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# Expression of urocortin and its receptors in the rat epididymis

Adriana De Luca [a,*], Giovanna Liguori [a], Caterina Squillacioti [a], Salvatore Paino [b], Giuseppe Germano [a], Sabrina Alì [a], Nicola Mirabella [a]

[a] Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Via Veterinaria 1, I-80137 Naples, Italy

[b] Department of Sciences of Animal Production, University of Basilicata, Via Nazario Sauro, I-85100 Potenza, Italy

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ABSTRACT

Urocortin (UCN; 40 aa) is a corticotrophin-releasing hormone (CRH)-related peptide. The biological actions of CRH family peptides are mediated by two types of G-protein-coupled receptors, CRH type 1 receptor (CRHR1) and CRH type 2 receptor (CRHR2). The biological effects of the peptides are mediated and modulated not only by CRH receptors but also by a highly conserved CRH-binding protein (CRHBP). The aim of the present study was to investigate the expression of UCN, CRHR1, CRHR2 and CRHBP by immunohistochemistry, Western blot, RT-PCR and real-time RT-PCR in the rat epididymis. Urocortin, CRHR1 and CRHR2, but not CRHBP, were expressed in all segments of the rat epididymis. Specifically, UCN- and CRHR2-immunoreactivities (IRs) were distributed in epididymal epithelial cells of the caput, corpus and cauda. CRHR1-IR was found in the fibromuscular cells surrounding the epididymal duct and in the smooth musculature of the blood vessels throughout the organ. UCN and CRHR2 mRNA expression levels were higher in the caput and corpus than in the cauda, while CRHR1 mRNA level was higher in the cauda than those in the caput and corpus. In summary, UCN, CRHR1 and CRHR2 are expressed in the rat epididymis. It is suggested that CRH-related peptides might play multiple roles in the maturation and storage of spermatozoa.



## 1. Introduction

Urocortin (UCN) is a peptide (40 amino acids, aa) belonging to the corticotrophin-releasing hormone (CRH) family and it exhibits 45% homology to CRH [1–3]. Urocortin was identified by Vaughan et al. [4], and similar to CRH, was found to stimulate adrenocorticotrophic hormone (ACTH) *in vitro* and *in vivo* production by corticotrophs [5]. Additional mammalian CRH-like peptides include UCN2 (stresscopin-related peptide, 38 aa) and UCN3 (stresscopin) which share more than 80% of homology [6]. The biological effects of CRH and UCNs

* *Corresponding author*. Tel.: +39 0812536114; fax: +39 0812536097.
E-mail address: adrideluca@unina.it (A. De Luca).

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are mediated by distinct G-protein-coupled receptors, CRH receptor type 1 (CRHR1) and 2 (CRHR2) [7,8]. CRHR1, primarily expressed in the central nervous system (CNS) and the anterior pituitary, is an important mediator of the activation of the hypothalamic–pituitary–axis (HPA) [9]. CRHR2 is expressed in extra-CNS sites and has 70% sequence identity to CRHR1. CRHR2 exhibits high affinity toward UCNs and no toward CRH. Its activation suppresses multiple metabolic functions including feeding in fasted mice [10], feeding-induced edema and gastric emptying [11]. The biological effects of UCN and other CRH-related peptides are also modulated by CRH-binding protein (CRHBP). CRHBP is a 37 kD glycoprotein identified in mammals and non-mammalian vertebrates and is highly conserved during phylogenesis [12]. It was isolated from human plasma [13,14], the rat brain and the anterior pituitary [15,16]. CRHBP binds human CRH (hCRH) and UCN with an equal or greater affinity than CRH receptors [4,17] and modulates the access of CRH to CRHRs [12]. UCN and CRHRs gene expression and/or immunoexpression have been detected in several peripheral organs belonging to the digestive, cardiovascular, immune, endocrine and reproductive systems [18–22].

In the male reproductive system, the presence of UCN was reported in the human prostate, suggesting its role in the autocrine/paracrine regulation of prostatic function [23]. Moreover, expression of UCN and CRH receptors was found in the rat, mouse and human testis [22,24,25] and they are believed to play a role in the regulation of spermatogenesis, sperm motility and testosterone releasing. To highlight other sites of urocortin action and to indicate other possible urocortin functions, we investigated the expression of UCN, CRHR1, CRHR2 and CRHBP in the rat epididymis.

## 2. Materials and methods

### 2.1. Animals and tissue collection

A total of 20 adult male Sprague-Dawley rats (13 weeks of age; body weight: 380 ± 20 g; purchased from Harlan Italy) were used. The rats were housed in temperature- and light-controlled rooms and were given *ad libitum* access to food and water. The animals received humane care, and the study protocol was in compliance with our institution's ethical guidelines. All procedures followed Italian laws regarding animal use in research (art. 7 D. Lgs. 116/92). All surgical procedures were carried out aseptically under anesthesia induced with urethane (1.2 g/kg). The animals were terminated under urethane anesthesia, and the removed epididymides were divided into the following three segments: caput (including initial segment), corpus and cauda. In addition, the brain was collected as a positive control. For Western blot and real-time RT-PCR, the samples were immediately frozen on dry ice and stored at −80 °C. For immunohistochemical studies, the samples were immediately fixed in Bouin's fluid.

### 2.2. Immunoprecipitation and Western blot

Frozen tissues were homogenized in buffer (50 mM Tris–HCl, pH 7.00; 150 mM NaCl; 2% Triton; 5 mM EDTA; 10 μg/mL leupeptin; 0.1 U/mL aprotinin; 1 mM PMSF) using an Ultra-Turrax homogenizer and centrifuged at 16 000 × *g* for 20 min at 4 °C. The protein concentration in the resulting supernatants was determined by a Bio-Rad assay (Hercules, CA, USA). Equal amounts of proteins were immunoprecipitated overnight at 4 °C with anti-UCN, -CRHR1, -CRHR2 and -CRHBP antisera (1 μg antibody/200 μg protein) previously bound to protein A/G agarose beads. A fraction of the supernatant was used as an immunoprecipitation input control (loading control). Beads were sedimented by brief centrifugation and washed extensively with ice-cold homogenization buffer. Proteins solubilized in boiling sodium dodecyl sulphate (SDS) sample buffer (2% SDS; 5% L-mercaptoethanol; 66 mM Tris, pH 7.5; 10 mM EDTA) were separated on 18% and 12% SDS-polyacrylamide gels (Bio-Rad), After electrophoresis, the proteins were transferred to nitrocellulose using a semi-dry apparatus (Bio-Rad). The membrane was incubated for 1 h at 42 °C in 5% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) in TBST (150 mM NaCl; 20 mM Tris–HCl, pH 7.4; 0.3% Tween-20), washed with TBST and incubated for 2 h at room temperature (RT) in antisera diluted 1:500 in TBST containing 1% BSA. The following primary antisera were used: polyclonal goat anti-UCN (sc-1825, Santa Cruz Biotechnology, CA, USA; diluted 1:1000), anti-CRHR1 (sc-12383, Santa Cruz Biotechnology; diluted 1:1000), anti-CRHR2 (sc-1826, Santa Cruz Biotechnology; diluted 1:1000) and polyclonal rabbit anti-CRHBP (SAB1300862, Sigma; diluted 1:1000). The membrane was washed three times with TBST, incubated for 1 h with anti-goat or anti-rabbit IgG conjugated to peroxidase (Vector Laboratories, Burlingame, CA, USA) diluted 1:2000 in 1% BSA containing TBST and washed three times with TBST. Proteins were visualized by an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). Marker proteins (Prosieve, Lonza) were used to estimate the molecular weight of each band. β-Actin acted as the loading control for the immunoprecipitated proteins. A fraction of the supernatant (50 μg) was separated by SDS-PAGE and immunoblotted with an anti-β-actin primary antibody (Mouse mAb JLA20 cp 01; Calbiochem, San Diego, CA; diluted 1:5000) followed by a peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Vector Laboratories; diluted 1: 2000).

### 2.3. Immunohistochemistry

Fresh tissue fragments were fixed by immersion in Bouin's fixative (6–24 h), processed for paraffin embedding in a vacuum and cut at a thickness of 3–6 μm. The avidin–biotin–peroxidase complex (ABC) method was performed by using the Vectastain ABC kit (Vector Laboratories) [20]. Primary antisera were the same as those used in the previous section and were directed against UCN, CRHR1, CRHR2 and CRHBP. The first three were diluted 1:200 and the fourth 1:500. The secondary antibodies were biotinylated anti-goat or anti-rabbit IgG (Vector Laboratories; diluted 1:200). The specificity of the primary immunoreactions was tested by replacing each antibody with a buffer, preabsorbing the antibody with an excess of the appropriate antigen (100 μg antigen/ml antiserum as the final dilution) or using a dot-blot assay [20]. No immunoreaction was detected in control tests. The slides were

observed with a Leica DMRA2 microscope (Leica Microsystems, Wetzlar, Germany).

### 2.4. RNA extraction, cDNA synthesis and real-time RT-PCR

Samples of the epididymis and brain (positive control) were homogenized in ice-cold TRI-Reagent (Sigma) using an Ultra-Turrax homogenizer. After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in RNAase-free DEPC water. Total RNA was measured with an Eppendorf Biophotometer (Eppendorf AG, Basel, Switzerland). For cDNA synthesis, 1 μg of total RNA was retro-transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) using random hexamers as primers. For conventional and real-time PCR reactions, specific primers were designed from the published mRNA Genbank gene sequences using Primer ExpressT software (PE Applied Biosystems) and are presented in Table 1. The PCR cycle conditions were as follows: 94 °C (30 s), 60 °C (30 s), 72 °C (1 min) for 35 cycles; 72 °C (5 min). The PCR products of rat urocortin, CRHR1, CRHR2 and CRHBP were purified using GFX PCR DNA and Gel Purification Kit (28-9034-70, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and sequenced. To assess the expression profiles of these genes, quantitative RT-PCR was used. The real-time PCR reactions contained 1 μL cDNA (40 ng/well) and 24 μL of SYBR Green Master Mix (Applied Biosystems) containing specific primers. The PCR conditions were 50 °C for 2 min and 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. The β-actin gene was also amplified in separate tubes under the same conditions to serve as an active endogenous reference to normalize quantification of the mRNA target. Real-time detection was performed on an ABIPRISM 7300 Sequence Detection System (Applied Biosystem), and data from the SYBR Green I PCR amplicons were assessed with ABI 7300 System SDS Software. The relative quantification method 2-ΔΔCt ($2^{-\Delta\Delta Ct}$) was used for the normalization of gene expression as described more fully elsewhere [20]. For statistical analyses, the data were expressed as mean ± SD. Significant differences in the UCN, CRHR1 and CRHR2 mRNA levels between the calibrator sample (caput) *vs.* corpus and cauda were determined by one-way ANOVA followed by Tukey's HDS test for independent samples. The level of statistical significance was set at $p < 0.05$ for all experiments.

## 3. Results

The results of the Western blot analysis are shown in Fig. 1. Protein bands corresponding to UCN (16 kD), CRHR1 and CRHR2 (both 55 kD) were found within caput, corpus and cauda of epididymis. There was no CRHBP protein expression in the epididymis, although the protein was expressed in the brain (a positive control).

Urocortin-immunoreactivity (IR) (Fig. 2A–C) was detected in the apical portion of the principal epithelial cells of the caput (Fig. 2A), corpus (Fig. 2B) and cauda (Fig. 2C). CRHR2-IR (Fig. 2D–F) had the same distribution as UCN-IR. CRHR1-IR was found in many fibromuscular cells surrounding the epididymal duct throughout the organ (Fig. 2G–I) and in the smooth musculature of the blood vessels (inset of Fig. 2G and H). No CRHBP-IR was found in the rat epididymis (Fig. 2L–N).

Urocortin, CRHR1 and CRHR2 mRNAs were expressed in all segments of the rat epididymis (Fig. 3A), while the presence of CRHBP mRNA was not found (data not shown). Real-time PCR confirmed that UCN, CRHR1 and CRHR2 mRNAs were expressed in all studied segments of the rat epididymis. The levels of UCN and CRHR2 mRNA expression were higher in the caput and corpus than in the cauda (Fig. 3B and C). The levels of CRHR1 mRNA expression were lower in the caput and corpus than in the cauda (Fig. 3D).

## 4. Discussion

In the present study, we demonstrated the presence of UCN, CRHR1 and CRHR2 in the epididymis of the rat by multiple techniques. CRHBP was not found in the examined organ. The protein band of approximately 16 kD detected by Western blot is consistent with the mammalian UCN precursor, which is a 122-aa protein [2,4]. In addition, 55 kD protein bands are

**Table 1 – Primer sequences used in RT-PCR.**

| Gene | Genbank accession number | Primer sequences | Product size (bp) |
|---|---|---|---|
| UCN | NM_019150 | Forward: 5′-CCATCGACCTCACCTTCCA-3′<br>Reverse: 5′-CTTGCCCACCGAATCGAATA-3′ | 110 |
| CRHR1 | NM_030999 | Forward: 5′-GGTGACAGCCGCCTACAATT-3′<br>Reverse: 5′-AAGGTACACCCCAGCCAATG-3′ | 200 |
| CRHR2 | NM_022714 | Forward: 5′-TGGTGCATACCCTGCCCTAT-3′<br>Reverse: 5′-GTGGAGGCTCGCAGTTTTGT-3′ | 200 |
| CRHBP | NM_139183.2 | Forward: 5′-TGGACTGGACACCTCCAAGAT3′<br>Reverse: 5′-ACTCCGGGATGCTGTTTCTG-3′ | 200 |
| β-Actin | V_01217 | Forward: 5′-GCTACAGCTTCACCACCAC-3′<br>Reverse: 5′-TACTCCTGCTTGCTGATCCAC-3′ | 498 |

UCN: urocortin; CRHR1: corticotrophin-releasing hormone type 1 receptor; CRHR2: corticotrophin-releasing hormone type 2 receptor; CRHBP: corticotrophin-releasing hormone-binding protein.

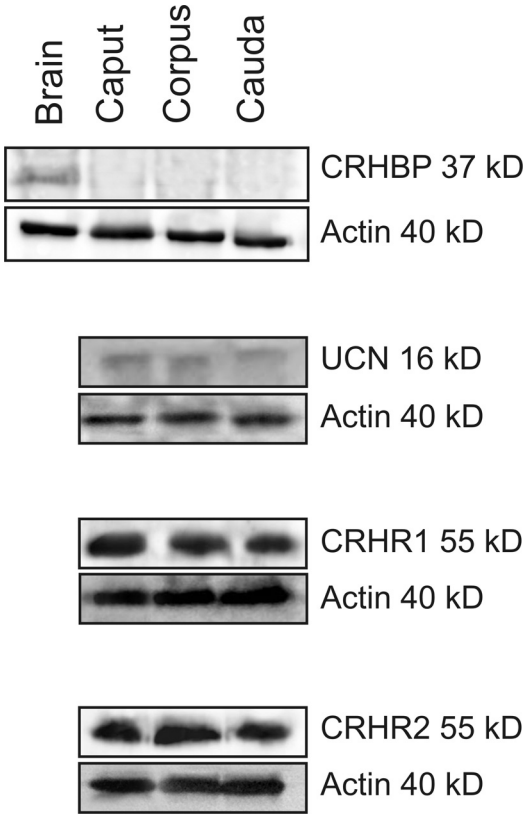


**Fig. 1 – Expression of urocortin (UCN), corticotrophin-releasing hormone receptor type 1 (CRHR1), corticotrophin-releasing hormone receptor type 2 (CRHR2) and corticotrophin-releasing hormone binding protein (CRHBP) in all segments of the rat epididymis (Western blot). Tissue extracts were immunoprecipitated and immunoblotted with anti-UCN, -CRHR1, -CRHR2 and -CRHBP antibodies; rat brain was used as positive control. β-Actin was the loading control for the immunoprecipitated proteins. kD: kilodalton.**

consistent with mammalian CRH receptors [7]. Although CRHBP was found to be expressed in the brain of several mammalian species [15,16,26,27], it was not detected in the epididymis in the current study. Therefore, we suggest that CRHBP does not play a role in antagonizing the effects of CRH-related peptides in the epididymis as it does in the brain.

In the present study, both UCN- and CRHR2-IRs were present in principal epithelial cells throughout the epididymis. Until now, only CRHR2 mRNA was reported to be present in the rodent epididymis [9,28]. The presence of UCN, CRHR1 and CRHR2 mRNAs and IRs were described in the mouse testis [24]. UCN, CRHR1 and CRHR2 IRs were found to be distributed in the testicular germ cells, where UCN seemed to inhibit spermatozoa motility and acrosome reaction. In the human testis, UCN and CRHRs mRNAs and IRs were found to be separately located among germ, peritubular myoid and Leydig cells, and UCN was suggested to have receptor-independent effects on germ cell differentiation and division [25]. In the rat testis, the presence of UCN mRNA and IR appears to be restricted to Leydig cells [22,29].

The results of the present study raise a question about the possible roles of UCN in the epididymis. The presence of the cognate receptor CRHR2 suggests that UCN affects functions of the epididymal epithelial cells *via* an autocrine mechanism. UCN may be involved in the growth modulation of epididymal epithelial cells. In fact, in the human endometrium, CRH affected cell growth *via* CRHR1-mediated activation of the cAMP–PKA pathway [30]. In non-reproductive epithelial cells, CRH-related peptides were reported to influence sebocyte proliferation [31] and to inhibit apoptosis in gastric cells [32]. Moreover, UCN was found in normal human as well as adenocarcinomatous human prostates [23], whereas CRHR2 was found in normal prostates but not in prostate cancer [25,33]. A role for UCN in modulating epididymal hormonal secretion has also been postulated. CRH and UCN stimulate pituitary proopiomelanocortin (POMC) gene expression [4,5,34]. It is well known that the rodent epididymis expresses POMC [35,36]. Thus, the presence of UCN in the rat epididymis shown in the present study suggests a possible effect of the peptide on epididymal POMC-derived hormone secretion, as it was reported in the placenta [37] and the pituitary gland [4,5,34].

CRH-related peptides have been demonstrated to modulate uterine contractility during pregnancy [38,39]. The presence of CRHR1-IR in the fibromuscular stromal cells surrounding the epididymal duct suggests a role for CRH-related peptides in the regulation of the contractility of the epididymis. Moreover, evidence indicates that, although the epididymis displays rich innervation, other local, non-neuronal factors participate in nerve-independent epididymal contractility [40,41]. CRHR1-IR was also observed in the vascular smooth musculature, thus suggesting an involvement of CRH-related peptides in the modulation of the rat epididymal blood flow. This hypothesis is supported by the finding that UCN is a dilator of rat [42] and mouse [43] arteries.

RT-PCR analysis revealed that UCN, CRHR1 and CRHR2, but not CRHBP, mRNAs were expressed in the rat epididymis, thus confirming immunohistochemical data. The levels of UCN and CRHR2 mRNA expression were higher in the caput and corpus than in the cauda. These results suggest that UCN and CRHR2 may play an important role in the epithelial cell-mediated maturation of spermatozoa that initially occurs in the caput and corpus [44]. UCN may influence the maturation of spermatozoa *via* different indirect mechanisms such as regulating the growth of epididymal epithelial cells and their hormonal secretion. The levels of CRHR1 mRNA expression were lower in the caput and corpus than in the cauda, and we detected CRHR1-IR only in muscle cells. CRH-related peptides have been demonstrated to modulate uterine contraction through different signaling pathways. CRHR1 maintains myometrial quiescence, whereas CRHR2 promotes smooth muscle contractility [38,39]. The cauda of the epididymis is the major site of spermatozoa storage in the male reproductive tract. CRH-related peptides most likely enhance the passage of spermatozoa through the epididymal duct by inhibiting the contraction of its fibromuscular cells and facilitating enlargement of the cauda, which is the site of germ cell storage.

In conclusion, these results clearly demonstrate that UCN, CRHR1 and CRHR2 are expressed in the rat epididymis and suggest an existence of a local regulatory system based on

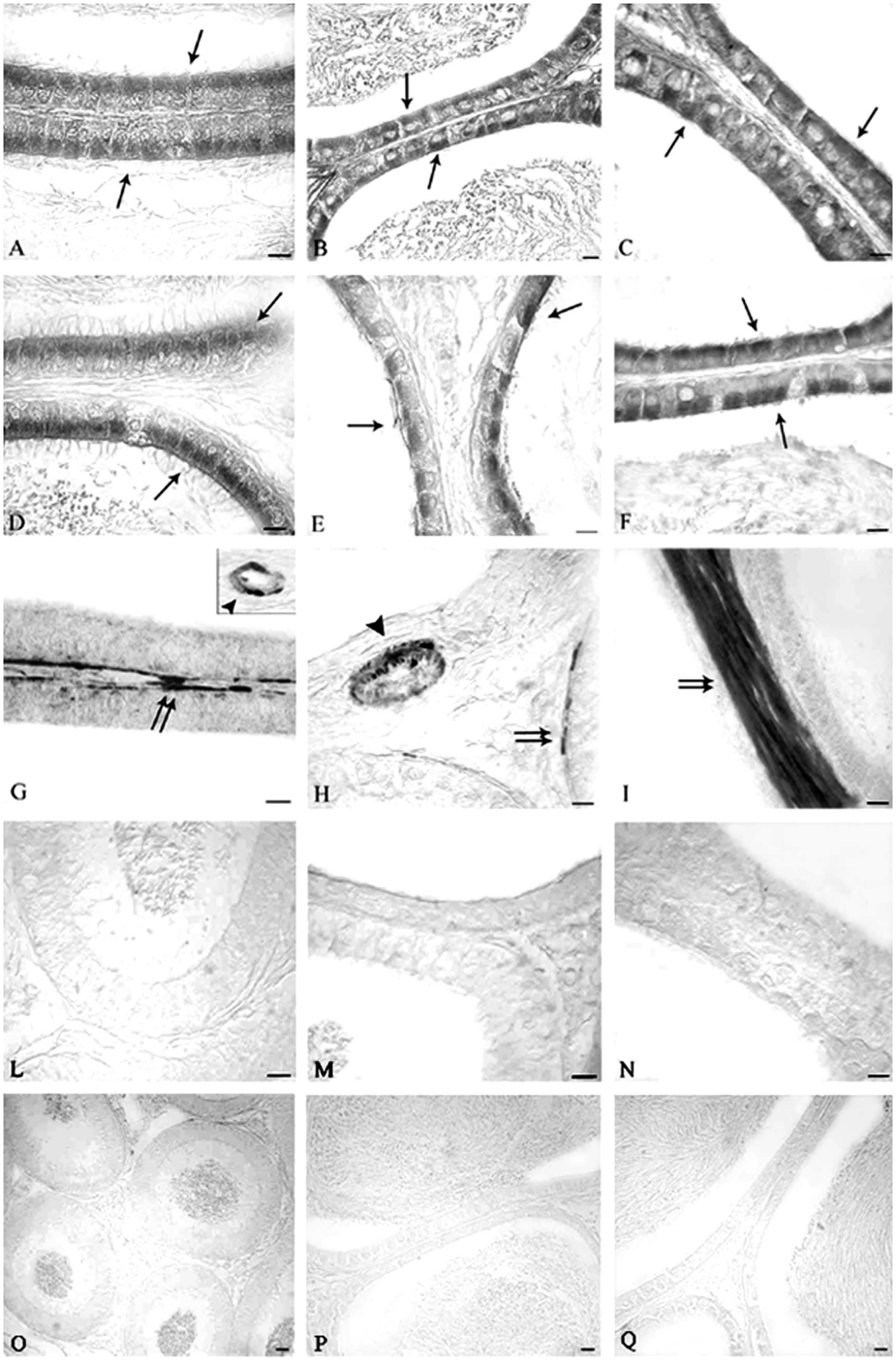


**Fig. 2 – Inmmunolocalization of urocortin (UCN) (A–C), corticotrophin-releasing hormone receptor type 2 (CRHR2) (D–F), corticotrophin-releasing hormone receptor type 1 (CRHR1) (G-I) and corticotrophin-releasing hormone binding protein (CRHBP) (L–N) in the rat epididymis. UCN-immunoreactivity (IR) and CRHR2-IR were found in the apical area of principal cells (arrows) of the caput (A,D), corpus (B,E) and cauda (C,F). The principal cells of the epididymal duct were devoid of CRHR1-IR, but were present in fibromuscular cells of the three epididymal segments (double arrows). The smooth musculature of a few blood vessels present in the interstitial connective tissue of the caput (G, inset) and corpus (H) shows CRHR1-positive staining (arrowhead). No CRHBP-IR was found in the epididymal duct (L–N). Negative controls of the caput (O), corpus (P) and cauda (Q). Bar = 20 μm.**

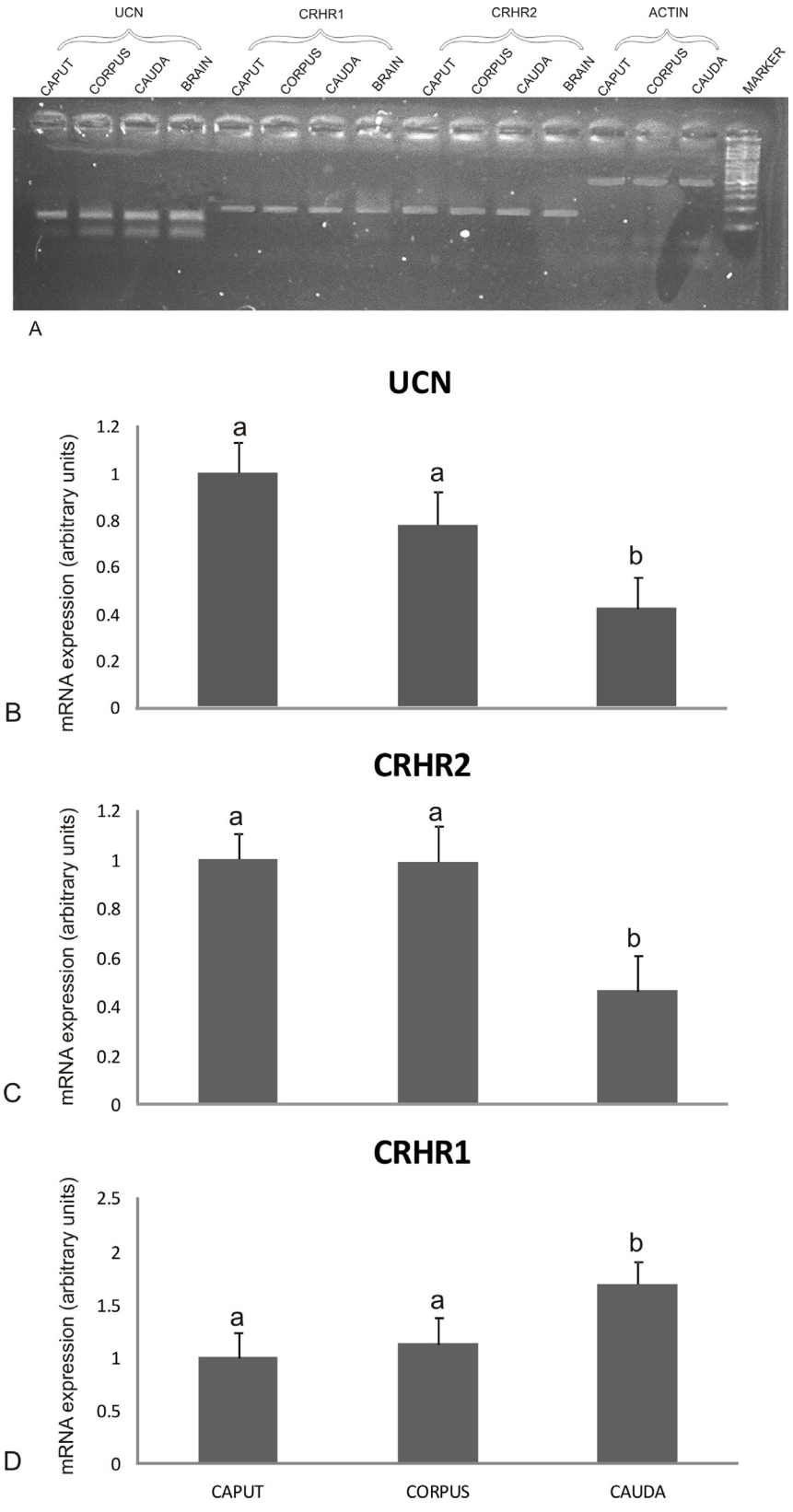


**Fig. 3 – Urocortin (UCN), corticotrophin-releasing hormone receptor type 1 (CRHR1) and corticotrophin-releasing hormone receptor type 2 (CRHR2) mRNA expression in the rat epididymis detected by RT-PCR (A) and real-time RT-PCR (B–D). (A) UCN (110-bp) CRHR1 (200-bp) and CRHR2 (200-bp) mRNAs were expressed in the caput, corpus and cauda of the rat epididymis. Marker: molecular markers of 100-bp ladder. (B–D) The statistical differences in UCN, CRHR1 and CRHR2 mRNA levels between the calibrator sample (caput) and the corpus and cauda were determined by one-way ANOVA followed by Tukey's HDS test. Different letters depict significant differences between the examined groups ($p < 0.05$).**

CRH-related peptides and CRH receptors. In particular, the urocortinergic system could modulate epididymal physiology and spermatozoa maturation, storage and deposition.

## Conflict of interest

None declared.

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