrocallista nimbosa* as a cardioexcitatory molecule thirty years ago (Price and Greenberg, 1977), and a structurally similar RFamide peptide (LPXRFamide peptide) was first discovered in the chicken brain (Dockray et al., 1983). The quail LPXRFamide peptide was named as gonadotropin-inhibitory hormone (GnIH) based on its inhibition of gonadotropin release in the quail anterior pituitary (Tsutsui et al., 2000; Ubuka et al., 2013). Subsequently, GnIH was successively identified from the brain of various mammalian species, including the rat (Ukena et al., 2002), mouse (Hinuma et al., 2000), cow (Yoshida et al., 2003), Syrian hamster (Kriegsfeld et al., 2005), sheep (Clarke et al., 2008), human (Ubuka et al., 2009a), rhesus macaque (Ubuka et al., 2009b) and Siberian hamster (Ubuka et al., 2012a). In mammals, LPXRFamide peptides were termed RFamide-related peptides (RFRPs) transcribed from the *RFRP* or *NPVF* gene, which was orthologous to GnIH (also called mammalian RFRP-3), (Smith et al., 2010). Three mammalian RFRPs (RFRP-1, -2 and -3) were encoded by LPXRFamide precursor and the C-terminal of RFRP-1 and RFRP-3 was an LPXRFamide (X = L or Q) motif instead of RFRP-2 which possessed a C-terminal RSamide or RLamide sequences. In recent years, studies have reported only two RFRP sequences (RFRP-1 and RFRP-3) encoded in rodents (Ubuka et al., 2012b).

In mammals, the *RFRP* is exclusively expressed in the dorsomedial and ventromedial areas of the hypothalamus (DMH/VMH) (Janati

Abbreviations: RFRP-3, RFamide-related peptide-3; GnIH, gonadotropin-inhibitory hormone; GnRH, gonadotropin-releasing hormone; RFRPs, RFamide-related peptides; DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus.

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* Corresponding author.
E-mail address: xulx@mail.qfnu.edu.cn (L.-X. Xu).

[1] Joint first authors.

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et al., 2013). For example, the RFRP-3 immunoreactive cell bodies mainly locate within the DMH, and some cells extend to the dorsal region surrounding the VMH for the rodents (Janati et al., 2013). In sheep, the RFRP-3-immunoreactive fibers also exist in the outer zone of the median eminence (Clarke et al., 2008). Prominently, in sheep and rodents (Kriegsfeld et al., 2005; Poling et al., 2012; Rizwan et al., 2012; Smith et al., 2010; Ubuka et al., 2012a) RFRP-3-immunoreactive fibers contact apparently with the 20–40% of gonadotropin-releasing hormone (GnRH) neurons. In situ hybridization results indicated that RFRP-3 receptor (GPR147) was expressed in the 25–30% of GnRH neurons in various rodent species (Poling et al., 2012; Rizwan et al., 2012). RFRP-3 receptor exists in most of GnRH immunoreactive neurons in the preoptic area (POA) using double-label immunocytochemistry for GnRH and RFRP-3 receptor (Ubuka et al., 2012a). GnRH is a pronounced stimulator responsible for the control of reproduction since it can promote synthesis and secretion of gonadotropins via secretion into the hypophysial portal system. Therefore, we hypothesize that RFRP-3 might play a role in mammalian reproduction via GnRH neurons (Clarke et al., 2009; Simonneaux et al., 2013).

Studies have demonstrated a potent inhibitory action of RFRP-3 on GnRH neurons. The GnRH neuronal activation was assessed by colocalization with the immediate early gene c-Fos (Anderson et al., 2009). Sheep GnRH neurons were restrained directly by RFRP-3 (Lai et al., 2012). Anderson et al. showed that the chronic icv infusion of RFRP-3 in female rats caused a dose-dependent suppression on the activation of GnRH neurons (Anderson et al., 2009). However, the above reports were contradictory with the novel observations in the male Syrian hamster and male Siberian hamster. In male Syrian hamsters, GnRH neuron activity was stimulated by acute icv administration of RFRP-3 (Ancel et al., 2012). Meanwhile, in male Siberian hamsters, administration of RFRP-3 in short days conditions stimulated gonadotropin secretion (Ubuka et al., 2012a). Firing rate of the 41% GnRH neurons within 1 μM RFRP-3 was suppressed, 12% was increased, and 47% had no significant change (Ducret et al., 2009). Dramatically, RFRP-3 was assumed as a hypothetical inhibitor on GnRH but had a stimulatory effect on GnRH in the male Syrian and Siberian hamsters. The effects of RFRP-3 on GnRH resulted in a sharp contrast, which drives further studies on the effects of RFRP-3 on the secretion of GnRH.

As the dominant rodent pest in northern China farmland, the striped hamster has higher reproductive activities in spring (March–April) and autumn (August–September) (Luo et al., 2000). It could breed three times every year, and give birth to a litter of four to nine offspring for every parity (Mu et al., 1999). Therefore, the striped hamster was considered to be a model to study the reproductive mechanism. The effect of RFRP-3 on the reproductive activity in the striped hamster is less understood. In this study, to determine the effects of RFRP-3 on the GnRH system in the striped hamster, we first characterized the *RFRP* cDNA sequence and predicted endogenous mature peptides from the striped hamster. The differential expression of *RFRP* at various developmental stages and sexes were also examined for the striped hamster. Furthermore, we also analyzed the correlation of the relative expression levels of *RFRP* mRNA in the hypothalamus with that of GnRH in different developmental stages (7-week-, 13-week- and 1.5-year-olds) for both sexes. Our results showed the significant evidence to further understand the mechanism of RFRP-3 acting on reproduction and the fluctuation mechanism of the striped hamster populations in abundance.

2. Materials and methods

2.1. Animals and tissue preparation

The striped hamsters used in this study were captured by live-trap method using an iron cage in the fields of PuWang area in Yinan County, Linyi City, Shandong Province, which is a long-term research station for agricultural rodents established by our research team. The trapped individuals were appraised and numbered, then fed in the animal feeding room of experimental center at Qufu Normal University. Composite rat food particles used to feed the striped hamsters, were purchased from Jining Medical College. In addition, they were also feed with tap water ad libitum. In feeding rooms, the natural light irradiation was given and the temperature was maintained at 22–25 °C. All experiments were performed in accordance with the rules for experimental animals of Qufu Normal University and the institutional practices of the regional Animal Ethics Committee of China.

Our study was conducted to monitor the effects of RFRP-3 on GnRH for the striped hamsters. The earliest estrous time for female striped hamsters is 2 months old, and the earliest sexual maturity time for male individuals is 1.5 months old. The striped hamsters that are 7-week-olds are usually thought to be subadults (Zhang and Wang, 1998) and neither females nor males have actual reproductive behaviors. The 13-week-old estrous females were reproductively competent and the brains were collected on the day of vaginal estrous. Identification of the estrous cycle were carried out by vaginal smears, which were conducted for the female striped hamster from 8 to 9 am every day for two consecutive weeks, and the cells in vaginal tissue were observed using a microscope. In the estrous period, there are more horny epithelial cells and less nucleated epithelial cells in the vagina (Byers et al., 2012). When the striped hamster is signed in estrous period, the brain tissue is removed immediately. The 13-week-old male striped hamsters were usually considered as adults (Zhang and Wang, 1998) which are reproductively competent based on estrous behavior. Most (96.23%) 1.5-year-old striped hamsters were non-breeding, but very few (3.77%) individuals have breeding phenomenon (823 female striped hamsters of 1.5 years old were used to detect estrous behavior by vaginal smears, 792 individuals were non-breeding phenomenon, only 31 have breeding phenomenon which comes from unpublished data). The striped hamsters of 1.5 years old in this study were actually non-breeding. Therefore, the examined striped hamsters in this study were divided into breeding hamsters (13-week hamsters) and non-breeding hamsters (7-week- and 1.5-year-old hamsters).

Tissues were obtained from the males and females of 7 weeks (non-breeding), 13 weeks (breeding) and 1.5 years old (non-breeding) ($n = 6$ per group), respectively. Animals were sacrificed via ether anesthesia and each hypothalamic tissue was collected and stored at −80 °C until assay.

2.2. Total RNA extraction and RT-PCR

Total RNA from each tissue was obtained and extracted using TRIzol reagent (TaKaRa). RNA concentration and purity were assessed by the D_{260}/D_{280} value using ultraviolet spectrophotometer (Germany Eppendorf). The integrity of RNA was determined by Agarose Gel Electrophoresis (AGE). According to the instructions of TaKaRa RNA PCR Kit (AMV) Ver.3.0 kit (TaKaRa), the equal quantities of all RNA samples were reverse transcribed simultaneously for each tissue by using AMV reverse transcriptase (TaKaRa) and an oligodeoxythymidylate (oligo(dT) 15) primer. All cDNAs were then stored at −20 °C.

2.3. Gene cloning

The intact complete coding sequence (CDS) of *RFRP* cDNA in the striped hamsters was amplified by PCR using primers (Supplemental Table 1) designed by Primer 5 and Oligo 7 software. The primer design was based on the sequence alignments of *RFRP* cDNA sequences from *Rattus norvegicus*, *Mus musculus* and *Mesocricetus auratus* (respective GenBank ID: NM_023952, BC116907, DQ371799), which are evolutionarily close to the striped hamsters. Fragment 1 cDNA was obtained with the F1 (5′-GAGACACATAGACACCAGGCT-3′) and R1 (5′-CCCCCCAATCTT TTAGTTC-3′) primers, complementary to targeted nucleotides 1–259, followed by further amplification of the F2 (5′-AACTAAAAGATTGGGG GGC-3′) and R2 (5′-AGGATGGGTTCCAGGTTTC-3′) primers, complementary to targeted nucleotides 242–643. The reaction system