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Urocortins are Present in the Rat Testis

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Abstract

The synthesis and release of testosterone (T) depends both on circulating luteinizing hormone (LH) and on an array of testicular factors whose role remains incompletely understood. Corticotropin-releasing factor (CRF) had been reported in the rat testes, where it was thought to inhibit T secretion. However, the discovery that the CRF-related peptides urocortins (Ucns), of which there are currently 3 subtypes (Ucn 1, 2 and 3), cross-react with many reagents previously used to detect CRF, has cast doubt on this concept. Here we show that while CRF was readily measurable in rat hypothalami (which served as controls), signals for this peptide were barely detectable in total RNA extracted from the testes. On the other hand, microarray, RT-PCR and real-time quantitative RT-PCR (qRT-PCR) analyses all indicated strong signals for Ucn 1 in the male gonads, with weaker levels of Ucn 2 and 3 mRNA gene expression. Results obtained for Ucn 1 gene expression were corroborated by immunohistochemical detection, which appeared restricted to Leydig cells. Finally, to investigate possible changes in testicular Ucn 1 levels induced by homeostatic challenges, we measured them in rats exposed to alcohol. We observed that indeed, the intragastric injection of this drug significantly increased testicular Ucn 1, but not Ucn 2, Ucn 3, CRF, CRFR1 or CRFR2 mRNA levels. Collectively, these results provide novel information regarding the presence of CRF-like peptides in the adult male rat testis.

Keywords

Urocortin; testosterone; Leydig cells; gene expression; alcohol

Introduction

The ability of various stressors to inhibit reproductive functions is well recognized {see for example (Breen and Karsch, 2006; Ferin, 1993; Lopez-Calderon et al., 1991; Rivest and Rivier, 1995; Rivier and Rivest, 1991)}. However, the mechanisms responsible for this influence remain surprisingly elusive, particularly in response to stimuli of short duration. We know, of course, that stressors interfere with the synthesis and release of the

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hypothalamic peptide gonadotropin-releasing hormone (GnRH) via the release of a variety of neurotransmitters such as corticotropin-releasing factor (CRF) (Rivest et al., 1993; Rivest and Rivier, 1995), prostaglandins (Rivest and Rivier, 1993), opiates, and catecholamines (Dobson et al., 2003; Kalra and Kalra, 1983). Interestingly however, stress-induced decreases in plasma levels of the pituitary hormone luteinizing hormone (LH) are usually relatively slow, and moreover often follow, rather than precede, significant changes in testosterone (T) levels, which can be very rapid (Maric et al., 1996; Orr et al., 1994; Srivastava et al., 1993). This led to the concept that while an important site of action of chronic stressors is hypothalamic GnRH neurons, shorter stimuli may release into the general circulation factors that can penetrate the gonads and act directly on sex steroid-producing cells, or are produced by the gonads themselves. Indeed, the rodent testis harbors a wide array of neurotransmitters and peptides believed to participate in the activity of Sertoli and Leydig cells (Gnessi et al., 1997), including vasopressin (Foo et al., 2001; Howl et al., 1995; Lefebvre and Zingg, 1991), pro-opiomelanocortin-related entities (Bardin et al., 1987; Boitani et al., 1985; Eskeland et al., 1989; Gerendai et al., 1984; Li et al., 1989; Valenca and Negro-Vilar, 1986) and “CRF” (Audhya and Hollander, 1987; Audhya et al., 1989; Dufau et al., 1993; Fabbri et al., 1990; Tinajero et al., 1992; Tortorella et al., 1993; Ulisse et al., 1989; Yoon et al., 1988). In support of the hypothesis that at least some of these compounds might influence gonadal activity, we recently demonstrated that the intratesticular injection of CRF-related peptides, and in particular Ucn 1, significantly inhibited the T response to human chorionic gonadotropin (hCG) (Rivier, 2008).

Several years ago, Dr. Dufau and colleagues reported that CRF exerted a strong inhibitory effect on rat Leydig cell activity (Dufau et al., 1993; Fabbri et al., 1990; Tortorella et al., 1993; Ulisse et al., 1989), while others proposed a stimulatory effect of this peptide on steroidogenesis in the mouse testis (Heinrich et al., 1998; Huang et al., 1995). In addition to this apparent species difference, other results appeared inconsistent. For example, both CRF (Fabbri et al., 1990; Ulisse et al., 1990) and its antibodies (Gerendai et al., 1993) were shown to inhibit T release through a local effect. Another potentially conflicting issue pertained to the nature of the “CRF” that was found in testes and/or thought to influence its activity. Indeed, after CRF was first isolated and characterized (Vale et al., 1981), other structurally related peptides were discovered and named urocortins (Ucns). These Ucns, of which there are currently three (Ucn 1, 2 and 3), show subtype-specific homologies with r/hCRF (Czimmer et al., 2006; Martínez et al., 2004) and affinities to CRF receptors (CRFR) (Donaldson et al., 1996; Vaughan et al., 1995a). Ucn 1 gene expression and/or immunoreactivity have been detected in a variety of peripheral organs (Donaldson et al., 1996; Kageyama et al., 1999; Oki and Sasano, 2004; Vaughan et al., 1995a), with one report stating its presence in mature mouse spermatozoa (Tao et al., 2007). When we became interested in trying to resolve issues linked to the presence and role of CRF-related peptides in the rat testes, we realized that the commercial antibodies that had been used to measure what was then identified as “CRF” in the testes (Tinajero et al., 1992), showed substantial cross-reactivity with Ucn 1, as tested in our laboratory (Vaughan and Vale, unpublished). Also, the CRF antagonist used to investigate the role of this peptide (Frungeri et al., 2002) blocks the receptors that are activated by both Ucn 1 and CRF. It is therefore possible that at least in these studies, the presence of “CRF” in the testes, as well as effects originally attributed to this peptide, were in fact due to Ucn 1. On the other hand, extraction of ovine testes yielded a sequence that was virtually identical to that of ovine hypothalamic CRF (Audhya et al., 1989), which suggested that in this case, true CRF may indeed have been present.

In view of this controversy, we decided to rely on current methodology to examine the presence of CRF-related peptides in the adult male rat testis. Our interest in this question was based in part on our earlier observation that the intratesticular injection of Ucn

significantly interfered with the Leydig cell response to human chorionic gonadotropin [Rivier, 2008 #17080]. Using reverse transcription polymerase chain reaction (RT-PCR), qRT-PCR and microarray analysis, we detected Ucn 1, but very little CRF in testicular extracts. Immunohistochemistry was then used to confirm the presence of Ucn 1 in structures thought to be Leydig cells, whereas no CRF immunoreactivity could be detected. Finally, to determine whether testicular Ucn 1 levels were influenced by homeostatic threats, we measured them in rats injected with alcohol intragastrically (ig). On the basis of qRT-PCR and microarray analysis, we found that this drug, which as expected (Rivier et al., 1990; Rivier and Lee, 1996) upregulated CRF mRNA levels in the hypothalamus, also increased testicular gene expression of Ucn 1, but not CRF, Ucn 2, Ucn 3, CRFR1 or CRFR2.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (200-220 g, Harlan Sprague Dawley, Inc, San Diego, CA) were kept under standard light (lights on 0600-1800) and feeding (rat chow and water *ad libitum*) regimens. Ig surgery was performed under isoflurane anesthesia 7-10 days before the bioassays. The animals were singly housed after the surgery to prevent chewing of the cannulae. All protocols were approved by the Salk Institute IACUC.

Reagents

Alcohol was diluted with saline to a final injected concentration of <20% (V/V). The dose chosen (4.5 g/kg, ig) induces a moderate degree of intoxication, and blood alcohol levels (BALs) in the range of those typically achieved during binge drinking (Ogilvie and Rivier, 1997), i.e., the range of BALs was 200-250 mg% 1-2 hr after ig alcohol. It also significantly inhibits T release and decreases levels of testicular steroidogenic enzymes (Herman et al., 2006; Selvage et al., 2004).

In vivo protocols

On the morning of the assays, the ig cannulae were connected to saline-filled tubing and the rats were allowed to rest undisturbed for 3 h. Alcohol or its vehicle were then slowly infused ig according to previously published protocols (Ogilvie et al., 1998; Ogilvie et al., 1997; Ogilvie and Rivier, 1997). For microarray analysis, RT-PCR and qRT-PCR, the animals were decapitated 1 and 2 hr after ig injection, and the testes and the brains were quickly removed. RNA was extracted and sent to Gyeongsang National University (Korea) for the microarray experiments, while other RNA samples were processed for RT-PCR and qRT-PCR at the Salk Institute (La Jolla, CA). For Ucn 1 immunohistochemistry, the animals were perfused 1 and 2 hr after the injection of alcohol or vehicle and the testis were processed as described below.

Leydig Cell Isolation

The Leydig cell isolation process has been previously described by Herman and Rivier (2006). Briefly, the testes from rapidly decapitated animals were quickly removed, decapsulated and cleared of large blood vessels. The samples were dispersed in Medium 199 (Invitrogen, San Diego, CA) that contained 0.1% w/v collagenase and 1.25% w/v BSA (81003, Fraction V, MP Biomedicals, Solon, OH). They were then washed in Medium 199 with BSA and penicillin/streptomycin (GIBCO, Invitrogen, San Diego, CA), suspended in an isotonic Percoll gradient and centrifuged at 4°C for 45 min at 6,500 × g. Semi-purified Leydig cells were then removed from the gradient, washed in heparin dissociation buffer, pelleted and frozen until RNA extraction.

RNA isolation and cDNA synthesis

Total RNA was extracted from rat testes or semi-purified Leydig cells using TRIzol reagent (Invitrogen, Carlsbad, CA). To rid the sample of genomic DNA contamination, 30 µg of total RNA was treated with RQ1 DNase (Promega, Madison, WI) for 30 min at 37°C then cleaned on an RNeasy Mini Column (Qiagen, Valencia, CA). The concentration and purity of the extracted RNA were determined by measuring the absorbance values at 260 nm and 280 nm with a Beckman DU600 spectrophotometer. One µg of total RNA was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) at 37°C for 2 hr. The recommended protocol from Applied Biosystem's RT Kit was followed.

RT-PCR

Rat testes cDNA from the reverse transcription reaction was subjected to PCR containing primers specific to Ucn 1, Ucn 2, Ucn 3, CRF, CRFR1, CRFR2 or the endogenous control GAPDH. The primers used in this study are summarized in Table 1. The 50-µl PCR reaction mixture contained a 1X High Fidelity buffer containing 60 mM Tris-SO₄ (pH 8.9) and 18 mM (NH₄)₂SO₄, 2 mM of MgSO₄, 0.2 mM of each deoxynucleotide, 1 U of Platinum *Taq* High Fidelity (Invitrogen, Carlsbad, CA), 20 pmoles of both forward and reverse primers, and 2 µl of rat testes cDNA. Negative controls for each primer set were performed by adding water to the reaction in place of cDNA or by the reaction without reverse transcriptase. After preheating the reaction at 94°C for 2 min, denaturing, annealing, and elongation were carried out at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, respectively. PCR was carried out for 35 cycles for Ucn 1, Ucn 2, Ucn 3, CRFR1, CRFR2 and 25 cycles for GAPDH. Five µl of the PCR amplification product was subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. A 100-bp DNA ladder (Invitrogen, Carlsbad, CA) was used to estimate the band sizes. Images were then taken of the gels and analyzed by Image J software.

Real-time quantitative RT-PCR (qRT-PCR)

After synthesis of rat testes cDNA, 2 µl of the 20 µl RT reaction was added to a 10 µl fast RT-PCR reaction. The fast RT-PCR reaction contained TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and a FAM dye-labeled TaqMan MGB probe at 250 nM and two unlabeled PCR primers at 900 nM each for Ucn 1, Ucn 2, Ucn 3, CRFR1, CRFR2, or the endogenous control, β-actin. Primers and probes for the gene expression assay of Ucn 1 (Rn00569682_m1), Ucn 2 (Rn00591306_s1), Ucn 3 (Rn02091611_sl), CRFR1 (Rn00578611_m1), CRFR2 (Rn00575617_m1) and the β-actin endogenous control (4352931E) were pre-designed by Applied Biosystems. Each sample was run in triplicate on an Applied Biosystems 7900HT Fast Real-Time PCR System containing a 384-well block with cycling conditions of 95°C for 20 sec followed by 40 cycles of denaturing at 95°C for 1 sec and annealing and extension at 60°C for 20 sec. Relative expression levels of Ucn 1, Ucn 2, Ucn 3, CRFR1, and CRFR2 were measured using the $2^{-\Delta\Delta Ct}$ method and the expression levels were calculated as a percentage of the expression levels in vehicle biological replicates.

Microarray RNA Quantity, Integrity and Purity

Total RNA quantity and purity were assessed by measurement of OD_{260/280} using a NanoDrop spectrophotometer. RNA with an A_{260/280} ratio of >1.8 is considered acceptable for the microarray study. RNA length distribution and integrity were assessed by capillary electrophoresis with fluorescence detection (Agilent Bioanalyzer 2100) using the Agilent Total RNA Nano chip assay (Agilent Technologies, Palo Alto, CA) for presence of 28S and 18S rRNA bands.

Microarray platform

Gene expression analysis was conducted using the Agilent Whole Rat Genome 4x44K Oligo Microarray Kit. The microarray was designed with four replicates of each probe distributed across the array, that is, 4x44K Multiplex slide format, each of which contains more than about 44,000 rat genes and transcripts sequences including control spots.

RNA Label and Hybridization

Fluorescence-labeled cRNA probes for oligo microarray analysis were prepared by amplification of total RNA in the presence of aminoallyl-UTP using Amino allyl MessageAmpTM aRNA kit (Ambion Inc., Austin, TX), followed by the coupling of Cy3 or Cy5 dyes (Amersham Pharmacia, Uppsala, Sweden).

Hybridizations were performed at 65°C for 17 hr in a rotating hybridization oven using the Agilent 60 mer oligo microarray processing protocol. Slides were washed as indicated in this protocol and then scanned with a GenePix 4000B Array Scanner (Axon Instruments, Union City, CA).

Microarray data analysis

Scanned images were analyzed with GenePix Pro 6.0 software (Axon Instruments, Union City, CA) to obtain gene expression ratios. Transformed data were normalized by LOWESS regression and analyzed with GeneSpring GX 7.3 software program (Agilent Technologies Inc., Santa Clara, CA). With the 1-color default normalization, (per chip: normalize to a median or percentile and per gene: normalize to median), GeneSpring GX first divides each raw intensity value by the median of the chip. Then each value is further divided by the median value of each gene across samples, resulting in the final normalized value.

Gene expression levels and trends in the change of gene expression ratios for hypothalami and testicular CRF and/or CRF-related peptides were analyzed from the normalized microarray data. The criteria used to detect differences in gene expression ratios, which were based on previously published validation (Bullen et al., 2004; Mariani et al., 2003; Sirianni et al., 2005), were >1.4 and <0.6, respectively. When changes answered these criteria, the corresponding signals were further analyzed by RT-PCR, qRT-PCR to confirm the significance of these gene regulation trends. Relative expressions of CRF, Ucn 1 and Ucn 2 in the testis were selected from the normalized values in the control group.

Immunohistochemistry

The rats were injected intraperitoneally (ip) with 100 IU heparin 15 min before perfusing with Bouin's solution for 20 min. Testes were removed and then transferred to 75% ethanol. Testes were embedded into paraffin wax using standard methods at UCSD histology (UCSD, La Jolla, CA). Paraffin embedded testes were cut transversely at a thickness of 5 μ m on a rotating microtome (Leica RM2035, Bannockburn, IL) and mounted onto slides. The slides were deparaffinized in xylene and rehydrated through descending grades of alcohol. Slides were then placed in citrate buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0) and microwaved for 10 min and then allowed to cool to room temperature. Briefly, the sections were rinsed with 0.01 M phosphate buffered saline (PBS) between each incubation step. After an initial rinse in PBS, sections were incubated with 0.3% H₂O₂ for 10 min to block for endogenous peroxidase. The sections were then treated with a blocking solution containing 5% normal goat serum for 1 hr before an overnight incubation with rabbit anti-Ucn 1 (Neat, Abcam) or rabbit anti-steroidogenic acute regulatory protein (StAR) (1:1000, Dr. W. L. Miller) primary antibody at 25°C. The sections were then incubated with secondary antibody (biotinylated goat anti-rabbit 1:500, Vector labs, Burlingame, CA) followed by 1 hr incubation with avidin-biotin complex (Vectastain ABC kit, Vector Labs,

Burlingame, CA). Following several rinses with PBS, sections were developed with an Ni-DAB solution for 5 min. The immunoperoxidase developing reaction took place with a mixture of 7.5 mg DAB and 0.3 g nickel-ammonium sulfate in 50 ml of 50 mM Tris-HCl, pH 8.0, to which 10 μ l of H₂O₂ had been added. The sections were rinsed with PBS, dehydrated and coverslipped with DPX mounting media (Fluka Biochemika, Ronkonkoma, NY).

Negative control sections that were incubated with the secondary, but not primary antibody, showed no significant immunoreactivity for either Ucn 1 or StAR. Some testicular sections were stained with hematoxylin (H) and eosin (E) to examine the morphology.

Statistical analysis

Data were expressed as the mean $\pm$ SEM and were analyzed using one- or two-way ANOVA followed by the least square means *post hoc* test. Differences were considered significant from $P \leq 0.05$.

Results

Measurement of CRF-like peptides

Vehicle injection—In the hypothalamus, both CRF and Ucn 1 mRNA levels were readily measured by RT-PCR (Fig. 1), which corresponds to previous reports based on different technologies (Kozicz et al., 1998; Lee and Rivier, 1994; Sawchenko et al., 1984; Vale et al., 1981; Vaughan et al., 1995a). In the testes, on the other hand, CRF was virtually undetectable by microarray (relative expression levels/control: 0.038) and only very low signals were detected by RT-PCR (Fig. 1). However, in agreement with a previous report (Perrin et al., 1995), we found modest levels of CRF receptors type 1 and 2 by RT-PCR or qRT-PCR (not shown). Signals for Ucn 1 were strong when measured by microarray (relative expression levels/control: 1.360) or RT-PCR (Fig. 1), while signals for Ucn 2 were weak (relative expression levels/control: 0.085).

Alcohol injection—In the hypothalamus, microarray data indicated that alcohol induced the expected (Rivier and Lee, 1996) statistically significant increases in CRF gene expression (3.33 and 2.75 fold 1 and 2 hr post-drug administration, respectively) but interestingly, not Ucn 1 or Ucn 2 mRNA levels (Table 2). In contrast, in the testes, this drug significantly ($P < 0.05$ – < 0.01) upregulated Ucn 1 gene expression regardless of whether RNA levels were measured by microarray (Table 2), RT-PCR or qRT-PCR (Fig. 2). While it also significantly increased Ucn 2 mRNA levels measured by microarray, these data were not corroborated by RT-PCR or qRT-PCR ($P > 0.05$) (Table 3). This discrepancy might be due to the relatively low expression levels of testicular Ucn 2 mRNA levels, compared to Ucn 1 mRNA levels; testicular Ucn 2 microarray data should thus be viewed with caution. Finally, we did not detect any alcohol-induced changes in testicular mRNA levels for CRF, CRF receptors or Ucn 3 measured by microarray (Table 2), RT-PCR or qRT-PCR (Table 3). The representative RT-PCR gel pictures of Ucn 2 and Ucn 3 are shown also in Figure 2.

Ucn 1 immunoreactivity (ir) is present in Leydig cells

In order to validate results obtained by microarray or qRT-PCR methodologies and to establish posttranslational processes, we used immunohistochemistry to detect Ucn 1 in the testes after having delineated their morphology with an hematoxylin (H) and eosin (E) stain (Fig. 3, A and D). To further determine if staining for Ucn 1-ir within the lymphatic space of the testes was specific to Leydig cells, we used a rabbit polyclonal antibody against the steroidogenic enzyme StAR. Because of technological issues, results were obtained with serial sections of the gonads, rather than with double immunocytochemistry in identical

sections. Whereas StAR-ir was observed in the majority of Leydig cells of the testicular lymphatic space (Fig. 3, B and E), Ucn 1-ir was only localized to a fraction of the cells serially adjacent to those stained positively for StAR (Fig. 3, C and F). Therefore, even though we cannot unambiguously conclude that Ucn 1 was present in StAR-positive cells because double staining was not obtained in identical sections, these results strongly suggest that Ucn 1-ir is localized in some (though not all) Leydig cells. To confirm the presence of Ucn 1 mRNA levels in Leydig cells, we then ran qRT-PCR for Ucn 1 in semi-purified Leydig cells. Ucn 1 gene expression levels in these cells were comparable to those for testicular Ucn-1 gene expression, i.e., the Ct values of Leydig cells and testicular RNA were both within 33-35 cycles, which is covered by the range of the standard curves. In addition, the same size RT-PCR band was obtained from Leydig cells and testicular RNA. These results suggest that indeed, Ucn 1 is present in Leydig cells.

Discussion

As indicated in the Introduction, studies initiated in our laboratory after the identification of the urocortins as new members of the CRF family (Lewis et al., 2001; Reyes et al., 2001; Vaughan et al., 1995b) suggested that the presence and role of CRF itself in the testes might need to be reconsidered. In this paper, we used current technology to measure mRNA levels for CRF and Ucn, as well as their receptors, in the adult male gonad. This approach indicated that only very low signals for CRF gene expression were detected by microarray or RT-PCR methodologies. In the present work, once we detected significant amounts of Ucn 1 mRNA levels by microarray, we used qRT-PCR and RT-PCR to confirm these results and to demonstrate that this gene was present in the testes. Furthermore, we also report the presence of Ucn 1 immunoreactivity in structures directly adjacent to those positive for the steroidogenic enzyme StAR, and therefore believed to be Leydig cells. Previous studies have reported Ucn 1-ir in spermatozoa (Tao et al., 2007) but to our knowledge, the present work is the first to suggest that this peptide may also be found in Leydig cells. With regard to Ucn 2 or 3, published studies performed on peripheral organs indicated their presence in the gastrointestinal tract, the heart, the pancreas, the adrenals, the ovaries and the skin, among others (Lewis et al., 2001; Li et al., 2003; Martinez et al., 2004; Suda et al., 2004; Takahashi et al., 2004; Yamauchi et al., 2005). Using both microarray and qRT-PCR technology, we report here that additionally, low but detectable levels of Ucn 2 and Ucn 3 genes were found in the rat testes.

A final series of experiments was designed to determine whether testicular Ucn levels could be altered by homeostatic threats known to influence Leydig cell activity. We chose the ig injection of alcohol according to a protocol previously shown to interfere with T release (Herman et al., 2006; Rivier, 1999; Selvage et al., 2004). Measurement of hypothalamic signals was used for the sake of comparison. While hypothalamic CRF gene expression was significantly upregulated by alcohol, Ucn 1 was not. There are reports of temporal discrepancies between changes in CRF and Ucn 1 mRNA levels (Smagin et al., 2001). Consequently, as both salt loading (Hara et al., 1997) and restraint (Smagin et al., 2001) appear able to increase hypothalamic Ucn 1 levels, the fact that we only studied the response of this peptide to alcohol at two closely related time points, might have caused us to miss them. In contrast to findings in the brain, both microarray and qRT-PCR methodology indicated that alcohol significantly increased Ucn 1 in the testes. As the intratesticular injection of Ucn 1 blunts the T response to hCG (Rivier, 2008), the upregulated gonadal levels of this peptide might have represented a possible mechanism mediating the inhibitory effect of this drug on Leydig cell activity (Herman et al., 2006; Rivier, 1999; Selvage et al., 2004). However, as we were unable to reverse the influence of alcohol by blocking CRF receptors (Rivier, 2008), the role played by CRF-like peptides in this model remains unresolved.

In conclusion, we report here that microarray and qRT-PCR technologies indicated the presence of the Ucn 1 gene in testicular extracts, where its gene expression is upregulated by alcohol. Additionally, using immunohistochemistry, we localized this peptide within structures thought to be Leydig cells. These results demonstrate that while earlier reports had suggested that CRF was found in the adult rat's gonad, the subsequent discovery of the Ucn family and the development of techniques able to accurately detect these peptides, indicate the predominance of Ucn 1 signals.

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Figure 1.

CRF mRNA expression was present in the hypothalamus, but only very low signals were found in the testes (A). In contrast, Ucn 1 mRNA expression was found in both the hypothalamus and the testes (B). Representative gel picture from RT-PCR is shown with 1-4 lanes from rat testes and a negative control (without cDNA or without reverse transcriptase) and two rat hypothalami (HT) as positive controls.

Figure 2.

Alcohol (4.5 g/kg, ig) significantly ($P < 0.05-0.01$) increased Ucn 1 mRNA expression in the testes 2 hr later, as measured by RT-PCR (A) and qRT-PCR (B). Representative gel pictures from RT-PCR are shown on the top (Ucn 1) and the bottom (Ucn 2 and Ucn 3). Each bar represents the mean $\pm$ sem of 5-6 rats. *, $P < 0.05$, **, $P < 0.01$ vs. vehicle.

Figure 3.

A, D: Cross section of H and E-stained rat testis showing the morphology of seminiferous tubules and Leydig cells. *B, E:* Immunohistochemical staining for StAR was used to identify the location of Leydig cells in the testis (StAR-ir positive cells are indicated by black arrows in E). *C, F:* Some (black arrowheads in F) but not all (white arrowheads in F) structures thought to be Leydig cells are positive for Ucn 1 immunoreactivity. A-C, low-power view of rat testis and boxes indicate the higher magnified views in D-F. Scale bars indicate 50 μ m in pictures (A, B, C), and 20 μ m in pictures (D, E, F), which are enlarged boxes to show details.

Table 1

Primer sequences for Ucn 1, Ucn 2, Ucn 3, CRFR1, CRFR2 or the endogenous control GAPDH.

Target Gene	GenBank Accession	Forward primer 5'-3'	Reverse primer 5'-3'	Product Size (b.p.)
Ucn 1	NM_019150	CTCTCCATCTTGCACTGGAT	TCAGGAGAACGACAATCGCC	237
Ucn 2	NM_133385	GGTCCTGATGTTGGATAGGG	ATGACCTGTCCGAGCCTTG	238
Ucn 3	XM_574076	AGCACTTCCACCCTAGAGCA	CTTTCGACATGGGCCTGTTC	387
CRF	NM_OB1019	AGAAAGGGGAAAGGCAAAGA	GTTGGAGTCGGCTAAGACTA	309
CRFR1	NM_030999	GCCGCCTACAATTACTTCCA	GGAAAGTCCCGAAGAAACAC	501
CRFR2	NM_022714	CTGGAACCTCATCACCACCT	CAGTTAGGACCTCTCTGCT	546
GAPDH	NM_017008	AGACAGCCGCATCTTCTTGT	TACTGAGATGGGTGCCGTTT	207

Table 2

Microarray analysis for the hypothalamus and the testes.

Gene	Hypothalamus		Testes	
	Ig 1 h ratio (EtOH/CON)	Ig 2 h ratio (EtOH/CON)	Ig 1 h ratio (EtOH/CON)	Ig 2 h ratio (EtOH/CON)
CRF	3.33	2.75	1.35	1.61
CRFR1	0.82	0.81	0.95	1.18
CRFR2	1.17	1.23	0.76	0.83
Ucn 1	0.83	0.67	1.68	2.03
Ucn 2	1.43	1.33	1.95	2.64
CRFBP	0.81	0.92	1.68	1.73
AVPR1a	1.36	2.21	2.6	2.12
AVPR1b	0.7	0.87	2.29	2.66
AVPR2	1.04	1.09	0.98	1.08
AVP-induced 1	1.14	1.11	0.98	0.78
Oxytocin	1.13	1.31	1.15	1.17
Oxytocin R	0.71	0.09	1.32	1.56
NPY	1.07	1.2	0.71	0.73
NPYRY2	0.6	1.08	1.03	1.27
NPYRY5	1	1.25	0.85	0.87
POMC	1	0.9	0.89	0.79
ADH6	1.76	0.87	1.59	1.69

Results are expressed as the ratio of alcohol-treated to control. N = 4-6 rats per group.

Table 3

RT-PCR and qRT-PCR analysis for the testes.

		RT-PCR (% of vehicle)	qRT-PCR (% of vehicle)
Ucn 2	vehicle	100 ± 4.08	100 ± 17.6
	EtOH - 1 hr	89.34 ± 7.81	89.83 ± 13.4
	EtOH - 2 hr	94.33 ± 11.91	124.86 ± 42.15
Ucn 3	vehicle	100 ± 3.9	100 ± 14.2
	EtOH - 1 hr	93.54 ± 15.66	88.2 ± 8.6
	EtOH - 2 hr	102.7 ± 13.44	108.6 ± 19.5
CRFR1	vehicle	100 ± 6.42	100 ± 15
	EtOH - 1 hr	109.27 ± 13.93	99.07 ± 17.37
	EtOH - 2 hr	105.13 ± 7.58	114.52 ± 8.99
CRFR2	vehicle	100 ± 7.71	100 ± 9.37
	EtOH - 1 hr	106.93 ± 17.37	93.32 ± 15.16
	EtOH - 2 hr	89.17 ± 7.75	118 ± 12.25

Results are expressed as % of vehicle (mean ± sem). N = 4-6 rats per group.