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New insights into melatonin/CRH signaling in hamster Leydig cells

Soledad P. Rossi [a], María E. Matzkin [a,b], Claudio Terradas [c,d], Roberto Ponzio [e], Elisa Puigdomenech [d], Oscar Levalle [c], Ricardo S. Calandra [a], Mónica B. Frungieri [a,b,*]

[a] Laboratorio de Esteroides, Instituto de Biología y Medicina Experimental, CONICET, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina
[b] Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121 Buenos Aires, Argentina
[c] División de Endocrinología, Hospital Durand, Facultad de Medicina, Universidad de Buenos Aires, Díaz Vélez 5044, 1405 Buenos Aires, Argentina
[d] Instituto Médico PREFER, Facultad de Medicina, Universidad de Buenos Aires, Güemes 2348, 1650 San Martín, Provincia de Buenos Aires, Argentina
[e] Instituto de Investigaciones en Reproducción, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121 Buenos Aires, Argentina

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ABSTRACT

We have previously described that melatonin inhibits androgen production in hamster testes via melatonin subtype 1a (mel1a) receptors and the local corticotrophin-releasing hormone (CRH) system. This study attempted to determine the initial events of the melatonin/CRH signaling pathway.

In Leydig cells from reproductively active Syrian hamsters, Western blotting, reverse transcription quantitative polymerase chain reaction (RT-qPCR) and a colorimetric assay demonstrated that melatonin and CRH activate tyrosine phosphatases and subsequently reduce the phosphorylation levels of extracellular signal-regulated kinase (erk) and c-jun N-terminal kinase (jnk), down-regulate the expression of c-jun, c-fos and steroidogenic acute regulatory (StAR), and inhibit the production of testosterone. These effects were prevented by a highly selective CRH antagonist, thus indicating that melatonin does not exert a direct role. Specific mitogen-activated protein kinase kinase (MEK) and jnk blockers inhibited expression of c-jun, c-fos, StAR and the production of testosterone, confirming that these are events triggered downstream of erk and jnk. In Leydig cells from photoperiodically regressed adult hamsters, CRH inhibited the production of androstane-3α,17β-diol (3α-diol), the main androgen produced, through the same signaling pathway.

Testicular melatonin concentration was 3–4-fold higher in reproductively inactive hamsters than that detected in active animals.

Since melatonin, CRH, and their receptors are present not only in hamster testes but also in testicular biopsies of infertile men, we can conjecture about the relevance of this previously uncharacterized pathway in human fertility disorders.

In summary, our study identifies crucial intracellular events triggered by melatonin/CRH in the testis that lead to a down-regulation of the steroidogenic process.

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1. Introduction

Melatonin is an indolamine neurohormone that is synthesized from tryptophan via 5-hydroxytryptophan, serotonin, and *N*-acetylserotonin in the pineal gland of most vertebrates [47]. This hormone is involved in the physiological regulation of daily and annual rhythmicity, sleep, mood and behavior, and also affects the immune system and aging, possibly as a potent free radical scavenger [23].

Seasonal rhythms, synchronized with the environment mainly by light, and particularly those associated with the reproductive system, have long been studied. The light signal is received by the photoreceptors of the retina and, through a circuitous connection of neurons, the information is transferred to the pineal gland, resulting in the suppression of melatonin synthesis [24,39,40,45,47]. In seasonal breeders, the nocturnal increase in melatonin can be interpreted as anti- or pro-gonadotropic depending on the duration, magnitude and/or window of sensitivity to the nocturnal melatonin peak [1,33,45,54]. The Syrian hamster is a long day (LD) seasonal breeder and, as a consequence, the hypothalamic–pituitary–testicular axis undergoes cyclic variations consisting of activation at the beginning of spring and suppression at the beginning of fall, coinciding with the preparation for hibernation [3]. In male hamsters kept under standard laboratory conditions including an artificial illumination of 14 h per day, secretion of gonadotropins, prolactin and testosterone remains almost unchanged throughout the adult life [3,18]. In contrast, exposure of

* Corresponding author at: Laboratorio de Esteroides, Instituto de Biología y Medicina Experimental, CONICET, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina. Fax: +54 11 4786 2564.
E-mail address: mfrungieri@fmed.uba.ar (M.B. Frungieri).

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adult hamsters to short day (SD) photoperiods (less than 12.5 h of light per day) for a period of 8–16 weeks, results in marked gonadal regression as a consequence of a severe decline in serum LH, FSH and PRL levels [3,18]. Testicular regression involves profound morphological changes in the tubular as well as in the interstitial compartments. The number of Leydig cells per testis fluctuates only very slightly during the involution phase but a significant reduction in the absolute volume and surface area of nearly all of the Leydig cells organelles including mitochondria and smooth endoplasmic reticulum which are the main sites of androgen biosynthetic enzymes, has been described [3,52,53].

It is well established that pineal-derived melatonin, acting mainly through the suprachiasmatic nucleus and the pars tuberalis, influences the synthesis and release of the hypothalamic GnRH, the adenohyposhyseal gonadotropin hormones and therefore, the function of the testis. Nevertheless, melatonin can directly exert its action on the testis. We have previously described the existence of a melatonergic system in the testis of the Syrian hamster that, working in concert with the primary effect of melatonin on the hypothalamic–pituitary axis, acts as a local modulator of steroidogenesis. The effect of melatonin on gonadal activity involves the interaction between melatonin subtype 1a (mel1a) receptors and the testicular corticotrophin-releasing hormone (CRH) system, leading to down-regulation of steroidogenic acute regulatory (StAR) protein and key steroidogenic enzymes [P450 side chain cleavage, 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD)] expression [14].

In the present work, we extended our previous studies in order to identify the signaling pathway associated with the action of melatonin/CRH in hamster Leydig cells. For such purpose, Leydig cells were purified from reproductively active or inactive adult Syrian hamsters, and incubated in the presence or absence of melatonin and CRH. Subsequently, the mitogen activated protein (MAP) kinases cascades, the activity of phosphatases, the expression of immediate early genes and StAR, and the production of androgens were examined.

2. Materials and methods

2.1. Animals

Male Syrian hamsters (*Mesocricetus auratus)* were raised in our animal care unit [Charles River descendants, Animal Care Lab., Instituto de Biología y Medicina Experimental (IBYME), Buenos Aires, Argentina] and kept from birth to adulthood in rooms at 23 ± 2 °C under a LD photoperiod (14 h light, 10 h darkness; lights on 7:00–21:00 h). Animals had free access to water and Purina formula chow. Adult hamsters aged 90 days were kept under a LD photoperiod or transferred to a SD photoperiod (6 h light, 18 h darkness; lights on 9:00–15:00 h) for 16 weeks. It is worth noting that hamsters from our colony reach maximum testicular regression after 16 weeks in a SD photoperiod (see additional information in Frungieri et al. [14]).

Hamsters were killed by asphyxia with carbon dioxide (CO_2) according to protocols for the use of laboratory animals, approved by the Institutional Animal Care and Use Committee (IBYME), following the National Institutes of Health (NIH) guidelines, USA. At the time of sacrifice, trunk blood was collected and testes were dissected. Left testes were used for Leydig cell purification. *In vitro* incubations of Leydig cells were performed followed by determination of mRNA expression [by reverse transcription quantitative polymerase chain reaction (RT-qPCR)], protein expression (by immunoblotting), tyrosine phosphatase activity (by a commercial non-radioactive assay), and androgen levels in the incubation media [by radioimmunoassay (RIA)].

Right testes were used for quantification of melatonin concentrations, using an enzyme-linked immunosorbent assay (ELISA).

2.2. Hamster Leydig cell purification and "in vitro" incubations

Hamster testes were used to isolate Leydig cells. Briefly, decapsulated testes were incubated in a shaking water bath at 34 °C for 5 min in the presence of 0.2 mg/ml collagenase type I (Sigma–Aldrich, St. Louis, MO, USA). At the end of the incubation, collagenase activity was stopped by adding medium 199 (Sigma–Aldrich), and the tubules were allowed to settle for 1 min. Supernatants were transferred to 75 cm^2 sterile flasks, and placed in an incubator at 37 °C under a humid atmosphere with 5% CO_2 for 10 min. The unattached cells were then recovered by swirling, followed by a gentle washing with medium 199, and filtered by a 100 μm Nylon cell strainer (BD Biosciences, Bedford, MA, USA). Attached cells, 95% enriched with macrophages positive for Indian Ink, ED-1 antigen and ED-2 antigen, were discarded.

Filtered cells were used for Leydig cell isolation under sterile conditions using a discontinuous Percoll density gradient as previously described [17]. Cells that migrated to the 1.06–1.12 g/ml density fraction were collected and suspended in medium 199. An aliquot was incubated for 5 min with 0.4% Trypan-blue and used for cell counting and viability assay in a light microscope. Viability of Leydig cell preparations was 97.5–98.5%. In order to evaluate enrichment in Leydig cells, 3β-HSD activity was measured as previously described by Levy et al. [31]. Cell preparations were 87–90% enriched with hamster Leydig cells. Less than 0.006% of the contaminating cells were macrophages positive for Indian Ink, ED-1 and ED-2 antigens, whereas no mast cells were detected. The remaining cell types had the morphology of either peritubular cells or endothelial cells. Petri dishes with 1 ml medium 199 containing 5×10^4 cells (for tyrosine phosphatase activity determination), 5×10^5 cells (for RT-qPCR and androgen determinations) or 1.5×10^6 cells (for immunoblotting) were incubated for 1 and 3 h at 37 °C under a humid atmosphere with 5% CO_2 and in the presence of the following chemicals from Sigma–Aldrich: melatonin (1 and 10 μM), CRH (1 and 10 μM), α-helical CRH [9–41] (a CRH receptor antagonist) (10 μM), U0126 (a specific mitogen-activated protein kinase kinase (MEK) 1/2 inhibitor) (10 μM), SP600125 (a specific jun N-terminal kinase (jnk) inhibitor) (20 μM), okadaic acid (an inhibitor of serine/threonine phosphatases) (1 μM) and sodium orthovanadate (an inhibitor of tyrosine phosphatases) (100 μM).

In this study, U0126 and SP600125 stock solutions were prepared in dimethyl sulfoxide (DMSO) (ICN Biomedicals Inc., Aurora, OH, USA). These solutions were then further diluted in medium 199. An appropriate volume of DMSO (5–10 μl DMSO/ml medium 199) was added to control experiments to account for possible effects of DMSO. Melatonin was dissolved in medium 199, which was then used as vehicle for control incubations.

After incubations, cells were used for RNA extraction followed by RT-qPCR, protein extraction followed by immunoblotting or determination of tyrosine phosphatase activity. Media were frozen at −20 °C until androgen levels were determined by RIA.

2.3. Immunoblotting

Approximately 1.5×10^6 hamster Leydig cells were homogenized in 20 mM Tris–HCl (pH 8), 137 mM NaCl buffer containing 10% glycerol, 1% lysis buffer (NP40, Sigma–Aldrich), and 1% of a pre-formed mixture of protease inhibitors (P8340, Sigma–Aldrich). Protein concentrations were measured by the method of Lowry et al. [34]. Samples were heated at 95 °C for 5 min under reducing conditions (10% 2-mercaptoethanol). Leydig cell protein homogenates (approximately 200 μg) were loaded onto 10% tricine–SDS–polyacrylamide gels, electrophoretically separated, and blotted

onto nitrocellulose as previously described [15]. Blots were incubated with mouse monoclonal anti-actin antibody (1:5000, Calbiochem, La Jolla, CA, USA), mouse monoclonal anti-phospho extracellular signal-regulated kinase (erk) 44/42 MAP kinases antibody (1:250, Cell Signaling Technology Inc., Beverly MA, USA), rabbit polyclonal anti-erk 44/42 MAP kinases serum (1:500, Cell Signalling Technology Inc.), rabbit polyclonal anti-phospho P38 MAP kinases serum (1:100, Cell Signaling Technology Inc.), rabbit polyclonal anti-P38 MAP kinases serum (1:500, Cell Signalling Technology Inc.), mouse monoclonal anti-phospho jnk MAP kinases antibody (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse monoclonal anti-jnk MAP kinases antibody (1:200, Santa Cruz Biotechnology Inc.), and rabbit polyclonal anti-phospho c-jun serum (1:500, Santa Cruz Biotechnology Inc.).

Subsequently, blots were incubated with peroxidase-labeled secondary antibodies (1:2000 goat anti-mouse IgM, Calbiochem, for actin, 1:1000 sheep anti-mouse IgG, GE Healthcare, Wauwatosa, WI, USA, for phospho erk 44/42 MAP kinases, phospho jnk MAP kinases and for jnk MAP kinases, 1:2500 goat anti-rabbit IgG, Sigma–Aldrich, for phospho P38 MAP kinases, phospho c-jun, erk 44/42 MAP kinases and for P38 MAP kinases). Signals were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

2.4. RT-qPCR analysis

Total RNA was prepared from hamster Leydig cells using the QIAGEN RNeasy mini kit (QIAGEN Inc., Valencia, MO, USA). The RT reaction was performed using dN6 random primers as previously described [15]. A cDNA amount corresponding to 400 ng total RNA was used for RT-qPCR.

qPCR assays were performed using oligonucleotide primers for c-fos (5′-CCAAGCGGTAGGTAGAGCC and 5′-GCAGACCCCCAGTCAAA TCCA), c-jun (5′-GCATGAGGAACCGCATCGC and 5′-CGTTAGCATGAG TTGGCAC), StAR (5′-AACGGGGATGAGGTGCTGAG and 5′-CCACTCT CCCATTGCCTC) and 18S rRNA (5′-ACACGGACAGGATTGACAGATT and 5′-CGTTCGTTATCGGAATTAACCA). 18S rRNA was chosen as the housekeeping gene. Reactions were conducted using SYBR Green PCR Master Mix and the ABI PRISM 7500 sequence detector System (Applied Biosystems, Bedford, MA, USA). The reaction conditions were as follows: one cycle of 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 60 °C and 1 min at 72 °C for c-fos, one cycle of 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 45 s at 55 °C and 1 min at 72 °C for c-jun, and one cycle of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 55 °C and 1 min at 60 °C for StAR and 18S. Following the mathematical model of Pfaffl [43], the relative levels of c-jun mRNA expression and c-fos mRNA expression were determined for each sample by calculating $(E\ \text{target})^{\Delta Ct(\text{target})}/(E\ \text{housekeeping})^{\Delta Ct(\text{houskeeping})}$, where E is the efficiency of the primer set, C_T the cycle threshold, and $\Delta Ct = Ct(\text{normalization cDNA}) - Ct(\text{experimental cDNA})$. The amplification efficiency of each primer set was calculated from the slope of the standard amplification curve of log microliters of cDNA per reaction *vs* the *C*t value over at least four orders of magnitude ($E = 10^{-(1/\text{slope})}$; E c-fos = 2.02, E c-jun = 2.13, E StAR = 1.97, and E 18S rRNA = 1.94 *vs* cDNA from hamster Leydig cells).

2.5. Androgen assays

Testosterone and androstane-3α,17β-diol (3α-diol) levels were determined in the incubation media by RIA according to the method previously described [17] without extraction, using antibodies obtained from Medicorp Inc. (Montreal, Canada). Testosterone was measured using an antibody to testosterone-7α-butyrate-BSA, which is known to have 35% cross-reactivity with dihydrotestosterone (DHT). A specific antibody to 3α-diol-15-CMO-BSA that has 5% cross-reaction with testosterone and DHT was used according to the method validated in hamsters by Frungieri et al. [18]. The minimal detectable assay concentration was 0.215 pmol/ml for testosterone and 0.425 pmol/ml for 3α-diol. The intraassay coefficients of variation were lower than 12% for testosterone and lower than 15% for 3α-diol. The interassay coefficients of variation were lower than 15% for both steroids.

The *in vitro* production of testosterone and 3α-diol from Leydig cells is expressed in terms of pmol per 10^6 Leydig cells.

2.6. Tyrosine phosphatase assay

Approximately 5×10^4 hamster Leydig cells were homogenized in 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 1% lysis buffer (NP40, Sigma–Aldrich), 10 μM phenylmethylsulfonyl fluoride (PMSF, Sigma–Aldrich), 10 μM ethylenediaminetetraacetic acid (EDTA, Sigma–Aldrich), and 10 μM ethylene glycol tetraacetic acid (EGTA, Sigma–Aldrich). Homogenates were then loaded onto a spin column containing Sephadex G25 to remove endogenous phosphate and other low molecular weight inhibitors from the sample. Tyrosine phosphatase activity was assayed using a non-radioactive assay system (Promega, Madison, WI, USA). This system determines the amount of free phosphate generated in the reaction by measuring the absorbance of a molybdate–malachite green–phosphate complex. The reaction was performed in a flat-bottomed 96-well culture plate. The reaction mix containing 5 μl homogenates (approximately 6 μg protein), 7.5 μl of 1 mM Tyr phosphopeptide-1 (END(pY)INASL), 7.5 μl of 1 mM Tyr phosphopeptide-2 (DADE(pY)LIPQQG) and 55 μl phosphate-free water was incubated for 1 h at 30 °C. The reaction was stopped with 75 μl molybdate dye/additive mixture. The optical density of the color was read at 620 nm using a microplate reader (Multiskan Ex, Thermo Electron Corporation, Waltham, MA, USA).

Tyrosine phosphatase activity is expressed as nanomole (nmol)/min/mg protein.

2.7. Melatonin assay

Serum melatonin levels as well as the content and concentration of testicular melatonin were determined in duplicate with an ELISA kit (IBL, Hamburg, Germany). Testes were homogenized in 0.1 M ammonium acetate buffer (pH 6.8) containing 1% of a pre-formed mixture of protease inhibitors (P8340, Sigma–Aldrich), 1 mM sodium orthovanadate, 1 mM PMSF and 50 mM sodium fluoride (NaF). Standards, controls, sera and testicular homogenates were extracted using C18 extraction columns according to the protocol of the manufacturer. Signals were densitometrically analyzed at 405 nm using a microplate reader (Multiskan Ex, Thermo Electron Corporation).

Serum melatonin levels are expressed as picomolar (pM). Melatonin concentration and content are expressed as femtomole (fmol) per g tissue and fmol per testis, respectively.

2.8. Patients and testicular samples

The study was designed in accordance with the Helsinki Declaration and its last modification in Tokyo 2004 on human experimentation and was approved by the Ethics Committee of the Durand Hospital (Buenos Aires, Argentina) and the Ethics Committee of the Instituto de Biología y Medicina Experimental (Buenos Aires, Argentina). Informed Consent was obtained from all the patients enrolled in the study.

Fifteen men (27–42 years old) presenting idiopathic infertility and non-obstructive azoospermia without an infection process were enrolled in this study and then assessed and diagnosed by

open testicular biopsy. Patients with known etiology of infertility, such as genitourinary infections, mumps orchitis, varicocele, hypogonadotropic hypogonadism, chromosome anomalies, obstruction or agenesia of seminal ducts were excluded. The diagnosis of azoospermia was based on the analysis of at least two semen samples collected at different times. All the patients underwent a detailed physical examination, endocrinology profile testing and a testicular biopsy. All of them presented normal karyotype. Non-obstructive azoospermia was defined by the presence of spermatogenic defects in the testicular biopsy without evidence of obstruction of the seminal ducts. All the patients agreed to have a small piece of testicular tissue (5 mm in diameter) taken for the additional studies in the present investigation. One-third of the tissue volume was immersed in Bouin's solution and subjected to histopathological diagnosis. The testicular histopathology was categorized according to the most advanced presence of spermatogenesis. Two pathologists evaluated all testicular slices. Biopsies were classified either as hypospermatogenesis or Sertoli cell only (SCO) appearance, according to McLachlan et al. [38]. The hypospermatogenesis group included men in which the biopsy specimens contained less than 17 mature spermatids per tubule [49,51].

Two-thirds of the volume of pathological samples was cryopreserved in liquid nitrogen for molecular biology studies. Ethical and legal considerations hampered the possibility to obtain testicular samples from healthy men.

2.9. Immunohistochemical analysis followed by laser capture microdissection and RT-PCR

Human testicular biopsies from patients with SCO syndrome and patients with hypospermatogenesis were examined by histological and immunohistochemical assays. Fifteen human testicular biopsies were evaluated. After fixation, tissues were dehydrated and embedded in paraffin wax, and 5-μm sections obtained from three different levels.

A polyclonal rabbit anti-3β-HSD serum (1:1000) kindly provided by Prof. Dr. J.I. Mason (University of Edinburgh, Scotland), and an avidin–biotin–peroxidase system (Vector Laboratories Inc., Burlingame, CA, USA) was used to detect 3β-HSD. For control purposes, the first antiserum was replaced by normal nonimmune sera. Subsequently, laser capture microdissection was performed. Briefly, the laser energy of the Applied Biosystems Arcturus laser capture microdissection equipment was used to circumscribe 3β–HSD-immunoreactive cells. A CapSure® HS LCM Cap (Applied Biosystems) containing a thermoplastic film was placed over the target area. The laser was pulsed through the cap. Therefore, the thermoplastic film formed a thin protrusion that bridges the gap between the cap and tissue and finally adhered to the target cell. Lifting of the cap removes the target cells now attached to the cap. Approximately two hundred 3β-HSD-positive cells were captured on each cap. As negative control, 3β-HSD-immunoreactive cells were destroyed by a few laser shots and the remaining material was catapulted into the cap of a microfuge tube. In order to improve RNA yields, samples were digested for 16 h at 37 °C in the presence of 20 μg Proteinase K. Then, RNA was extracted using the Paradise Plus Reagent system (Applied Biosystems) following the manufacturer's instructions. Degradation of contaminating DNA was performed by incubation of the cell extract mixtures with RNase-free DNase (1 U per μg RNA) at room temperature for 20 min.

The RT reaction was performed using dN6 random primers as previously described [37]. PCR conditions were 95 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 55–60 °C (annealing temperature) for 1 min, 72 °C for 1 min, and a final incubation at 72 °C for 5 min. When required, a second PCR amplification using nested primers was also carried out.

PCR assays were performed using oligonucleotide primers for 18S rRNA (5′-ACACGGACAGGATTGACAGATT and 5′-CGTTCGTTATC GGAATTAACCA), StAR (5′-GAGTGGAACCCCAATGTC and 5′-GCACC ATGCAAGTGGGAC), CRH (first set: 5′-AGAAACTCAGAGACCAAG and 5′-CATCAGTTTCCTGTTGCTG; hemi-nested set: 5′-TAGGAATG CCCTCGGCGG and 5′-CATCAGTTTCCTGTTGCTG), CRH subtype 1 receptor (CRH-R1) (first set: 5′-GTTGGTGACAGCCGCCTA and 5′-GAAGGAGTTGAAGTAGATGAA; nested set: 5′-TACTACGACAATGA GAAGTG and 5′-AGGGGCAGCAGCACCAGA), and mel1a receptor (first set: 5′-AGAACTCCCTCTGCTACG and 5′-CTGACGGACTGGGCG AAG; hemi-nested set: 5′-CCGTCCTGCCCAACCTCC and 5′-CTGAC GGACTGGGCGAAG).

When information about exon structure was available at GenBank, oligonucleotide primers were designed as homologous to regions of different exons. In this context, we designed oligonucleotide primers for StAR, CRH and CRH-R1 to be homologous to areas of different exons.

PCR products were separated on 2% agarose gels, and visualized with ethidium bromide. The identity of the cDNA products was confirmed by sequencing, using a fluorescence-based dideoxy-sequencing reaction and an automated sequence analysis on an ABI 373A DNA sequencer.

2.10. Statistical analyses

Statistical analyses were performed using ANOVA followed by Student's *t* test for two comparisons or Student–Newman–Keuls test for multiple comparisons. Data are expressed as mean ± S.E.M.

For immunoblotting studies, bands were quantified by densitometry and normalized to actin housekeeping gene, using SCION IMAGE (SCION Corporation, Frederick, MD, USA).

3. Results

3.1. Identification of the signaling mechanism by which melatonin and CRH inhibit testosterone production in Leydig cells of reproductively active adult Syrian hamsters

Western blot experiments were performed to determine whether the inhibitory effect of melatonin and CRH on testosterone production is mediated via MAP kinases.

Levels of phospho-erk and phospho-jnk were significantly decreased when hamster Leydig cells were incubated with melatonin or CRH (Fig. 1). Phospho-P38 levels were not affected by CRH, but markedly decreased when melatonin was added to the incubation media (Fig. 1). No significant differences were found between the results obtained when actin was used as internal standard and the data achieved when the corresponding total non-phosphorylated MAP kinase was chosen for standardization of immunoblotting assays (data not shown).

RT-qPCR assays showed that both melatonin and CRH inhibit the expression levels of the transcription factors c-fos and c-jun (Fig. 2A) and that c-jun phosphorylation was also inhibited by melatonin and CRH (Fig. 2B).

In order to distinguish between the signaling events associated with melatonin and CRH, purified hamster Leydig cells were incubated with melatonin or CRH in the presence or absence of a specific CRH antagonist (α-helical CRH [9–41]). When cells were treated with melatonin plus α-helical CRH [9–41], P38 phosphorylation remained unchanged (Fig. 3A). In contrast, the inhibitory effect of melatonin on erk, jnk and c-jun phosphorylation (Fig. 3A), c-jun, c-fos (Fig. 3B) and StAR mRNA expression (Fig. 3C), and testosterone production (Fig. 3C) was abolished by the CRH antagonist. Immunoblotting assays revealed no significant differences between the results obtained when actin was used as

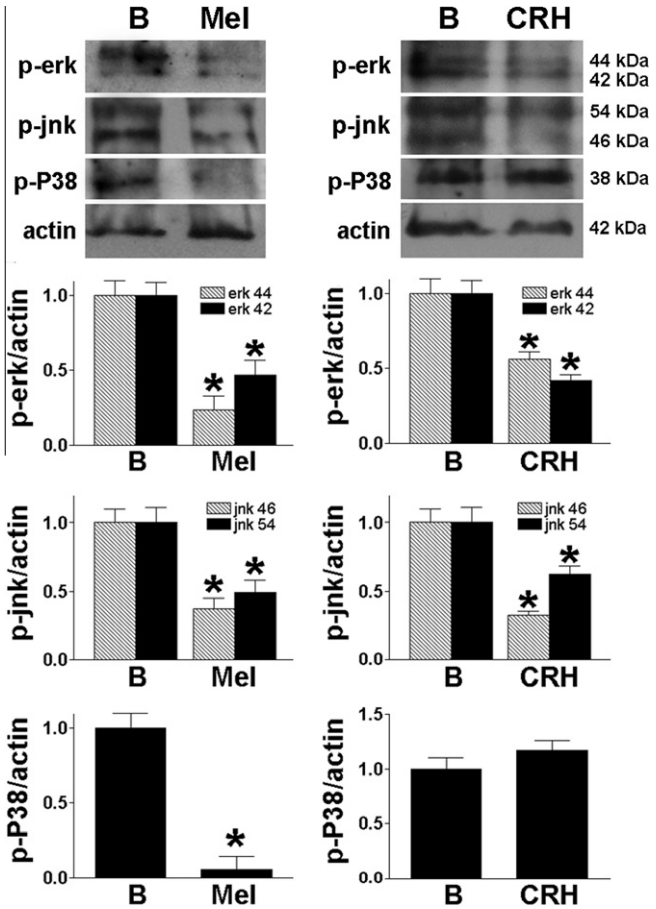

Fig. 1. Inhibitory effect of melatonin and CRH on erk, jnk and P38 phosphorylation in Leydig cells from reproductively active Syrian hamsters. Hamster Leydig cells were incubated in medium 199 for 1 (data not shown) and 3 h in the presence or absence of melatonin (Mel, 1 and 10 μM) or CRH (1 and 10 μM). For each experiment, cells were isolated from six adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness). Phospho-erk (p-erk, 42–44 KDa), phospho-jnk (p-jnk, 46–54 KDa), phospho-P38 (p-P38, 38 KDa), and actin (42 KDa) protein levels were determined by immunoblotting. These representative immunoblots show the results obtained from one of three experiments performed in different cell preparations that showed similar results. Bar plot graphs represent the mean ± S.E.M. and show the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to actin. $^{*}p < 0.05$, Student's *t* test. Immunoblotting assays revealed no significant differences between the results obtained when actin was used as internal standard and the data achieved when the corresponding total non-phosphorylated MAP kinase was chosen for standardization.

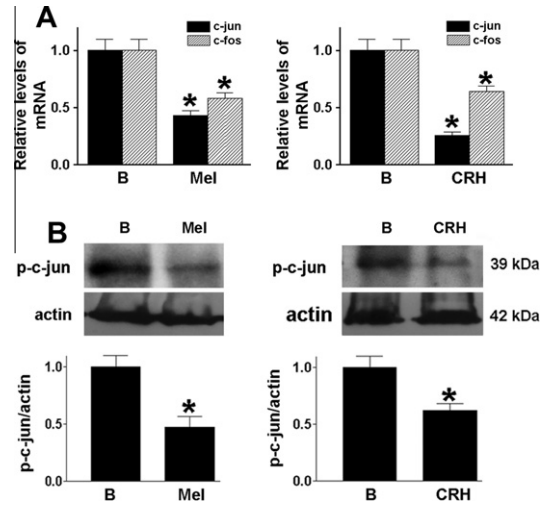

Fig. 2. Inhibitory effect of melatonin and CRH on c-jun and c-fos in Leydig cells from reproductively active Syrian hamsters. Hamster Leydig cells were incubated in medium 199 for 1 (data not shown) and 3 h in the presence or absence of melatonin (Mel, 1 and 10 μM) or CRH (1 and 10 μM). For each experiment, cells were isolated from six adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness). (A) C-jun and c-fos mRNA levels were determined by RT-qPCR. 18S rRNA was chosen as the housekeeping gene. The relative levels of c-jun and c-fos mRNA expression obtained in three independent experiments were determined as described in the Section 2. Bar plot graphs represent the mean ± S.E.M. $^{*}p < 0.05$, Student's *t* test. (B) Phospho-c-jun (p-c-jun, 39 KDa), and actin (42 KDa) protein levels were determined by immunoblotting. These representative immunoblots show the results obtained from one of three experiments performed in different cell preparations that showed similar results. Bar plot graphs represent the mean ± S.E.M. and show the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to actin. $^{*}p < 0.05$, Student's *t* test.

internal standard and the data achieved when the corresponding total non-phosphorylated MAP kinase was chosen for standardization (data not shown).

As expected, effects of CRH on MAP kinases, early immediate gene expression, StAR expression and testosterone production were blocked in the presence of α-helical CRH [9–41] (data not shown).

To determine the signaling events triggered downstream of erk and jnk dephosphorylation, hamster Leydig cells were incubated in the presence of U0126, a specific MEK1/2 inhibitor, and SP600125, a specific jnk inhibitor. Both blockers significantly inhibited c-jun and c-fos mRNA expression (Fig. 4A), c-jun phosphorylation (Fig. 4B), StAR expression (Fig. 4C), and testosterone production (Fig. 4C).

To investigate whether protein phosphatases are responsible for the melatonin-dependent dephosphorylation of MAP kinases, hamster Leydig cells were incubated with inhibitors of tyrosine and serine/threonine phosphatases (sodium orthovanadate and okadaic acid, respectively) in the presence or absence of melatonin. Melatonin reversed sodium orthovanadate-stimulated phosphorylation of erk and jnk and the release of testosterone into the incubation media, but did not affect okadaic acid-induced MAP kinases phosphorylation or testosterone production (data not shown). CRH reversed sodium orthovanadate-stimulated phosphorylation of erk and jnk and the release of testosterone into the incubation media, but did not alter okadaic acid-induced MAP kinases phosphorylation or testosterone production (data not shown). Since these preliminary results suggest the involvement of tyrosine phosphatases in the signaling pathway of melatonin/CRH, a commercial and non-radioactive assay was used to determine tyrosine phosphatase activity. Both CRH (Fig. 5A) and melatonin (Fig. 5B) significantly stimulated tyrosine phosphatase activity in hamster Leydig cells whereas α-helical CRH [9–41] prevented these stimulatory effects (Fig. 5A and B).

3.2. Identification of the signaling mechanism by which CRH inhibits 3α-diol production in Leydig cells of photoperiodically regressed adult Syrian hamsters

Tyrosine phosphatase activity was significantly higher in Leydig cells purified from photoperiodically regressed hamsters incubated in the presence of 1 μM CRH (7.42 ± 0.32 nmol/min/mg protein, mean ± S.E.M) than control cells under basal conditions (4.66 ± 0.05 nmol/min/mg protein, $p < 0.05$, Student's *t* test).

The signaling events triggered by CRH in Leydig cells purified from hamsters exposed to a SD photoperiod for 16 weeks were determined by western blot experiments, RT-qPCR assays and

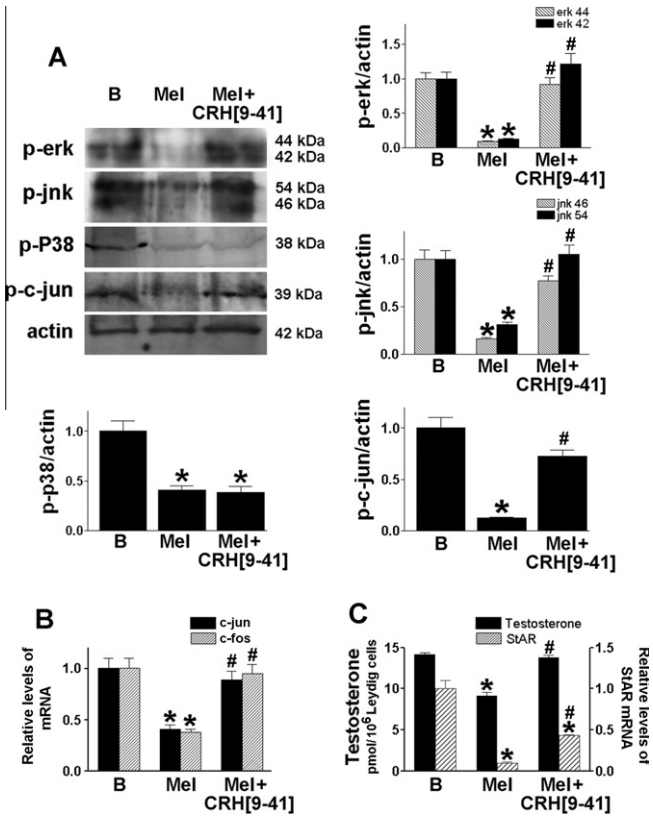

Fig. 3. The inhibitory effect of melatonin on erk, jnk, P38 and c-jun phosphorylation, c-jun, c-fos, and StAR expression, and *in vitro* testosterone production from Leydig cells of reproductively active Syrian hamsters is reversed by the competitive CRH receptor antagonist α-helical CRH [9–41]. Hamster Leydig cells were incubated in medium 199 for 1 (data not shown) and 3 h in the presence or absence of melatonin (Mel, 1 and 10 μM), either with or without α-helical CRH [9–41] (10 μM). For each experiment, cells were isolated from six adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness). (A) Phospho-erk (p-erk, 42–44 KDa), phospho-jnk (p-jnk, 46–54 KDa), phospho-P38 (p-P38, 38 KDa), phospho-c-jun (p-c-jun, 39 KDa), and actin (42 KDa) protein levels were determined by immunoblotting. These representative immunoblots show the results obtained from one of three experiments performed in different cell preparations that showed similar results. Bar plot graphs represent the mean ± S.E.M. and show the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to actin. $^{*}p < 0.05$, compared with the control group. $^{\#}p < 0.05$, compared with the melatonin-treated group. Student–Newman–Keuls test. Immunoblotting assays revealed no significant differences between the results obtained when actin was used as internal standard and the data achieved when the corresponding total non-phosphorylated MAP kinase was chosen for standardization. (B and C) C-jun, c-fos and StAR mRNA levels were determined by RT-qPCR. 18S rRNA was chosen as the housekeeping gene. The relative levels of c-jun, c-fos and StAR mRNA expression obtained in three independent experiments were determined as described in the Section 2. Bar plot graphs represent the mean ± S.E.M. $^{*}p < 0.05$, compared with the control group. $^{\#}p < 0.05$, compared with the melatonin-treated group. Student–Newman–Keuls test. (C) Testosterone production was determined by RIA. Bar plot graphs represent the mean ± S.E.M. from three independent experiments (five to six replicates per experiment) performed in different cell preparations. $^{*}p < 0.05$, compared with the control group. $^{\#}p < 0.05$, compared with the melatonin-treated group. Student–Newman–Keuls test.

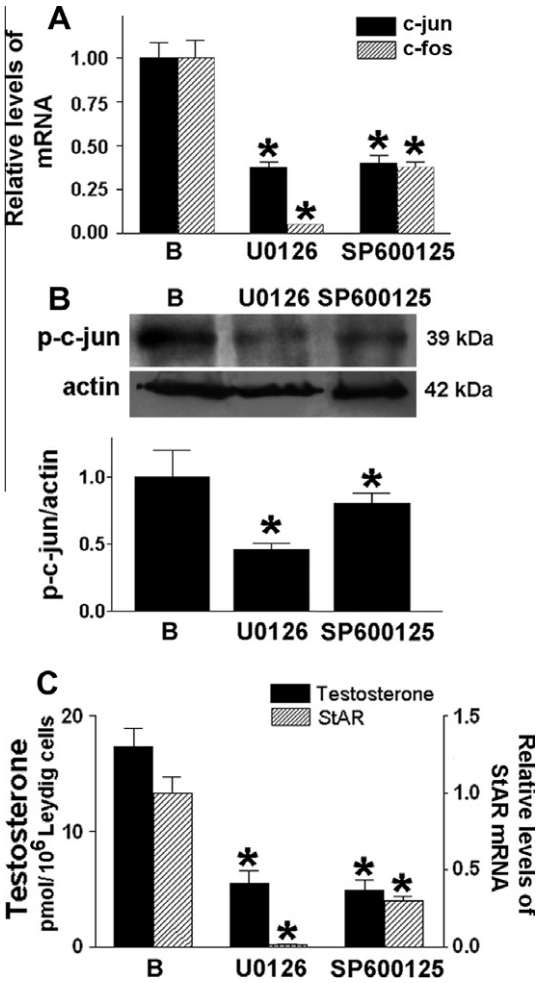

Fig. 4. Inhibitory effect of a specific MEK1/2 inhibitor (U0126) and a specific jnk inhibitor (SP600125) on c-jun phosphorylation, c-jun, c-fos and StAR expression, and *in vitro* testosterone production from Leydig cells of reproductively active Syrian hamsters. Hamster Leydig cells were incubated in medium 199 for 1 (data not shown) and 3 h in the presence or absence of U0126 (10 μM) or SP600125 (20 μM). For each experiment, cells were isolated from six adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness). (A and C) C-jun, c-fos and StAR mRNA levels were determined by RT-qPCR. 18S rRNA was chosen as the housekeeping gene. The relative levels of c-jun, c-fos and StAR mRNA expression obtained in three independent experiments were determined as described in the Section 2. Bar plot graphs represent the mean ± S.E.M. $^{*}p < 0.05$, Student–Newman–Keuls test. (B) Phospho-c-jun (p-c-jun, 39 KDa), and actin (42 KDa) protein levels were determined by immunoblotting. These representative immunoblots show the results obtained from one of three experiments performed in different cell preparations that showed similar results. Bar plot graphs represent the mean ± S.E.M. and show the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to actin. $^{*}p < 0.05$, Student–Newman–Keuls test. (C) Testosterone production was determined by RIA. Bar plot graphs represent the mean ± S.E.M. from three independent experiments (five to six replicates per experiment) performed in different cell preparations. $^{*}p < 0.05$, compared with the control group. Student–Newman–Keuls test.

RIA. CRH significantly inhibited the levels of phosphorylation of erk, jnk and c-jun (Fig. 6A), the mRNA expression of c-jun, c-fos and StAR (Fig. 6B and C), and the production of 3α-diol (Fig. 6C). In contrast, CRH did not affect testosterone production (Fig. 6C). Immunoblotting assays revealed no significant differences between the results obtained when actin was used as internal standard and the data achieved when the corresponding total non-phosphorylated MAP kinase was chosen for standardization (data not shown).

3.3. Quantification of intra-testicular levels of melatonin in Syrian hamsters

Table 1 summarizes the testicular weight (g), melatonin concentration (fmol/g tissue) and melatonin content (fmol/testis) in adult hamsters exposed to a LD photoperiod or a SD photoperiod for 16 weeks that were killed two hours after lights were turned on and two hours after lights were turned off. Under the same photoperiod, no significant differences were found between the groups

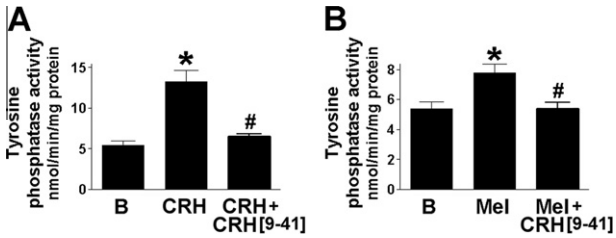

Fig. 5. The stimulatory effect of melatonin and CRH on tyrosine phosphatase activity in Leydig cells from reproductively active Syrian hamsters is reversed by the competitive CRH receptor antagonist α-helical CRH [9–41]. Hamster Leydig cells were incubated in medium 199 for 1 (data not shown) and 3 h in the presence or absence of CRH (1 and 10 μM) (A) or melatonin (Mel, 1 and 10 μM) (B), either with or without α-helical CRH [9–41] (10 μM). For each experiment, cells were isolated from six adult hamsters kept under a LD photoperiod (14 h light, 10 h darkness). Tyrosine phosphatase activity was determined by a commercial non-radioactive assay system. Bar plot graphs represent the mean ± S.E.M. from three independent experiments (five to six replicates per experiment) performed in different cell preparations. $^{*}p < 0.05$, compared with the control group. $^{\#}p < 0.05$, compared with the CRH- or melatonin-treated group. Student–Newman–Keuls test.

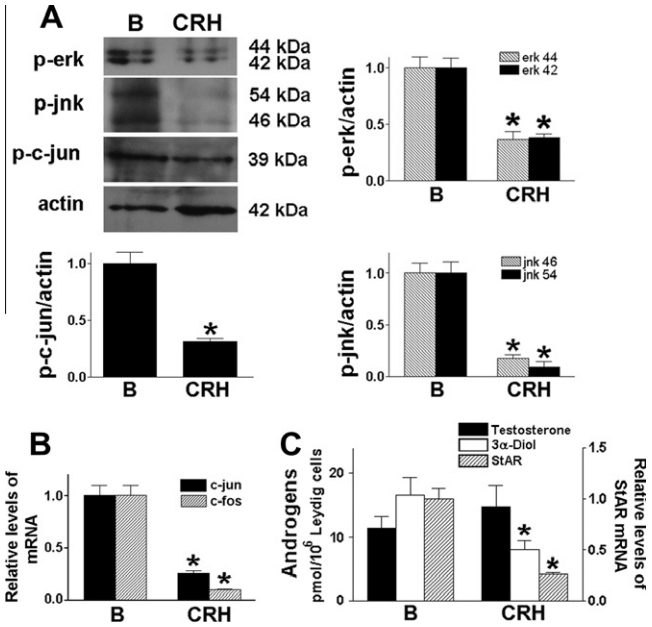

Fig. 6. The inhibitory effect of CRH on erk, jnk, and c-jun phosphorylation, c-jun, c-fos, and StAR expression, and *in vitro* 3α-diol production from Leydig cells of photoperiodically regressed Syrian hamsters. Hamster Leydig cells were incubated in medium 199 for 1 (data not shown) and 3 h in the presence or absence of CRH (1 and 10 μM). For each experiment, cells were isolated from four adult hamsters kept under a SD photoperiod for 16 weeks (6 h light, 18 h darkness). (A) Phospho-erk (p-erk, 42–44 KDa), phospho-jnk (p-jnk, 46–54 KDa), phospho-c-jun (p-c-jun, 39 KDa), and actin (42 KDa) protein levels were determined by immunoblotting. These representative immunoblots show the results obtained from one of three experiments performed in different cell preparations that showed similar results. Bar plot graphs represent the mean ± S.E.M. and show the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to actin. $^{*}p < 0.05$, Student's *t* test. Immunoblotting assays revealed no significant differences between the results obtained when actin was used as internal standard and the data achieved when the corresponding total non-phosphorylated MAP kinase was chosen for standardization. (B and C) C-jun, c-fos and StAR mRNA levels were determined by RT-qPCR. 18S rRNA was chosen as the housekeeping gene. The relative levels of c-jun, c-fos and StAR mRNA expression obtained in three independent experiments were determined as described in the Section 2. Bar plot graphs represent the mean ± S.E.M. $^{*}p < 0.05$, Student's *t* test. (C) Testosterone and 3α-diol production was determined by RIA. Bar plot graphs represent the mean ± S.E.M. from three independent experiments (five to six replicates per experiment) performed in different cell preparations. $^{*}p < 0.05$, Student's *t* test.

killed two hours after lights were turned on and two hours after lights were turned off (Table 1).

Testicular weight and melatonin content were significantly higher in reproductively active hamsters than those determined in regressed animals (Table 1). In contrast, testicular melatonin concentration was much higher in hamsters exposed to a SD photoperiod for 16 weeks than that detected in adult animals kept under a LD photoperiod (Table 1).

3.4. Identification of the main components of the melatonergic/CRH system in the human testis

To examine whether this newly identified pathway may be of relevance to human fertility disorders, we studied human testicular biopsies from men presenting SCO syndrome, and moderate and severe hypospermatogenesis. Melatonin testicular concentrations were determined using a commercial ELISA kit. No significant differences were found between the groups (Fig. 7A). By laser capture microdissection and RT-PCR, expression of CRH and specific receptors for melatonin and CRH (mel1a and CRH-R1) was detected in human Leydig cells isolated from the testis of patients revealing hypospermatogenesis or SCO syndrome (Fig. 7B).

4. Discussion

We have previously seen that, in the Syrian hamster, melatonin increases intracellular CRH concentration through mel1a receptors [14]. It has been described that Leydig cells secrete quantifiable levels of CRH to the incubation media [11]. Subsequently, CRH, via CRH-R1 receptors, down-regulates the expression of StAR and key steroidogenic enzymes (P450 side chain cleavage, 3β-HSD and 17β-HSD), leading to a significant decrease in the production of testosterone in hamster Leydig cells [14].

In the present study, we focused on the characterization of the signaling transduction pathway triggered by melatonin and CRH in the hamster testis.

To this end, we initially analyzed MAP kinases phosphorylation in Leydig cells from reproductively active hamsters incubated in the presence or absence of melatonin and CRH. The MAP kinases family includes three major members: erk, P38 and jnk.

Results showed that both melatonin and CRH reduce the phosphorylation levels of erk and jnk, which directly or indirectly play roles in the regulation of cell cycle, apoptosis, inflammation, cell differentiation and proliferation. A similar action of melatonin has been previously described in other experimental models [8,9,13,29]. It has also been reported that CRH inhibits jnk phosphorylation in the hippocampus [12], and that CRH/urocortin exerts a differential regulation of erk phosphorylation/dephosphorylation in cells expressing CRH-R1 or CRH-R2 receptors [25].

Whereas melatonin also reduced P38 phosphorylation, which is usually associated with cell differentiation and apoptosis, CRH had no effect. In agreement with our results, decreased levels of P38 phosphorylation after melatonin treatment have been observed in several tissues and cells [9,13,29,36,41,42,62].

It is worth noting that erk phosphorylates and regulates the transcription of c-fos, whereas c-jun is mainly activated by the jnk pathway. We therefore analyzed whether melatonin and CRH regulate c-jun and c-fos. Both hormones inhibited the expression of c-jun and c-fos and reduced the phosphorylation levels of c-jun in hamster Leydig cells. In principle, reduced c-jun phosphorylation could result from either modification of the activity of kinases/phosphatases or the lower c-jun mRNA expression detected in the presence of melatonin and CRH.

To our knowledge, there are no available commercial antibodies that specifically recognize phospho-c-fos in hamster tissues. We

Table 1
Melatonin concentration (fmol/g tissue) and content (fmol/testis) in testes of adult Syrian hamsters exposed to a long day (LD) photoperiod or a short day (SD) photoperiod for 16 weeks. Animals kept under a LD photoperiod were killed at 9:00 h (two hours after lights turned on) and 23:00 h (two hours after lights turned off). Hamsters exposed to a SD photoperiod for 16 weeks were killed at 11:00 h (two hours after lights turned on) and 17:00 h (two hours after lights turned off).

Photoperiod (h light: h darkness)	Time of sacrifice (h)	Melatonin (fmol/g tissue)	Melatonin (fmol/testis)	Testis weight (g)
LD 14: 10	9:00 (two hours after lights turned on)	8.21 ± 1.16[a]	13.58 ± 2.79[a]	1.66 ± 0.09[a]
LD 14: 10	23:00 (two hours after lights turned off)	6.81 ± 1.03[a]	12.09 ± 1.99[a]	1.77 ± 0.04[a]
SD 6: 18	11:00 (two hours after lights turned on)	22.61 ± 5.90[b]	5.91 ± 0.10[b]	0.20 ± 0.03[b]
SD 6: 18	17:00 (two hours after lights turned off)	28.67 ± 4.81[b]	6.09 ± 0.10[b]	0.23 ± 0.04[b]

The data represent the mean ± SEM (n = 5–6 animals per group).
Values within the same column with different superscripts are significantly different, $p < 0.05$, Student–Newman–Keuls test.

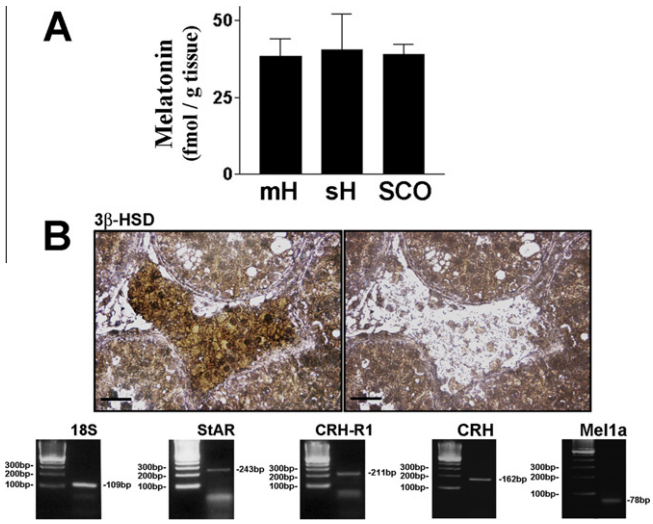

Fig. 7. Concentration of melatonin and expression of CRH, CRH-R1 receptors and mel1a receptors in human testes. (A) Melatonin concentrations in testicular biopsies from patients suffering moderate hypospermatogenesis (mH), severe hypospermatogenesis (sH) or SCO syndrome were determined by a commercial ELISA kit. Bar plot graphs represent the mean ± S.E.M (n = 5 testicular biopsies per group). (B) Using laser microdissection, 3β-HSD immunoreactive interstitial cells showing characteristic features of Leydig cells were isolated from the testis of a patient with severe hypospermatogenesis and subjected to RT-PCR studies. Expression of StAR, CRH, CRH-R1 receptors and mel1a receptors in these cells was detected. Bar, 40 μm. Similar results were obtained when biopsies from patient suffering moderate hypospermatogenesis or SCO syndrome were used (data not shown).

thus tested two different commercial antibodies (Santa Cruz Biotechnology Inc., catalog number sc52, and Oncogene Research Products, catalog number PC05), which are known to react with phospho-c-fos in several species including humans, rats and mice. However, these antibodies were not functional in hamster Leydig cells.

C-fos and c-jun are immediate early genes which are activated transiently and rapidly in response to a wide variety of cellular stimuli leading to the transcriptional regulation of a diverse range of genes. In this context, c-fos and c-jun have been proposed to mediate the long-term responses to some hormones and growth factors which Leydig cells are exposed to *in vivo* [22]. Furthermore, these immediate early genes have been recently linked to the molecular events involved in the transcription of the StAR gene [35].

To distinguish between melatonin- and CRH-initiated signaling events, MAP kinases phosphorylation, early immediate gene and StAR expression, and testosterone release to the incubation media were evaluated in the presence and absence of α-helical CRH [9–41], a highly selective CRH antagonist. Results revealed that melatonin-dependent dephosphorylation of erk, jnk and c-jun, down-regulation of c-fos, c-jun and StAR expression, as well as inhibition of testosterone production, are events related to the stimulatory role of melatonin on CRH production [14] and not a direct mechanism of this indolamine on MAP kinases, transcription factors and/or the steroidogenic pathway.

We also observed that U0126 and SP600125, specific inhibitors of MEK1/2 and jnk, respectively, markedly inhibited c-jun phosphorylation, c-fos, c-jun and StAR expression, and testosterone production. Taken together, these findings allow us to propose that the melatonin/CRH-dependent reduction of erk and jnk phosphorylation leads to the inhibition of c-fos and c-jun, and consequently, to a diminished synthesis of testosterone in Leydig cells of reproductively active Syrian hamsters.

Melatonin dephosphorylation of P38 was not prevented by the CRH antagonist. In consequence, it seems reasonable to assume that this hormone affects P38 by a CRH-independent mechanism.

Several reports indicate that P38 activation is involved in the induction of apoptotic events [28,30]. Although we did not investigate the pathway in detail, a pilot study demonstrated that melatonin treated Leydig cells purified from reproductively active hamsters expressed a significantly lower proapoptotic Bax/Bcl-2 ratio compared with the control group. Nevertheless, whether melatonin-induced dephosphorylation of P38 is associated with an anti-apoptotic role of this hormone in hamster Leydig cells should be further investigated.

On the other hand, melatonin induces CRH production in hamster Leydig cells through mel1a receptors [18], and unpublished data from our group indicates that SB203580, a specific inhibitor of P38, induces CRH mRNA expression in these cells. Therefore, the stimulatory effect of melatonin on CRH synthesis might occur via the P38 pathway. In this context, SB203580 has been shown to regulate CRH expression and the activity of the CRH promoter in other experimental models [26,60].

It has been extensively demonstrated that many cellular processes are regulated by a delicate balance between protein kinases and protein phosphatases [20]. In order to investigate whether protein phosphatases are responsible for the melatonin-triggered dephosphorylation of MAP kinases, we initially used sodium orthovanadate and okadaic acid, two well-known inhibitors of tyrosine and serine/threonine phosphatases, respectively. Considering that melatonin and CRH significantly decreased the phosphorylation levels of erk, jnk and c-jun in the presence of sodium orthovanadate, but that these hormones had no effect in the presence of okadaic acid, we hypothesized that melatonin/CRH could be involved in the activation of tyrosine phosphatases. Afterwards, we provided a direct demonstration for our assumption by determining tyrosine phosphatase activity. Both melatonin and CRH significantly stimulated the activity of tyrosine phosphatases in hamster Leydig cells. The effect of melatonin was prevented by the CRH antagonist. Thus, it is reasonable to assume that melatonin regulates the activity of tyrosine phosphatases indirectly through its previously described stimulatory action on CRH production [14]. Our results are in line with a previous report pointing out the

participation of tyrosine dephosphorylation in the melatonin-triggered sensitization of adenylate cyclase in pars tuberalis of sheep, a well-known seasonal breeder [2].

The duration of melatonin secretion from pineal gland to circulation is proportional to the duration of the night. In agreement with previous reports [21,46], unpublished data from our group suggest that circulating melatonin shows daily variations reaching peak levels (117.04 ± 13.88 pM, mean ± S.E.M.) at 23:00 h (two hours after lights are off). Then, circulating melatonin concentration begins to fall and reaches minimum levels (59.08 ± 12.63 pM, mean ± S.E.M.) at 9:00 h (two hours after lights are on).

In this study we determined, for the first time, the concentration and content of melatonin in testes of adult Syrian hamsters. In contrast to the circadian rhythm described in circulation, the levels of testicular melatonin at 9:00 h (two hours after the lights were on) were similar to those observed at 23:00 h (two hours after the lights were off). Thus, the levels of testicular melatonin do not seem to reflect the daily secretion of this hormone from the pineal gland.

Testicular melatonin content accompanied the decline observed on testis weight in hamsters exposed to a SD photoperiod for 16 weeks. Hamsters appear to produce significantly more melatonin during winter season under natural conditions, as well as during the artificial exposure to SD photoperiods [21,46]. Since the melatonin released from the pineal gland to the circulation is taken up by peripheral tissues including gonads [7,23,44–46,61], it could account for the 3–4-fold higher melatonin concentration observed in testes of photoperiodically regressed hamsters compared to those values detected in gonads of active animals. In addition, testes are also able to locally synthesize melatonin [19,27,55,56].

We have previously described that melatonin stimulates CRH production in Leydig cells from reproductively active hamsters. In contrast, CRH concentration in Leydig cells from regressed hamsters is unaffected by melatonin. CRH production in Leydig cells from reproductively inactive hamsters doubles that detected in untreated Leydig cells from active animals and is similar to that observed in melatonin-treated Leydig cells purified from adult hamsters kept under a LD photoperiod [14]. These data let us speculate that Leydig cells of regressed adult hamsters exposed to light deprivation, which implies an extended night melatonin secretion from pineal gland into circulation and an increased testicular melatonin concentration, are already challenged to produce a higher amount of CRH than those synthesized from Leydig cells of reproductively active hamsters.

The results of the present study showed that in Leydig cells purified from hamsters exposed to a SD photoperiod for 16 weeks, CRH does not affect the release of testosterone to the incubation media but inhibits the production of 3α-diol, the main androgen produced from testes of regressed hamsters [16], through the same signaling pathway as those previously described in cells of reproductive active animals.

Taken together, previous data [14] and the present results describe the existence of melatonin and CRH, and the expression of mel1a receptors and CRH-R1 receptors in hamster Leydig cells [14]. Melatonin locally stimulates CRH production in hamster testes. Thus, activation of tyrosine phosphatases by CRH, leads to reduced phosphorylation levels of erk and jnk, and subsequently to down-regulation of c-jun, c-fos and StAR expression, and inhibition of testosterone production.

In most target cells, activation of CRH-R1 receptors by CRH leads to stimulation of the Gs-protein/adenylyl cyclase/protein kinase A cascade. Signal transduction of CRH-R1 also involves MAP kinases pathways that play an essential role in CRH action. In this context, CRH differentially regulates cell growth and apoptosis by modulating MAP kinases activity in pituitary corticotrophs and the hippocampus [32,59,63]. It has been described that CRH-induced erk and p38 phosphorylation leads to release of the adrenocorticotropic hormone (ACTH) in corticotrophs [5,58]. CRH induces erk phophorylation in hippocampal CA1-CA3 areas and in the basolateral amygdalar complex, structures that are known to be involved in learning and memory, stress-related behaviors and sensory processing of multimodal stimuli [5]. In contrast, CRH inhibits glutamate-mediated jnk phosphorylation exerting a neuroprotective effect in the hippocampus [12]. Although CRH is the key regulator of the hypothalamic–pituitary–adrenal axis, this hormone also modulates the inflammatory response directly, via its effect on mast cells and macrophages. In this regard, CRH induces VEGF release in human mast cells via selective activation of the cAMP/protein kinase A/P38 signaling pathway [6], and suppresses tumour necrosis factor α (TNFα) secretion in mouse macrophages through a P38-dependent induction of cyclooxygenase 2 (COX2) [57].

Having in mind the above-mentioned reports, and the inhibitory role exerted by CRH on testicular erk and jnk phosphorylation previously described in this study, we could conjecture about a plastic modulation of MAP kinases activity by CRH, which could account for the differential effects observed in different cell types. Thus, specific CRH signaling pathways could be restricted to specific cell populations. In addition, different kinetics of MAP kinases activation might take place in response to CRH depending of the tissue and the cellular microenvironment.

To our knowledge, this is the first time that melatonin concentration is quantified in human testicular biopsies of infertile men. Laser microdissection technique followed by RT-PCR enabled us to detect the expression of CRH as well as of mel1a and CRH-R1 receptors in Leydig cells of men with impaired spermatogenesis or SCO syndrome. SCO syndrome was first described by Del Castillo et al. [10]. According to the original description it has been used when no spermatogenic cells have been seen in a testicular biopsy specimen. In contrast, if spermatogenic cells, spermatogonia, spermatocytes, spermatids or spermatozoa are found in the seminiferous tubules, although in minimal numbers, the state is not SCO, but hypospermatogenesis or germ cell arrest [50].

Thus, all components identified to be involved in melatonin/CRH-induced down-regulation of androgen production in hamster Leydig cells are also present in testes of infertile men. Nevertheless, Leydig cells are not the unique target for melatonin in the testis. Unpublished data from our group demonstrate the existence of mel1a receptor in immune cells (macrophages and mast cells) in testicular biopsies from patients with hypospermatogenesis and SCO. Thus, melatonin might participate not only in the regulation of steroidogenesis but also in the modulation of inflammatory processes in the human testis.

These data allow us to conjecture about the potential relevance of melatonin and/or CRH in some fertility disorders. Drugs targeting melatonin, CRH, and their receptors are being developed or are already in clinical use for a variety of conditions. For instance, antipsychotic drugs that inhibit the human CRH promoter are available [4], and exogenous melatonin is commonly prescribed for treatment of sleep disturbances [48]. The impact of these drugs on male (in)fertility and/or their future as potential therapeutic targets should be considered. Nevertheless, further investigations are required to determine the role of melatonin and CRH in the human testes in its proper perspective and establish whether our results in hamsters can be extended to non-seasonal reproductive species including humans.

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