Seasonal changes in the expression of PACAP, VPAC1, VPAC2, PAC1 and testicular activity in the testis of the muskrat (*Ondatra zibethicus*)

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) plays an important role in the steroidogenesis and spermatogenesis in the testis through its receptors PAC1, VPAC1 and VPAC2. In this study, we investigated the seasonal expressions of PACAP, PAC1, VPAC1, VPAC2, luteinizing hormone receptor (LHR), follicle stimulating hormone receptor (FSHR), steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3β-HSD) and CYP17A1 in the testis of male muskrat during the breeding season and non-breeding season, respectively. Histologically, we observed the presence of Leydig cells, Sertoli cells and various types of germ cells in the testis during the breeding season, yet only Leydig cells, Sertoli cells, spermatogonia and primary spermatocyte during the non-breeding season. In addition, the immunohistochemical localizations of PACAP and VPAC1 were identified in the Leydig cells, spermatogonia and spermatozoa during the breeding season, while only in the Leydig cells and spermatogonia during the non-breeding season, and PAC1 and VPAC2 were localized in the Leydig cells in both seasons, among which LHR, StAR, 3β-HSD and CYP17A1 were also expressed. Meanwhile, the protein and mRNA expression levels of PACAP, PAC1, VPAC1, VPAC2, LHR, FSHR, StAR, 3β-HSD and CYP17A1 in the testis during the breeding season were significantly higher than those during the non-breeding season. These results suggested that PACAP is involved in the regulation of steroidogenesis and spermatogenesis *via* an endocrine, autocrine or paracrine manner in the testis of muskrat.

Key words: Pituitary adenylate cyclase-activating peptide (PACAP); PACAP receptors; steroidogenesis; testis; *Ondatra zibethicus*.

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated from an ovine hypothalamic extract based on its ability to stimulate cyclic AMP production by rat anterior pituitary cells cultures 20 years ago,[1] PACAP belongs to the vasoactive intestinal polypeptide (VIP)-secretin-glucagon peptide superfamily.[2] There are two biologically active PACAP isoforms, the 27 amino acids (PACAP27) and 38 amino acids (PACAP38), of which PACAP38 accounts for 90% of the protein in most tissues.[3] The structure of PACAP has been conserved well during evolution over the past 700 million years, suggesting that PACAP plays an essential physiologic role.[4] Although PACAP was first identified as a hypothalamic-releasing factor, it was subsequently known to act as a paracrine/autocrine factor in many tissues.[5] There are three distinct 7-transmembrane receptors that can be activated by PACAP: the PACAP-specific PAC1, and the PACAP/VIP-indifferent VPAC1 and VPAC2.[6] Previous studies have concluded that, in addition to the distribution in the central nervous system,[7] PACAP receptor mRNAs have also been identified in many peripheral tissues such as pituitary, pancreas, liver, gonads, kidney and so on, through which the regulation of *PACAP* accompanies the entire reproductive development process of rodents.[8] *PACAP* has been proved to be an indispensable modulator of basic function of the hypothalamic-pituitary-gonadal axis.[9]

PACAP participates in the regulation of multiple functions of the testes directly or indirectly.[10] PACAP and its receptors are located in the testis of rodents, but there are differences in the expression and localization among species.[8] In rat, Hannibal *et al*. reported PACAP immunoreactivities are shown in spermatids near the lumen, in spermatogonia and primary spermatocytes.[11] Similarly, Li *et al*. found that *PACAP* and its receptors are co-expressed in spermatids.[12] The experiments performed by Daniel *et al*. in isolated rat germ cells *in vitro* and transgenic mice *in vivo* confirmed that the *PACAP* gene contains a testis-specific promoter. This promoter is intensely expressed in the postmitotic round spermatids during developmental stages I-VIII, which signifies that the activity of *PACAP* gene promoter is highly regulated during the spermatogenic cycle.[13] In addition, other studies in mouse consider the expression of *PACAP* and its receptors in germ cells, Leydig cells and vascular endothelial cells, and further inferred part of testicular PACAP may be derived from vessel cells or the bloodstream.[14] The highest concentration of PACAP was found in the hypothalamus, followed by the testis, but the total amount of PACAP in both testes exceed its content in the whole brain.[3] PACAP labeled with ^{125}I (I-P38)was used to analyze its ability to cross the vascular component of the blood-testis barrier and the results showed PACAP was rapidly degraded with 20.5% being intact in the testis and only 6.9% in in the circulation after 10 minutes after intravenous injection, which meant the circulating PACAP may conduce to the intratesticular pool and play an active role in the reproductive function of the testes.[15] These results demonstrate PACAP may participate in spermatogenesis *via* an endocrine, autocrine or paracrine manner. Furthermore, PACAP-knockout mice have been shown to exhibit multiple reproductive-related dysfunctions, such as steroidogenesis [evaluated by the levels of testosterone, steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3β-HSD), and CYP17A1],[16] decreased social interaction, and disturbed signaling in spermatogenesis. There is sufficient evidence to show that PACAP activates both cAMP- and phosphatidylinositol-dependent mechanisms in Leydig cells, and plays an irreplaceable role in testicular steroidogenesis.[17] Furthermore, PACAP is also involved in the regulation of gonadotropin secretion, and ulteriorly influences the reproductive function of testis.[10 18,19] Treatment with intravenous PACAP in

3-month-old male rats resulted in the pronounced increase of LH in the blood.[20]

The muskrat (*Ondatra zibethicus*) is a semiaquatic rodent native to North America, with important economic value and scientific research value.[21] The fur of the muskrat can be used as coat material, and the musk is an important medicinal and perfume raw material. As a seasonal breeding animal, the active reproductive status of the muskrat is limited to a specific period in an annual cycle lasting for 8 months (March to October), affected by the latitude and altitude. Our previous research demonstrated that the morphological size of testes in the breeding season is significantly greater than that in the non-breeding season, and the round and elongated spermatids can only be observed in the breeding season.[22] There have been many studies on the expression and function of *PACAP* in the testes, but most of them focus on conventional model animals such as mice and rats, while gaps remain in research on the role played by *PACAP* in testicular activity regulation in seasonal breeding animals. We suppose *PACAP* is involved in the regulation of the testicular activity mechanism of the muskrat in various ways through its receptors *via* an endocrine, autocrine or paracrine manner. so, the aim of the present study was to investigate the seasonal expressions of PACAP, PAC1, VPAC1, VPAC2, luteinizing hormone receptor (LHR), follicle stimulating hormone receptor (FSHR), StAR, 3β-HSD and CYP17A1, so as to elucidate the relationship among PACAP, PACAP receptors, steroidogenesis and spermatogenesis.

Materials and Methods

Animals

Sixteen adult male muskrats were obtained in January (n=8, non-breeding season), and June (n=8, breeding season), 2020 from Xinji Muskrats Breeding Farm, Hebei Province, China. All animals were treated according to the Policy on the Care and Use of Animals by the Ethical Committee and Animal Welfare Committee and all procedures were approved by Beijing Forestry University, China (EAWC_BJFU_2022003). Muskrats were anesthetized by proper amount of diethyl ether, weighed with an analytical balance and euthanized by decapitation. Every testis was collected from the muskrat and weighed with an analytical balance. For every testis of each muskrat, half of the testes were immersion-fixed immediately for 12 h in Bouin's solution for histological and immunohistochemical analysis, the other half was stored at -80°C for RNA isolation and protein extraction.

Histology

The testicular samples were dehydrated in ethanol series and embedded in paraffin in accordance with standard procedures. Serial sections (5 μm) were mounted on slides coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO, USA). Some of the sections were stained with hematoxylin and eosin (HE) for histological observations. The slides were examined using a photomicroscope (BX51, Olympus, Tokyo, Japan). We randomly chose 16 testicular sections from each testis of 8 muskrats in the breeding season and the non-breeding season which were cut on the middle level of the testis, and used ImageJ software (ImageJ bundled with 64-bit Java) to calculate the area of every seminiferous tubule.

Immunohistochemistry

After being deparaffinized and rehydrated, the testicular sections were incubated with 10% normal goat serum to reduce background staining caused by the secondary antibody. And then the sections were incubated with primary rabbit anti-rat polyclonal

antibody (1:400 dilution) against PACAP (bs-0190R), PAC1 (bs-0198R), VPAC1 (bs-2982R), VPAC2 (bs-0197R), LHR (bs-6431R), FSHR (bs-0895R), StAR (bs-20387R), CYP17A1(bs-3853R) and 3β-HSD (bs-3906R) for 12 h at 4°C, which were all bought from Bioss Biotechnology (Beijing, China). The negative control sections were treated with normal rabbit serum instead of the primary antibody. Subsequently, the sections were incubated with secondary antibody, goat anti-rabbit IgG conjugated with biotin and peroxidase with avidin, using a rabbit ExtrAvidin staining kit (Sigma Chemical Co.), followed by visualizing with 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 mL of 0.05 M Tris-HCl buffer, plus 30 μL H_2O_2. Finally, the sections were counterstained with hematoxylin solution (Merck, Tokyo, Japan). The immune-stained slides were examined using a photomicroscope (BX51, Olympus). A semi-quantitative score for positivity was analyzed by ImageJ software (ImageJ bundled with 64-bit Java) as described in Andrew *et al.*[23] and the result was determined as positive (+), strong positive (++), very strong positive (+++), and negative (–). Staining that was weak but higher than control was set as positive (+); the highest intensity staining was set as very strong positive (+++); staining intensity between + and +++ was set as strong positive (++).

Protein isolation and Western blotting analysis

First, the frozen testes from -80°C were diced into many small pieces by a clean razor blade respectively to increase tissue lysis efficiency. Then, the pieces of testis were homogenized in a homogenizer containing 800 μL RIPA Lysis Buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P 40, 0.5% sodium deoxycholate) with 10 μL PMSF (10 mg/mL) using high-speed homogenizer (Bread Ruptor12, OMNI International CO., Ltd., Kennesaw, GA, USA). Afterwards, the homogenates of testis were centrifuged at 12,000 × g for 6 min at 4°C and, the supernatant was collected. Then the concentration of protein extract is measured by BCA Protein Assay Kit (BN27109, Biorigin Biotechnology, Beijing, China). Protein extracts (25 μg) were mixed with an equal volume of 2 × Laemmli sample buffer. Equal amount of each sample was loaded and run on a 12% SDS-PAGE gel at 18 V/cm, and then transferred to nitrocellulose membranes using a wet transblotting apparatus (Bio-Rad, Richmond, CA, USA). The membranes were blocked in 3% bovine serum albumin (BSA) for an hour at room temperature (RT). Then the membranes were incubated overnight in the primary antibodies which were the same as used in immunohistochemistry, with polyclonal anti-β-actin antibody (1:1000 dilution) (AM1021B, Abgent Biotechnology, Suzhou, China) as the endogenous control. Secondary incubation of the membrane was then carried out using a 1:1000 dilution of goat anti-rabbit IgG (BN20601, Biorigin Biotechnology, Beijing, China) tagged with horseradish peroxidase for an hour. Finally, the membrane was washed in 50 mL TBS-T buffer (0.02 M Tris, 0.137 M NaCl and 0.1% Tween-20, pH 7.6) for three times, then was colored with 10 mg 3,3-diaminobenzidine (Wako) solution in 50 ml phosphate buffer (0.03 M) plus 30 μL H_2O_2. The intensities of the bands were quantified using ImageJ software (ImageJ bundled with 64-bit Java).

Total RNA isolation and preparation of cDNA

Total RNA was isolated from testicular tissues of the muskrat using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA). About 0.1 g testicular tissues were thawed and immediately homogenized in 1 mL TRIzolTM Reagent. The homogenate was incubated for 5 min at RT to ensure the nucleoprotein complexes completely dissociated. After the addition of 0.2 mL of chloroform, the mixture was vigorously shaken for 15 s at RT and then centrifuged at 12,000 x g for 20 min at 4°C. Subsequently, the aqueous phase was

transferred to a fresh tube, with an addition of equal volume of isopropanol. The sample was kept at RT for 10 min. Afterwards, RNA was precipitated by centrifugation at 12,000 x g for 20 min at 4°C. The integrity of RNA was tested by gel electrophoresis, and after measuring the concentration with spectrophotometer (NANODROP 8000, Thermo Fisher Scientific, Waltham, MA, USA), RNA was diluted to 250 ng/μL. The first-strand cDNA from total RNA was synthesized using StarScript II First-strand cDNA Synthesis Mix (GenStar, Beijing, China) and the cDNA was stored at -20°C.

Quantitative Real-time PCR

The PCR reactions were carried out in a 10 μL volume with Hieff UNICON Universal Blue qPCR SYBR Green Master Mix (Yeasen, Shanghai, China). Primer sequences for mRNA were summarized in Table 1, and the primer sequences for PAC1 was also confirmed in rat by Han *et al.*[24] We used Fast Real-Time System (Applied Biosystems, Foster City, CA, USA) and followed the process described below: 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. Then the melting curves were performed to test the homogeneity of the PCR products by increasing the temperature progressively to 95°C, afterwards decreasing it to 65°C for 60 sec and increasing it again to 95°C. The PCR efficiency of target and reference genes were similar and the negative control reactions were performed in the absence of reverse transcriptase in order to test the genomic DNA contamination. All samples were run in triplicate, including a negative control and the intra-assay variation was less than 10%. The expression level of each target mRNA relative to β-actin mRNA was determined using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). The normality and homoscedasticity tests were evaluated by D'Agostino & Pearson test and F test, respectively. The statistical significance between differences groups was compared using the Student's *t*-test and statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical values of p<0.05 were considered significant.

Table 1. Primers' sequence used for mRNA qRT-PCR.

Gene	Primer sequence	Product length (bp)
PACAP	F: 5'- CTACCAGATCGTCTCCCCCA -3' R: 5'- AACTAGGAGGGAGCGTGACA -3'	216
PAC1	F: 5'- GGTGAGATGGTCCTTGTAAGC -3' R: 5'- CCCACAAGCATCGAAGTAGT -3'	186
VPAC1	F: 5 - GAAGACCGGCTACACCATCG -3 R: 5 - CAGTGGTCTATCTCCCCGCT -3	198
VPAC2	F: 5 - TGCTCTACTCCAGCTCAGGT -3 R: 5'- ACTGGGGATACCCCATCCAA -3'	215
LHR	F: 5'- TCGTCGTCATCTGTGCTTGC -3' R: 5'- TGGTGACAGTGATAAGGGGC -3'	192
FSHR	F: 5'- ACAACTGTGCATTCAACGGAA -3' R: 5'- GTATGTTGACCTGGCCCTCAG -3'	194
StAR	F: 5'- GACTGGAGGTGCTGCTAGAC -3' R: 5'- ACCTCTACGCTTGGTACAGC -3'	221
3 -HSD	F: 5'- TCATGAAAGCACATGGTCAGATG -3' R: 5'- AGGAATGGACTTCTCTCCCCA -3'	156
CYP17a1	F: 5'- GCCACTATCCGAGAAGTGCT -3' R: 5'- GCAAGTAACTCTGCGTGGGT -3'	238

Results

Morphology

Morphological observations of the testes of the male muskrat were presented in Figure 1. The length of the testes during the breeding seasons was visibly longer than that during the non-breeding season (Figure 1 a,b). The whole view of the testes in the male muskrat during the breeding season and the non-breeding season were shown in Figure 1 c,d and the significant reduction of the morphological size of the testes during the breeding season was ovservated compared to the breeding season. The weight ratio of testes to body of each muskrat during the breeding season was conspicuously higher compared with that during the non-breeding season (Figure 1e).

Histology

Histological observations of the testes of the male muskrat were shown in Figure 2. Leydig cells, Sertoli cells, spermatogonia, primary spermatocyte, secondary spermatocyte, spermatids and spermatozoa were located in the testis of the muskrat in the breeding season (Figure 2 a,c), while only Leydig cells, Sertoli cells, spermatogonia and primary spermatocyte were identified in the testis of the muskrat in the non-breeding season (Figure 2 b,d). The

area of seminiferous tubules of the muskrat during the breeding season were significantly larger than that during the non-breeding season (Figure 2e), and these results indicate the reproductive status of the male muskrat changes with the seasons.

Immunohistochemistry

The immunohistochemical localizations of PACAP, PAC1, VPAC1, VPAC2, LHR, FSHR, StAR, 3β-HSD and CYP17A1 in the testis of the male muskrat were present in Figure 3. Compared with negative control sections (Figure 3 i,j), labelling for PACAP (Figure 3 a,b) and VPAC1 (Figure 3 e,f) mainly occurred within Leydig cells, spermatogonia and spermatozoa during the breeding season, while within Leydig cells and spermatogonia during the non-breeding season; the immunoreactivity of PAC1 (Figure 3 c,d)and VPAC2 (Figure 3 g,h) was localized in the Leydig cells in both seasons; positive staining for LHR (Figure 3 k,l), 3β-HSD (Figure 3 q,r) and CYP17A1 (Figure 3 s,t) were observed in the Leydig cells in both seasons; FSHR (Figure 3 m,n) was positioned in the Sertoli cells and StAR was localized in the Leydig cells and the Sertoli cells in both seasons. The staining results obtained from the images were quantified and summarized in Table 2.

Western blot

The protein levels of PACAP, PAC1, VPAC1, VPAC2, LHR, FSHR, StAR, 3β-HSD and CYP17A1 in the testis of the male
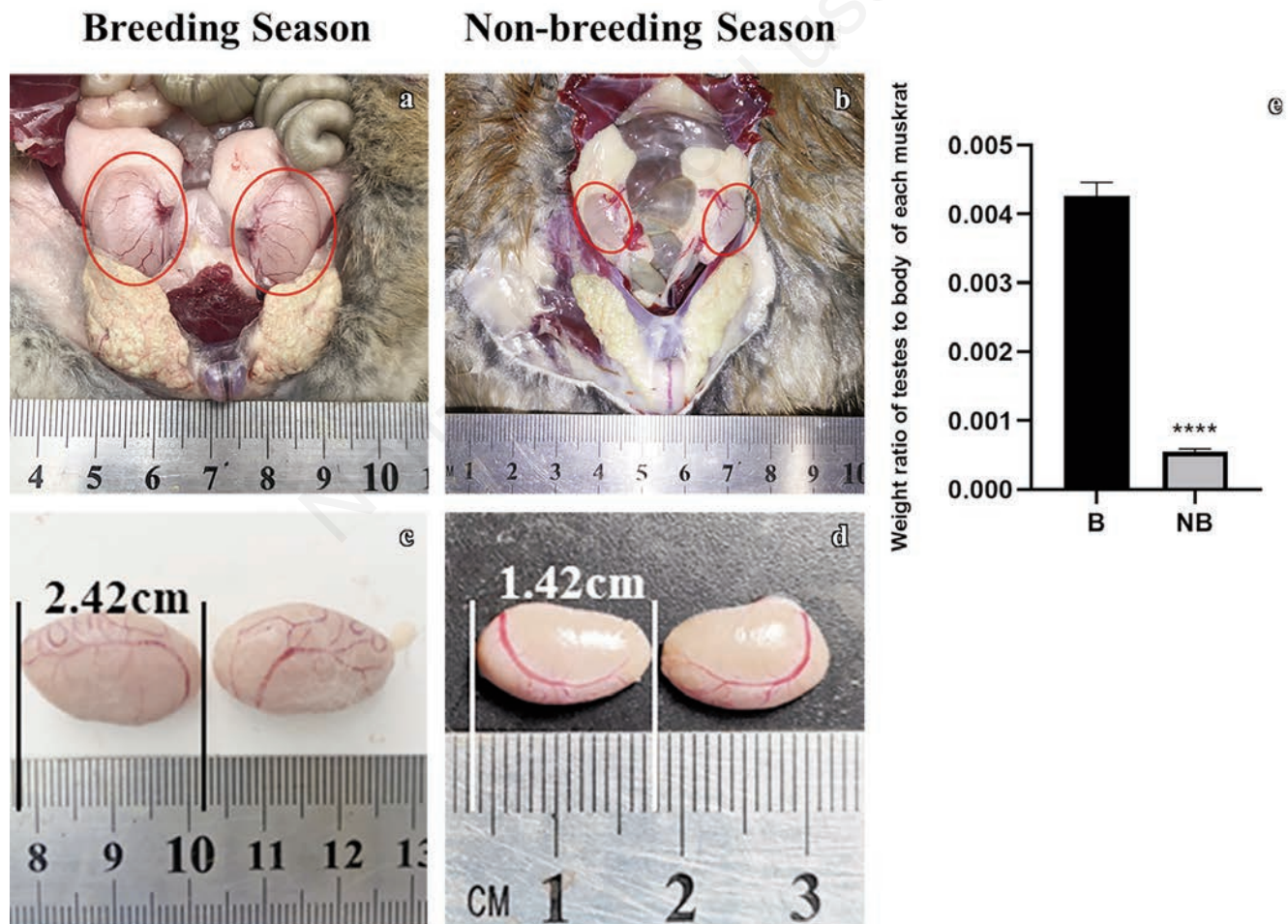

Figure 1. Anatomic localization and morphology of the testes in the muskrat. a,c) The testes of the muskrat during the breeding season. b,d) The testes of the muskrat during the non-breeding season. e) The weight ratio of testes to body of each muskrat. B, the breeding season; NB, the non-breeding season. The error bars represent means ± SEM (n=8, each period); ****p<0.0001.

muskrat during the breeding season and the non-breeding season were examined by Western blotting, and the results were shown in Fig. 4. The major bands for PACAP, PAC1, VPAC1, VPAC2, LHR, FSHR, StAR, 3β-HSD and CYP17A1 were detected at a molecular weight of about 19kDa, 58kDa, 47kDa, 48kDa, 74kDa, 78kDa, 32kDa, 40kDa and 57kDa respectively (Figure 4 a-i). The protein levels of PACAP, PAC1, VPAC1, VPAC2, LHR, FSHR, StAR, 3β-HSD and CYP17A1 during the breeding season were markedly higher than those during the non-breeding season. The quantification results were normalized to the expression level of β-actin.

Quantitative Real-time PCR

The qRT-PCR analyses of the mRNA levels of PACAP, PAC1, VPAC1, VPAC2, LHR, FSHR, StAR, 3β-HSD and CYP17A1 in the testis of the male muskrat during the breeding season and the non-breeding season were shown in Figure 5. The levels of PACAP and its receptors PAC1, VPAC1 and VPAC2 transcripts in the testis of the muskrat decreased significantly from the breeding season to the non-breeding season (Figure 5 a-d). Similarly, the mRNA expression levels of LHR, FSHR, StAR, 3β-HSD and CYP17A1 in the testis during the breeding season were notably higher than those during the non-breeding season (Figure 5 e-i).
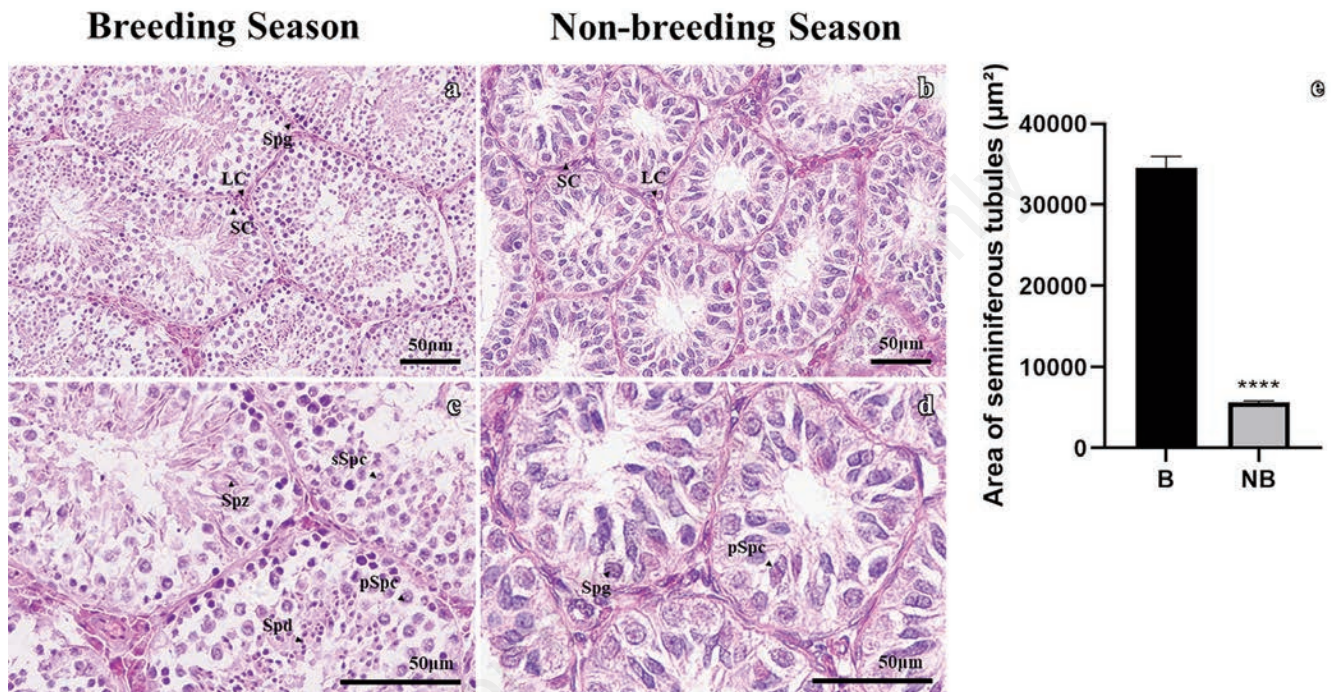

Figure 2. Histological structure of the testis in the muskrat by hematoxylin-eosin (H&E). a,c) histological observations of the testes during the breeding season. b,d) Histological observations of the testes during the non-breeding season. e) Area of seminiferous tubules (μm²) of the testis. B, the breeding season; NB, the non-breeding season; LC, Leydig cells; SC, Sertoli cell; Spg, spermatogonia; pSpc, primary spermatocytes; sSpc, secondary spermatocyte; Spz, spermatozoa. The histogram bars represent means ± SEM (n=8, each period); ****p<0.0001; scale bars: 50 μm.

Table 2. Immunohistochemical localizations of PACAP, PAC1, VPAC1, VPAC2, LHR, FSHR, StAR, 3β-HSD and CYP17A1 during the breeding and the non-breeding season.

Antibody	Breeding season				Non-breeding season			
	LC	SC	Spg	Spz	LC	SC	Spg	Spz
PACAP	++	-	++	++	+	-	+	-
PAC1	++	-	-	-	+	-	-	-
VPAC1	++	-	++	++	+	-	+	-
VPAC2	+++	-	-	-	+	-	-	-
LHR	+++	-	-	-	+	-	-	-
FSHR	-	+++	-	-	-	++	-	-
StAR	+++	+++	-	-	++	++	-	-
3β-HSD	+++	-	-	-	+	-	-	-
CYP17a1	+++	-	-	-	+	-	-	-
NC	-	-	-	-	-	-	-	-

Discussion

In this present study, we examined the seasonal expression of PACAP and its receptors in the testis of the muskrat, and also investigated several factors regulated by PACAP, such as FSHR, LHR, StAR, 3β-HSD and CYP17A1. The histological results showed that the seminiferous tubes during the breeding season are much more developed than those during the non-breeding season. Immunohistochemical data revealed that PACAP, and VPAC1 are expressed in the Leydig cells, spermatogonia and spermatozoa during the breeding season while they are only found in Leydig cell and spermatogonia during the non-breeding seasons; PAC1, VPAC2, LHR, 3β-HSD and CYP17A1 are located in the Leydig cell in both seasons; FSHR is detected in the Sertoli cell in both seasons; StAR is present in the Leydig cell and the Sertoli cell in both seasons. Moreover, the presence of PACAP, PAC1, VPAC1, VPAC2, LHR, FSHR, StAR, 3β-HSD and CYP17A1 protein levels and transcripts are described in the testis of the muskrat as being significantly higher during the breeding season than those during the non-breeding season. These results suggest that PACAP may increase the expression of LHR and FSHR in the muskrat during the breeding season, and participate in the steroidogenesis and spermatogenesis regulation in the testis of the muskrat *via* an endocrine, autocrine or paracrine manner.

Histologically, the the seminiferous tubes of the muskrat during the breeding seasons are more developed than that during the non-breeding seasons, which are manifested as the appearance of all types of spermatogenic cells, including mature spermatozoa and the larger area of the seminiferous tubes and the changes are similar to other studies in the seasonal breeding animals.[25,26] For instance, there are acute seasonal changes in testicular morphology and histology of the male wild ground squirrels during the breeding seasons and the non-breeding seasons, such as testicular size of single male wild ground squirrel and the types of cells shown in the testes.[25] In raccoon dogs (*Nyctereutes procynoides*), with the beginning of the breeding season, a variety of germ cells gradually appear and the weight and size of the testis increase.[26] Immunohistochemically, in adult male Sprague-Dawley rats, Shivers *et al*. observed that the PACAP binding site was localized in the testis and epididymis, using conventional methods of receptor autoradiography and radioligand receptor binding assays.[27] In rats, Shioda *et al*. found that PACAP was expressed in the germ cell and spermatogonia, while the most intense PACAP was demonstrated in the spermatids during their early developmental stages during the entire period of spermatogenesis.[28] Matsumoto *et al*. also confirmed the existence of PACAP specific receptor PAC1 in the Leydig cells through which the PACAP could regulate the development of immature Leydig cells.[29] Moreover, Shivers *et al*. and Li *et al*. demonstrated the only presence of VPAC1 and VPAC2 in the Leydig cell of rat.[3,12] More recently, Prisco *et al*. investigated the expression of PACAP and its receptors in the testis of *Mus musculus* by RT-PCR, *in situ* hybridization and immunohistochemistry, and demonstrated that PACAP and its receptors are localized in spermatocytes, spermatids, spermatozoa, Leydig cells and vascular endothelial cells.[14] In the pre-
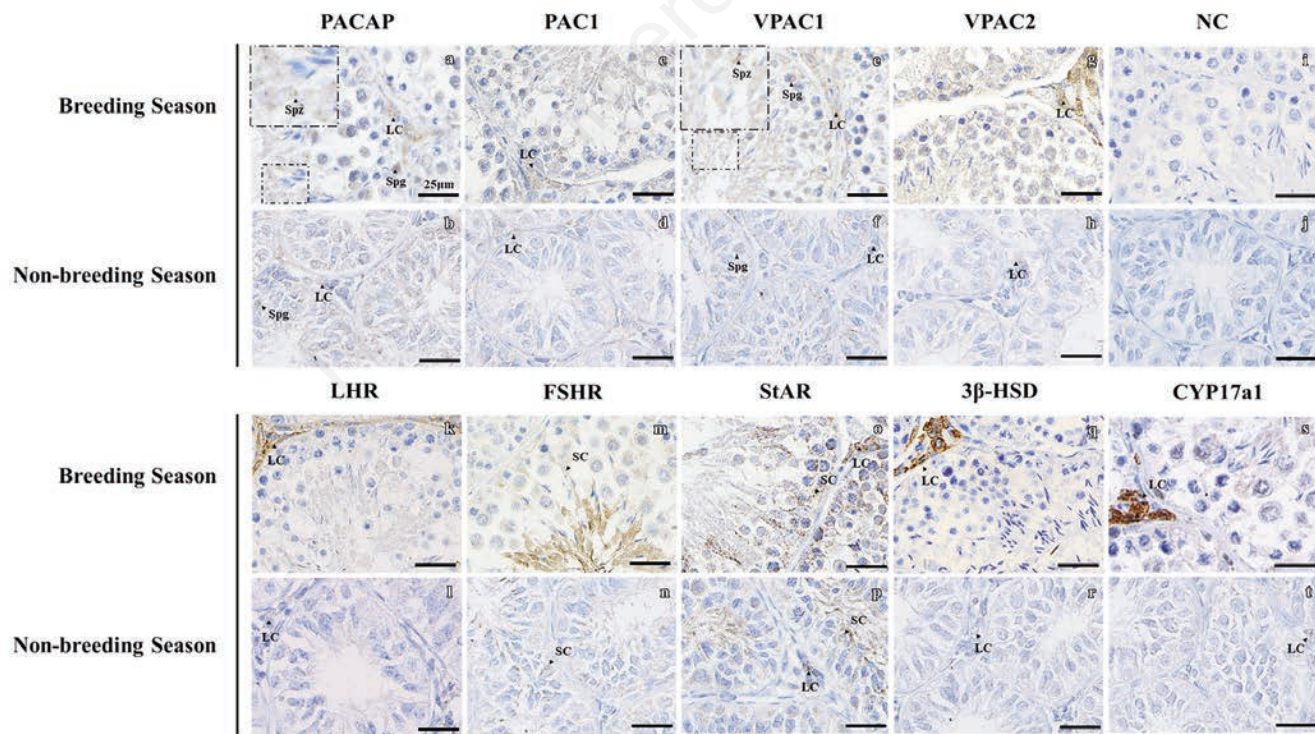

Figure 3. The immunohistochemical localizations PACAP (a,b), PAC1 (c,d), VPAC1 (e,f), VPAC2 (g,h), LHR (k,l), FSHR (m,n), StAR (o,p), 3β-HSD (q,r) and CYP17a1 (s,t) in the testis of the muskrats during the breeding season (a,c,e,g,k,m,o,q,s) and the non-breeding season (b,d,f,h,l,n,p,r,t). The negative control sections the testis during the breeding season (i) and the non-breeding season (j). B, the breeding season; NB, the non-breeding season; LC, Leydig cells; SC, Sertoli cell; Spg, spermatogonia; Spz, spermatozoa. Scale bars: 25 μm.

sent study, in the testis of the muskrat, a typical seasonal breeding rodent, we further confirmed the expression of PACAP and its receptors: there are PACAP, PAC1, VPAC1 and VPAC2 expressions in Leydig cells, PACAP and VPAC1 in spermatogonia and spermatozoa (in particular at the level of the tails). These results strongly suggest that PACAP could control spermatogenesis or spermiogenesis in paracrine, autocrine and intracrine manner and may also participate in sperm motility regulation as described in human.[30] Moreover, there is evidence that PACAP and PAC1 are widely present during the reproductive cycle of male lizard, and may take part in the control of spermatogenesis.[3]. However, we did not observe the expression of PACAP and its receptors in the Sertoli cell as already confirmed in the immature mouse and the mature rat.[28,32] There is also evidence that PACAP could stimulates cAMP accumulation in Sertoli cells cultured from 15-day-old rats.[33] Differently, the influence of PACAP on mouse Sertoli cells have yet to be demonstrated.[14] We hypothesize the distinct expression might due to species or growth stage differences, and further study is still needed to figure out the role PACAP played in the Sertoli cell.
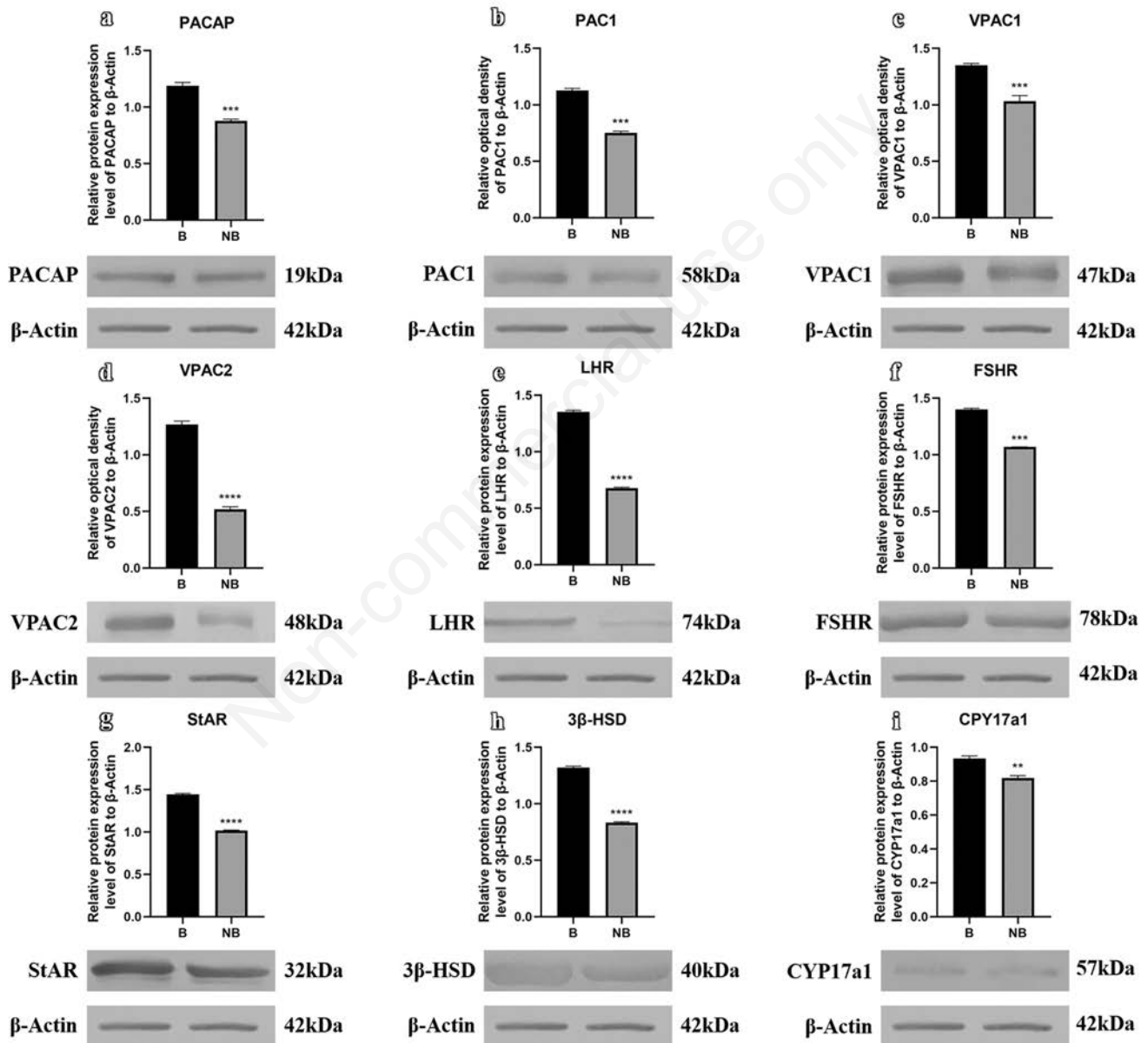

Figure 4. The protein expressions of PACAP, PAC1, VPAC1, VPAC2, LHR, FSHR, StAR, 3β-HSD and CYP17a1 in the testis of the muskrat during the breeding season and the non-breeding season by Western blot analysis. β-Actin blots were used as controls to correct for loading in each lane. The expression levels of PACAP (a), PAC1 (b), VPAC1 (c), VPAC2 (d), LHR (e), FSHR (f), StAR (g), 3β-HSD (h) and CYP17a1 (i) in the testis of the muskrat during both seasons were determined by densitometric analysis. B, the breeding season; NB, the non-breeding season. Data were shown as the mean ± SEM (n=4 for each period); **p<0.01; ***p<0.001; ****p<0.0001.

Changes in the levels of PACAP during the breeding season and the non-breeding season may affect testicular function. In our study, qPCR results showed that the mRNA levels of PACAP, PAC1, VPAC1 and VPAC2 are significantly higher during breeding season than those during the non-breeding season. The protein levels of PACAP, PAC1, VPAC1 and VPAC2 are also markedly higher during breeding season than those during the non-breeding period. Previous study showed that the mRNA levels of PACAP in the testis of rat are first detected on day 20 after birth and then gradually increased until on day 60 to a maximum level, which are low when spermatogenesis is disrupted such as cryptorchidism.[34] On the contrary, studies on the αGSU-PACAP mouse found the overexpression of PACAP results in the delay of pubertal development and the suppression of testicular activity.[35] We suppose the
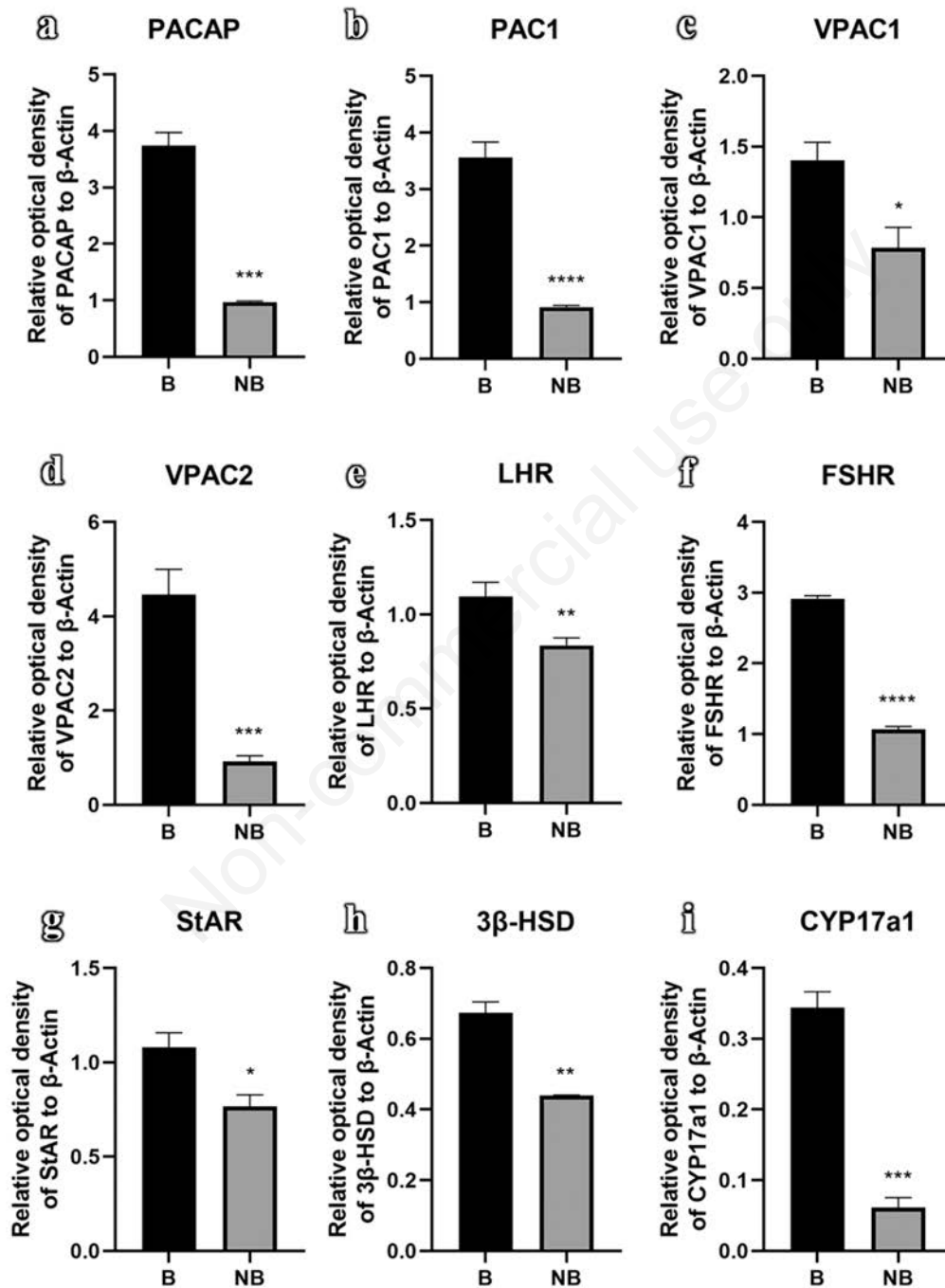

Figure 5. The mRNA expressions of PACAP (a), PAC1 (b), VPAC1 (c), VPAC2 (d), LHR (e), FSHR (f), StAR (g), 3β-HSD (h) and CYP17a1 (i) detected by quantitative real-time PCR in the testis of the muskrat during the breeding season and the non-breeding season. B, the breeding season; NB, the non-breeding season. Data were shown as the mean ± SEM (n 4 for each period); *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

appropriate up-regulation of the expression level of PACAP and its receptor may increase the testicular activity of the muskrat during the breeding season.

The regulation of testicular function by PACAP may be achieved through controlling the synthesis of steroids.[10] PACAP could work through PAC1 to enhance the production of cAMP and intracellular calcium mobilization and then directly interact with many cell types, such as gonadotrope, Leydig and germ.[5] Lacombe *et al*. found PACAP-knockout mice steroidogenesis was impaired and demonstrated PACAP could act on its receptors in Leydig cells to in stimulates steroidogenesis.[16] Furthermore, Romanelli *et al*. confirmed PACAP may play an amplificatory rolo on testicular steroidogenesis stimulated by hCG in the Leydig cell of rat, which can be prevented by the PACAP receptor antagonist.[17] Similarly, treatment with low concentrations of PACAP in the fetal rat leads to the significant stimulation of cAMP and testosterone production.[36] PACAP may also play as a paracrine factor to turn on and maintain early fetal testicular steroidogenesis before LH secretion in rat.[37] Seasonal patterns of steroid hormone receptors abundence are closely linked with seasonal reproduction mechanism, while sex steroid receptors are usually upregulated during the breeding season.[38] Taken together, in the muskrat, enhanced PACAP signal during the breeding season may act on the VPAC1 VPAC2 and PAC1 in the Leydig cell and stimulates the expression of StAR, 3β-HSD and CYP17A1 *via* paracrine, autocrine or intracrine manner. From another perspective, Halvorson *et al*. pointed out that a complex reciprocal regulatory relationship exists between PACAP and gonadotropins (especially LH and FSH).[18] Preceding study in the testes of rat found the concentration of PACAP is significantly reduced after hypophysectomy, and it can be restored by FSH administration, which suggests that pituitary gonadotropins may involve in the control of PACAP expression.[39] Subsequently, PACAP also takes part in the regulation of gonadotropin cyclicfluctuations during the estrus, and the levels of PACAP mRNA expressed in the PVN (paraventricular nucleus) and pituitary significantly increased before the gonadotropin surge in the proestrus of rat.[40] According to our qPCR and Western Blot results, which showed that the expression of LHR and FSHR in the Leydig cell and Sertoli cell increased during the breeding season, we believe that moderate PACAP, LH and FSH exert a mutually reinforcing effect on seasonal reproductive regulation of muskrats.

In conclusion, PACAP, VPAC1, VPAC2 and PAC1 are widely represented in the testis of the muskrat in both germ and somatic cells, and the expressions of PACAP, VPAC1, VPAC2 and PAC1 during the breeding season are significantly highly than those during the non-breeding season. The present investigation demonstrates that PACAP may play an important role in the regulation of steroidogenesis and spermatogenesis *via* an endocrine, autocrine or paracrine manner in the testis of the muskrat. The results of this study provides new insight regarding the regulatory mechanism of PACAP in seasonal breeding rodents, yet further research is still needed to investgate the function of PACAP in the hypothalamus and pituitary of the muskrat during the breeding season and the non-breeding season.

Acknowledgments

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