Separate Locations of Urocortin and its Receptors in Mouse Testis: Function in Male Reproduction and the Relevant Mechanisms

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Key Words

Urocortin (UCN) • Corticotrophin-releasing factor (CRF) • Sperm motility • Acrosome reaction (AR) • T-type calcium channel

Abstract

Urocortin (UCN), a newly identified corticotrophin-releasing-factor (CRF) related peptide, has been demonstrated to play important roles in female reproductive system. However, few studies were reported about its effects on male reproduction. This study aimed to investigate the expression profile of UCN and CRF receptors (CRFR) in mouse testis and functions of UCN in male reproduction. Expression of UCN and CRFR mRNA was detected by RT-PCR. Localization of UCN peptide was determined by immunohistochemistry and double-immunostaining. We found that both UCN mRNA and peptide were obviously expressed in mature spermatozoa, whereas CRFR1 and CRFR2 were expressed respectively in spermatocytes and spermatogonia. Double-immunostaining results showed that UCN expression decreased with acrosome reaction (AR) proceeding. UCN significantly inhibited AR initiated by progesterone with chlortetracycline staining and

decreased spermatozoa motility concentration-dependently. Pre-incubation of spermatozoa with astressin, a CRFR antagonist, did not affect these inhibitions. In addition, flow cytometry showed that UCN concentration-dependently decreased intracellular Ca^{2+} [Ca^{2+}]_i in spermatozoa. In summary, UCN located in mouse spermatozoa and exerted inhibitory effects on male reproductive functions including motility and AR. UCN's inhibition on [Ca^{2+}]_i via T-type calcium channels might be responsible for these effects.

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Introduction

Urocortin (UCN), an endogenous 40 amino-acid peptide belonging to corticotrophin-releasing factor (CRF) family, shares 43% homology with CRF at the amino acid level [1, 2]. This peptide was originally cloned from rat midbrain and thereafter human [3] and had receptors expressed in heart [4], lymphocytes [5] and various peripheral tissues, including placenta, ovary, and deciduas [6]. Previous reports showed that UCN was involved in

the modulation of a variety of biological activities, such as causing a dose-dependent increase in coronary, testicular artery blood flow [7] suppression of appetite [8], and maintenance of the placental function & labor [9]. CRF was observed to have modulating effects on male reproduction early in late 80's [10]. However, UCN, the newly identified member of CRF family, has never been reported to play a role in male reproduction.

In reproductive system, the localization of UCN in prostatic epithelial and fibromuscular stroma as well as in its vascular smooth muscle & endothelial cells suggests that UCN, produced locally, could participate in the prostatic function by an autocrine/paracrine mechanism [11]. Moreover, the gene expression and localization of UCN, and CRFR in syncytiotrophoblast, cytotrophoblast, and decidua were reported [12]. These reports indicate that it is possible that many actions mediated by CRF during pregnancy may be attributed to UCN. Furthermore, it was reported that UCN could activate the myometrial contractility [9]. In addition, presence of UCN in the seminal fluid [13] could be of relevance during fertilization and pregnancy by influencing sperm transport through the female genital tract. Therefore, these findings suggest that the CRF family, including UCN, CRF, and its receptors [14, 15], may play important roles in various biological functions during the course of reproduction. However, in male reproductive system, especially in the testis tissue, there has not been any evidence to show the existence and functions of UCN, CRFR1, and CRFR2.

Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) plays an essential role in the initiation of sperm-oocyte interaction, and is required for the acrosome reaction (AR) and sperm motility which are the key steps during reproduction [16]. Previous studies showed that AR was inhibited by calcium channel blockers or in Ca^{2+} -free medium [17]. T-type calcium channels are expressed during the meiotic and post-meiotic stages of spermatogenesis and are retained in mature sperm, and play an important role in the regulation of $[\text{Ca}^{2+}]_i$ during AR and sperm motility [18]. It was reported that the intake of calcium channel blockers could cause contraceptive effects in men [19]. These results indicate that the inhibition of calcium currents and decreasing $[\text{Ca}^{2+}]_i$ could be responsible for the antifertility activity of these drugs.

In the present study, the expression of UCN and its receptors was determined in mouse testis. Male reproductive functions including AR and sperm motility were then investigated in the presence of UCN. In addition, effects of UCN on $[\text{Ca}^{2+}]_i$ and T-type calcium channels were also examined.

Materials and Methods

Animal care and handling conformed to the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health, and the study was approved by the local institutional ethical committee.

Reagents and solutions

Urocortin (UCN), progesterone, astressin, CsF, CsCl, EGTA, ATP-Mg, HEPES, Phosphocreatine, Cs-methanesulfonate and Fluo-3 were obtained from Sigma (USA). The culture medium contained: DMEM (GIBCO) 13.4 g/L, NaHCO_3 3.7 g/L, 10% Foetus bovine blood serum (FBS), 100U/ml penicillin and 100i g/ml streptomycin, pH was adjusted with to 7.3.

Preparation of spermatozoa and spermatogenic cells

Spermatozoa were obtained from six ICR strain mice (4–6 month-old) (Shanghai Experimental Animal Center, Chinese Academy of Sciences, China) and were released into modified tyrode's solution (in mM: 114.54 NaCl, 2.68 KCl, 0.49 MgCl_2 , 25 NaHCO_3 , 0.36 NaH_2PO_4 , 20 HEPES, 5.56 glucose, 1.80 CaCl_2 , and 100 $\mu\text{g/ml}$ penicillin, 4 mg/ml bovine serum, pH 7.45). After incubation for 15 min at 37 °C, the motile spermatozoa were selected by centrifugation through a two-step Percoll gradient [20]. Briefly, aliquots of spermatozoa were layered over the upper step of a 35% and 70% Percoll gradient and centrifuged at $650 \times g$ for 20 min. The sediment was diluted in 10 volumes of modified Tyrode's solution, centrifuged again at $400 \times g$ for 8 min and then resuspended in the same solution. The concentration of spermatozoa was adjusted to $2-4 \times 10^7$ cells/ml. The spermatozoa were then incubated at 37 °C for about 1 h before use. Sperm concentration and motility were determined with a hemocytometer and the sample suspensions with less than 60% motility were discarded. By utilizing differential sedimentation velocity at unit gravity of cells that differ in volume, homogeneous populations of six kinds of spermatogenic cells were isolated successfully from the 3-month-old ICR mouse testes throughout the development period, as described in our previous study and others' [21, 22, 23]. The identity and purity of the isolated germ cell populations were verified based on distinctive morphological features characteristic of the respective cell types by light and electron microscopy as described in our previous reports [23, 24].

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with RNeasy mini kit (QIAGEN, USA) using the method provided by the manufacturer. Total RNA was denatured at 65 for 5 min and then reverse transcribed at 37 °C for 60 min in a total volume of 20 μl reaction buffer (Life Technologies, Inc., Grand Island, NY) containing 0.5 μg oligo-deoxythymidine (Amersham Pharmacia Biotech, Uppsala, Sweden), 10 nmol deoxy-NTPs, and 200 U SuperScript™ α reverse transcriptase. The reaction mixture was then heated to 95 °C for 5 min and immediately chilled on ice. Subsequently, the synthesized complementary DNA (cDNA) products (4 μl)

were subjected to PCR in a reaction mixture (20 µl) containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 2 mM MgCl₂, 4 nmol deoxy-NTPs, 5 pmol of each primer, respectively, and 0.5 U Taq DNA polymerase (Promega, WI, USA). The sequences of the primers employed in this study are summarized in Table 1. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was examined as an internal control. After preheating at 96 °C for 2 min, denaturation, annealing, and elongation were carried out at 96 °C for 15 sec, at 64 °C (CRFR1, R2α, and R2β) or 60 °C (GAPDH) for 30 sec, and at 72 for 1 min, respectively. PCR was carried out for 35 cycles (CRFR1, R2α, and R2β), or 22 cycles (GAPDH), respectively. Amplification products were subjected to electrophoresis in a 1% polyacrylamide gel, stained with ethidium bromide, and viewed on a UV box. Negative controls contained all reagents, except that 4 µl H₂O was substituted for reverse transcriptase in the RT reaction product. PCR analysis was repeated at least twice with the same samples to confirm reproducibility of the results.

Immunohistochemical staining

After fixation in 10% neutral buffered formalin, matrices were dehydrated through an ascending series of graded ethanol and embedded in paraffin wax. Sections of 5-mm-thick wax-embedded tissue were cut and placed on 3% aminopropylethoxysilane (APES)-coated glass slides. These sections were then de-waxed and rehydrated through descending grades of alcohol to distilled water. Immunohistochemistry was performed on the sections as previously described [5]. Briefly, sections were incubated in 1% hydrogen peroxide, washed in PBS and non-specific protein binding was blocked with rabbit or goat serum (Beijing ZhongShan Biotechnology Co., China). Sections were then incubated overnight in a humid chamber at 4 with affinity purified goat antibodies specific to CRFR1 (1:200, Santa Cruz, USA), anti-CRFR2 (1:200, Santa Cruz, USA), and rabbit antibody specific to anti-UCN (1:2000, Sigma, USA), respectively. Anti-UCN did not cross-react with CRF and urotensin. Following three PBS washes, sections were incubated with HRP conjugated rabbit anti-goat and anti-rabbit secondary antibody, respectively. Immunoreactive sites were visualized brown with diaminobenzidine and mounted for bright field or differential interference contrast microscopy. Negative controls were incubated with dilution lacking primary antibody and were otherwise subject to all the immunohistochemical procedures.

Double immunostaining for UCN

Double immunostaining for UCN was performed according to the method of [25] with some modifications. Sperm acrosomal status was evaluated by chlortetracycline fluorescence staining (described later in AR part), and the different reacted tissue sections were written down and subsequently incubated in 0.2 mol/L Tris HCl buffer (pH 8.2) for 5 min at room temperature. UCN immunostaining was subsequently performed on the same reacted tissue positions using alkaline phosphatase-conjugated anti-rabbit antibodies, and the immunoreactivity for UCN was visualized as red.

Gene	Oligo nucleotide primers for RT-PCR	Predicted size	Gen Bank
UCN	5'-ATGATACAGAGGGGACGCGCTAG-3' 5'-TCACCTTGCCACCGAATCG-3'	369 bp	BC116948
CRFR1	5'-ATGTTTCGGTGAGGGCTGCTACC-3' 5'-CCTGGAGTCCTTTCAGGGCTTC-3'	475 bp	BC105673
CRFR2α	5'-ATGGGGACCCAGGCTCTCTTC-3' 5'-CTCTACTAGGGCTCCGGGTGCG-3'	243 bp	NM009953
CRFR2β	5'-CCCTCACCAACCTCTCAGGTCC-3' 5'-CAGGTCATACTCTCTGCTGTGC-3'	248 bp	NM009953
GAPDH	5'-GGTCGGAGTCAACGGATTG-3' 5'-ATGAGGTCCACCACCTGTT-3'	969 bp	BC096440

Table 1. Primers used for RT-PCR analysis of UCN, CRFR1, CRFR2α, and CRFR2β

Measurement of sperm motility

The details of semen collection, sperm preparation had been described above. Sperm motility was assessed immediately after liquefaction or sample concentration by centrifugation. Samples were thawed at room temperature and placed in a humidified chamber at 37 under 5% CO₂ in air. Sperm suspensions were adjusted with modified sperm washing medium to a density of 1×10⁶/ml. Sperms from each sample were divided into four equal portions. The adjusted sperm suspension aliquots were centrifuged at 500 g for 5 min and the supernatants discarded as much as possible, then washed with PBS for swim-up preparation. The sperm pellets were resuspended in PBS including UCN. To test the effect of UCN on sperm motility, spermatozoa suspensions were equilibrated for 10 min. The Makler chamber was used for motility scoring. Motility was estimated under the light microscope using the ×400 magnification. Only spermatozoa with progressive motility (WHO categories 'a' and 'b') were assessed. Five fields were randomly selected during evaluation and 200 sperms were analyzed in each field.

Assay for acrosome reaction (AR)

Sperm acrosomal status was evaluated by chlortetracycline fluorescence staining as described previously [20]. Briefly, the stain solution was prepared by dissolving chlortetracycline (300 mM) in TN buffer (in mM: 130 NaCl, 20 Tris, 5.0 L-cysteine, pH 7.8) and was kept on ice and in the dark until use. At the time of assay, 50 µl sperm suspension and 50 µl chlortetracycline solution were successively dropped onto a warmed (37) slide and mixed. After incubation at 37 for 15 s, the sperm samples were fixed in 18 l solution containing 5% formaldehyde and 16% triethanolamine. Then sperm acrosomal status was examined under a fluorescence microscope in which an Hg excitation beam passed through a 405-nm filter and fluorescence emission was monitored using a DM 455 dichroic mirror (Olympus IX70, Japan). To calculate the percentage of

Fig. 1. Detection of UCN (A), CRFR1 (B) and CRFR2 (C) mRNA in spermatogonia, spermatocytes and spermatozoa. Total RNA (1 µg per lane) from testis tissue or sperms were characterized by RT-PCR using gene specific primers (Table 1). Different expression of UCN and CRF receptors were summarized (D). GAPDH in panel A, B, and C indicated positive mRNA preparation for spermatogonia, spermatocytes and spermatozoa, respectively. Negative controls were mRNA without prior RT.

AR, a total of 100 spermatozoa per experimental condition were assayed to assess the different chlortetracycline staining patterns recognized earlier: “F”, characteristic of uncapacitated, acrosome-intact spermatozoa; “B”, representing capacitated, acrosome-intact spermatozoa; and “AR”, corresponding to spermatozoa that have undergone the acrosome reaction [20, 26].

Determination of $[Ca^{2+}]_i$ by flow cytometry

All experiments were performed on a Coulter Elite flow cytometer equipped with an air-cooled 488 argon laser and a helium-cadmium UV laser. At least 10,000 cells were collected for each sample, and data were analyzed using the Coulter software package and MultiTime (Phoenix Flow). Cells, detached with trypsin/EDTA and suspended in DMEM, were loaded for 30 minutes at room temperature with UCN (0.01-1 µM) and alrestressin (1 µM) (pretreatment for another 30 min). Cells were then washed 3 times and re-suspended in the extracellular medium (in mM): NaCl 140, KCl 4.6, $CaCl_2$ 2, glucose 10. Experiments started 30 minutes later to ensure adequate loading of the dye, Fluo-3. Cells were analyzed on a flow cytometer with excitation at 325 nm and emission at 405 and 525 nm corresponding to the peak emission of the Ca^{2+} -bound and Ca^{2+} -free forms of the indicator, respectively. The fluorescence intensity plot was used as an index of $[Ca^{2+}]_i$.

Cells with fluorescence <50 were excluded. No autofluorescence of unloaded cells was detected in any of the experimental conditions.

Data analysis

Data were expressed as means ± standard error (S.E.M). The changes including $I_{Ca,T}$ induced by different concentrations of UCN vs. control were compared statistically by an analysis of variance (ANOVA) with repeated measures completed by the corrected Dunnett’s t-tests. The threshold of significance retained was $P < 0.05$ or $P < 0.01$. The statistical software used was Stata 8.0.

Results

Expression of UCN and CRFR in mouse testis

RT-PCR analysis demonstrated that UCN mRNA (specific predicted size of transcripts 369 bp) was detected in spermatozoa instead of in spermatogonia or spermatocytes (Fig.1A). CRFR1 mRNA (Fig.1B) (predicted size 475 bp) was detected in spermatocytes, while CRFR2α (Fig.1C) and CRFR2β (Fig.1D) (predicted

Fig. 2. Immunohistochemical detection of UCN (A and D), CRFR1 (B and E), and CRFR2 (C and F) in mouse testis. HE staining (upper panel, Left) confirmed the normal structure of testis tissue. Control (upper panel, Right) section was used to confirm the specificity of staining. Micrographs were shown at magnification $\times 400$ (middle panel), or $\times 1000$ (lower panel). Arrows indicated the strong special staining of immunohistochemistry.

Fig. 3. Immunolocalization of UCN in the sperm. Before capacitation (A), expression of UCN showed the strong fluorescence (D). After capacitation and before AR (B), UCN's expression was decreased (E), and after AR (C), expression of UCN showed little fluorescence (F). In the presence of antibody specifically recognizing UCN, lower panel showed different fluorescence intensity during different courses of AR.

size 243 and 248 bp, respectively) were expressed in spermatogonia. In spermatozoa, neither CRFR1 nor CRFR2 α/β was detected. PCR products were further analyzed by direct sequencing and confirmed to be identical to the registered cDNA sequences for UCN, CRFR1, CRFR2 α , and CRFR2 β (data not shown). GAPDH in

panel A, B and C were examined as an internal control for spermatogonia, spermatocytes and spermatozoa, respectively. The negative control showed no band. Different expression of UCN and its receptors were summarized in Fig.1E.

Fig. 4. Inhibition of UCN on progesterone-evoked AR. (A) Concentration-dependent inhibition of UCN on AR. Amiloride was also showed to inhibit AR induced by progesterone. (B) Astressin (1 μM) had no influence on the inhibition of UCN. Data in each column were obtained from six experiments with each 200 spermatozoa and represent means $\pm$ S.E.M. (* P <0.05, ** P <0.01 vs. AR evoked by progesterone).

Fig. 5. Measurement of spermatozoa motility ($n=6$). (A) Treated with UCN at the concentration of 0.01, 0.1, 1, or 10 μM. * P <0.05 vs. control at 0.5 h. # P <0.05 vs. control at 3 h. ** P <0.05 vs. control at 6 h. (B) Effects of astressin (1 μM) together with UCN (0.1 μM) on the sperm motility. * P <0.05 vs. control. % of sperm motility represented the percentage of sperm 'a' (rapidly progressive with velocity = 35 μm/s at 37°) + 'b' (slow/sluggish progressive with velocity = 10 μm/s but < 35 μm/s) grades.

Localization of UCN, CRFR1, and CRFR2 in mouse testis

To localize the protein expressions of UCN, CRFR1 and CRFR2 and their cell/tissue-specific distribution, we performed immunohistochemistry on mouse testis section (Fig.2). HE staining (Fig.2, upper panel, left) was used to confirm the normal structure in the prepared section. Control (Fig.2, upper panel, right) section, which was stained with secondary antibody only, was used to confirm the specificity of staining. By using anti-UCN, which had little cross-reactivity with CRF, UCN-immunoreaction was observed only in spermatids and mature spermatozoa (Fig.2A). Mouse testis tissue sections showed a strong staining predominantly from spermatocytes where special anti-CRFR1 was used (Fig.2B). Immunolocalization of CRFR2 was detected mainly from the spermatogonia

(Fig.2C). The lower panel (Fig.2D, E, and F) shows the different amplification ($\times 1000$) for the middle panel ($\times 400$), respectively.

Immunolocalization of UCN in spermatozoa

To localize the expression of UCN in spermatozoa and clarify the role of UCN during acrosome reaction (AR), UCN peptide was detected in spermatozoa undergoing random normal AR using double immunostaining (Fig.3). In the 'F' staining pattern (uncapacitated and acrosome intact) with fluorescence visible over the whole of the head (Fig.3A), UCN expressed the strong fluorescence (Fig.3D, red) in mouse sperm acrosome. In the 'B' staining pattern (capacitated and acrosome intact) with a fluorescence-free band in the post-acrosomal region (Fig.3B), UCN expression

Fig. 6. UCN concentration-dependently decreased intracellular Ca^{2+} in mouse spermatozoa. (A) Effects of UCN at 0.001, 0.01, 0.1, and 1 μM , or UCN (0.1 μM)+astressin (1 μM) on $[\text{Ca}^{2+}]_i$ by flow cytometry. No fluorescence was detected in the control without loading the Fluo-3 (Blank). (B) Statistical data of UCN on the intracellular Ca^{2+} levels (* $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, $n = 6$).

levels were notably reduced (Fig.3E, red). In the 'AR' staining pattern (capacitated and acrosome reacted) with very dull fluorescence over the whole of the head (Fig.3C), UCN was barely detectable (Fig.3F). These results showed that levels of UCN expression in spermatozoa decreased with AR proceeding.

Inhibition of UCN on sperm AR

To determine the function of UCN in male reproduction, aliquots of spermatozoa suspension were incubated in modified tyrode's solution in the presence or absence of different concentrations of UCN. Progesterone (15 μM), the physiological agonist of AR, was used to evoke the AR. Progesterone was added and after an additional 15-min incubation, the AR was assessed. Spontaneous AR occurred in $18.8 \pm 1.9\%$ of incubated cells whereas $58.6 \pm 3.2\%$ AR was observed in

progesterone-induced cells. The progesterone-evoked AR was inhibited by UCN concentrations ranging from 0.01 μM to 10 μM (Fig.4A). In the presence of a CRFR antagonist, astressin (1 μM), UCN (0.1 μM) inhibited AR by about 37.2% ($P < 0.05$, $n = 6$), which showed no difference from the inhibition in the absence of astressin (Fig.4B). Amiloride, an antagonist of T-type calcium channels, inhibits T-type calcium channels in spermatogenic cells with an IC_{50} of 245 μM [20]. In this study, the effect of amiloride on the AR was also examined. Amiloride (100 μM) significantly inhibited the progesterone-evoked AR by $30.6 \pm 4.3\%$ ($P < 0.05$, $n = 6$), implying the involvement of T-type calcium channels here.

Effect of UCN on sperm motility

We further investigated the effects of UCN on sperm motility. UCN significantly inhibited sperm motility

when added to mouse sperm suspension. Compared with sperm incubated in modified human tubal fluid medium (hTF), UCN at the concentration of 0.01 μM , 0.1 μM , 1 μM , and 10 μM inhibited the motility from $86.5 \pm 5.8\%$ to $76.7 \pm 3.5\%$, $67.2 \pm 8.2\%$, $50.6 \pm 9.1\%$, $43.7 \pm 3.7\%$ ($P < 0.05$, $n = 6$), respectively after being treated for 30 min (Fig. 5A). Further incubation for 3 h and 6 h showed time-dependent increase in inhibition. This inhibition was not prevented when CRFR was blocked by 1 μM astressin (Fig. 5B).

Determination of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) by flow cytometry

Functions including AR and sperm motility are thought to be highly associated with $[\text{Ca}^{2+}]_i$. We then explored whether the inhibitory effects of UCN in spermatozoa was via $[\text{Ca}^{2+}]_i$. To calibrate the system, blank (buffer without the Fluo-3) and control (buffer with Fluo-3) were measured (Fig. 6, upper panel). Exposure of the cells to UCN (0.01–1 μM) reduced $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner (Fig. 6, lower panel). Pre-treatment of spermatozoa with astressin, a non-selective CRFR antagonist, did not block the inhibition of $[\text{Ca}^{2+}]_i$ induced by UCN, which indicated that inhibition of UCN was independent of the CRFR pathway.

Discussion

CRF has been found to play a role in male reproductive system since 1980's. Recently, the new member of CRF family, UCN, was found to exist in seminal fluid [13]. These reports lead to our hypothesis that UCN might express in testis and function in male reproduction processes such as sperm motility and AR, which are associated with intracellular Ca^{2+} and T-type calcium channels [17].

UCN has been reported to function via its CRF receptors, including CRFR1 and CRFR2 [14, 15]. In the present study, both RT-PCR and immunohistochemistry results showed that UCN, CRFR1, and CRFR2 were located at the different positions in the mouse testis. UCN expressed only in mature sperm while CRFR1 and CRFR2 existed in spermatocytes and spermatogonia, respectively. Previous reports also showed that UCN could be synthesized and secreted by cardiac myocytes, functioning as a cardioprotective peptide via CRFR [27, 28]. However, UCN could also cause neural activation in cell groups that are involved in autonomic activation, which did not express either type of CRFR [29]. In the present study, UCN, but not CRFR, was expressed in

Fig. 7. Example traces showed the inhibitory effects of UCN (0.1 μM) on T-type calcium currents ($I_{\text{Ca,T}}$). $I_{\text{Ca,T}}$ were evoked by a 200 ms-long depolarizing step pulse from the holding potential of -90 mV to -20 mV at the frequency of 1 Hz.

spermatozoa, which implied that actions of UCN on spermatozoa may not be via CRF receptors. Our results in double-immunostaining further supported the expression of UCN in spermatozoa, i.e. the great majority of mature sperm cells express UCN. With AR proceeding, the expression of UCN declined, and after ascosome reaction, UCN could not be detected any more, which might be attributed to the simple loss or step by step degradation of UCN during AR as a necessity of reproduction process.

As well known, both motility and AR are highly associated with intracellular Ca^{2+} , Ca^{2+} channels and Ca^{2+} influx in spermatozoa AR [30]. AR is a key step in sperm-oocyte membrane fusion, which is inhibited by calcium channel blockers and in Ca^{2+} -free solution [16, 20, 31]. In the present study, UCN significantly inhibited the motility of the sperm and ascosome reaction in a concentration-dependent manner. Also, UCN inhibited the $[\text{Ca}^{2+}]_i$ increase in extracellular medium, which was not affect by astressin, a CRFR blocker. In male reproductive cells, intracellular Ca^{2+} is mainly through the voltage-gated T-type calcium channels [16, 32]. Our previous reports showed that UCN exerted the inhibitory effects on $I_{\text{Ca,T}}$ via a CRFR-independent pathway (example shown in Fig. 7) [33]. These results demonstrated that UCN might

decrease $[Ca^{2+}]_i$ via inhibiting T-type calcium channels directly, instead of binding to its receptors firstly. Our present results are consistent with some previous reports. It was reported that sperm-specific CatSper1 and CatSper2 proteins are critical to sperm-hyperactivated motility and male fertility [34]. Recent reports also showed that CatSper1 and CatSper2 can associate with and modulate the function of the T-type calcium channels (Ca_v3), which might be important in the regulation of sperm functions including sperm motility [35] and AR. Also, the inhibition of the progesterone-initiated AR by mibefradil may suggest the involvement of T-type calcium channels [36]. In addition, Alberto *et al* showed that a Ca_v3 expressed in the *Strongylocentrotus purpuratus* and participated in the regulation of sperm motility [32]. Although further investigations are needed, all of these reports hinted that T-type calcium channels may participate in some sperm functions like motility, capacitation, and AR. However, in human spermatozoa, studies on progesterone-induced AR in human sperm did not show the participation of T-type calcium channels [37]. In the present study, UCN was found to inhibit progesterone-induced AR of mouse sperm while decreasing $[Ca^{2+}]_i$ via T-type calcium channels in mouse spermatozoa, also suggesting an involvement of these channels in this AR-inhibitory effect. These contradictions

might be attributed to the different cell species/ages used for the experiments. The concrete mechanisms leading to the differences remain to be further explored.

In conclusion, UCN, CRFR1, and CRFR2 exist at separate locations in mouse testis. UCN inhibits T-type calcium channels in mouse spermatogenic cells, sperm motility and progesterone-evoked sperm AR, indicating that inhibition of Ca^{2+} channels may be a mechanism for the inhibitory effects of UCN on male reproductive functions. In our further studies, transfected HEK293 cells will be used to distinguish the inhibitory effects of UCN on $I_{Ca,T}$ through $Ca_v3.1$ or $Ca_v3.2$ channels, either of which is the main functional calcium channel in wild-type spermatogenic cells [38]. Also, why the spermatocytes and spermatogonia express different CRF receptor types (CRFR1 and CRFR2, respectively) is interesting but the significance of the different expression in the two cell phases needs further investigation.

Acknowledgements

This work was supported by grants from Natural Science Foundation of China (No.30572185), Key Subject Fund of China Ministry of Education (No. 03047). We thank Dr. Bi Ye for their excellent technical assistance.

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