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Direct Effect of Melatonin on Syrian Hamster Testes: Melatonin Subtype 1a Receptors, Inhibition of Androgen Production, and Interaction with the Local Corticotropin-Releasing Hormone System

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Besides the hypothalamus and pituitary, melatonin action at the testicular level has been recently suggested. Therefore, we investigated in the Syrian hamster, a well-characterized seasonal breeder, melatonin action on Leydig cells, testicular expression of melatonergic receptors, and possible interactions between melatonin receptors and the previously identified testicular serotoninergic and CRH systems. In isolated Leydig cells from active testes of adult hamsters kept in a long-day (14 h light, 10 h dark) photoperiod and from regressed testes of adult animals exposed to a short-day photoperiod during 16 wk (6 h light, 18 h dark), melatonin significantly reduced human chorionic gonadotropin-stimulated production of cAMP and the main androgens: testosterone and androstane-3α,17β-diol, respectively, and decreased the expression of steroidogenic acute regulatory protein, P450 side chain cleavage, 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase. In Leydig cells exposed to a short-day photoperiod during 16 wk, melatonin stimulated the conversion of testosterone into 5α-reduced androgens by inducing 5α-reductase isoform 1, and controlled androstane-3α,17β-diol production by inhibiting 3α-hydroxysteroid dehydrogenase expression. Melatonin subtype (mel1a) receptors were detected in Leydig cells. Although the local serotonin system did not mediate melatonin action on androgen production, melatonergic effect on steroidogenesis involved the interaction between mel1a receptors and the inhibitory CRH system. Moreover, melatonin significantly increased CRH mRNA levels and production in hamster Leydig cells expressing CRH subtype 1 receptors. Our studies indicate that melatonin may act as a local inhibitor of human chorionic gonadotropin-stimulated cAMP and androgen production through mel1a receptors, down-regulation of steroidogenic acute regulatory protein, and key steroidogenic enzymes expression and its interaction with the local CRH system. (*Endocrinology* 146: 1541–1552, 2005)

THE PINEAL HORMONE melatonin plays a key role mediating the influence of photoperiod on the reproduction of many mammalian species. The duration of daily melatonin secretion is negatively correlated with the time the seasonal animal is exposed to daylight. Light signals are received by the photoreceptors of the retina. Therefore, the neural information is transferred from the eyes to the pineal gland through a circuitous connection of neurons involving retinohypothalamic fibers, the suprachiasmatic nuclei, hypothalamus-pineal fibers, and the peripheral sympathetic nervous system, resulting in the suppression of melatonin synthesis (1–3). Interruption of the neural pathways anywhere between the suprachiasmatic nuclei and the pineal turns this gland nonfunctional in terms of its capacity to alter the reproductive system (2, 4). Melatonin exerts its effects via specific receptors. Melatonin receptors, which are coupled to G protein and characterized by a seven transmembrane-spanning domain, were initially classified as ML1 and ML2 subtypes by Dubocovich *et al.* (5), based on pharmacological and kinetic differences. Recent cloning studies identified three subtypes of ML1 receptors: melatonin subtype (mel)1a receptors (also known as ML_{1A} or mt_1), which are expressed in the suprachiasmatic nucleus of the hypothalamus and pars tuberalis of the pituitary (6, 7); mel1b receptors (also known as ML_{1B} or MT_2), which are expressed in brain and retina (5, 8); and mel1c receptors, which are not expressed in mammals but were found in *Xenopus*, chicken, and Zebra fish (9).

Melatonin is a transducer of the photoperiodic information that, acting mainly at the level of the brain and pituitary, exerts gonadotropic effects. Whether the day-

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Abbreviations: CRH-R1, CRH subtype 1 receptor; DHT, dihydrotestosterone; 3α-Diol, androstane-3α,17β-diol; EIA, enzyme immunoassay; hCG, human chorionic gonadotropin; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 5HT, 5-hydroxytryptamine; LD, long day; mel receptor, melatonin subtype receptor; *p*-MPPI, 4-iodo-*N*-[2-[4-(methoxyphenyl)-1-piperazinyl] ethyl]-*N*-2-pyridinyl-benzamide hydrochloride; P450scc, P450 side chain cleavage; 5α-R1, 5α-reductase isoform 1; 16SD, 16 wk in short day; StAR, steroidogenic acute regulatory.

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light signal is interpreted as anti- or progonadotropic will depend on the species (long-day seasonal breeders including the hamsters, short-day seasonal breeders such as sheep, or nonseasonal breeders like humans), the duration of night melatonin peak (the duration hypothesis), the magnitude of the night melatonin peak (the amplitude hypothesis), and/or the window of sensitivity to melatonin (the internal coincidence hypothesis) (10–13). The internal coincidence model is of interest because it has been proposed to explain a significant aspect of the seasonal reproductive cycle in the Syrian hamster. After long periods of short daylight, gonads of the Syrian hamster undergo spontaneous or endogenous recrudescence either in the total absence of light or under short-day conditions. Even when circulating levels of melatonin are high, the neuroendocrine-reproductive system merely ignores the message, perhaps as a consequence of the lack of coincidence between the high levels of melatonin and the melatonin sensitivity period of the reproductive system (13).

Thus, through the hypothalamic suprachiasmatic nucleus and the pars tuberalis, melatonin influences the synthesis and release of the hypothalamic GnRH and the adenohypophyseal gonadotropin hormones. Nevertheless, it has also been described that newly synthesized melatonin, which is released from the pineal gland into the circulation almost entirely at night, reaches peripheral tissues including the testes (13–15). New evidence suggests the existence of a local synthesis of melatonin in testes (16–19). In this context, it is important to mention previous studies describing melatonin action at gonadal level including the regulation of testicular growth, cAMP production, and testosterone production (20–22). In addition, 2-[^{125}I]iodomelatonin binding sites have been detected in rat and avian testes (23–25).

The Syrian hamster (*Mesocricetus auratus*) is a thoroughly studied seasonal breeder and, consequently, a useful model for investigating the role played by melatonin in the regulation of the reproductive function (26). In the present study, adult Syrian hamsters were either maintained in a long-day (LD, 14 h light, 10 h dark) photoperiod or exposed to a short-day photoperiod for 16 wk (16SD, 6 h light, 18 h dark) to reach the maximal testicular regression. This stage is accompanied by a marked decrease in serum levels of FSH, LH, and prolactin and a decline in blood and gonadal concentrations of testosterone, its hormonal precursors, and its metabolites (26–29). The specific aims of this study were: 1) to examine the role of melatonin on *in vitro* cAMP and androgen production from purified Leydig cells; 2) to analyze the action of melatonin on the androgen biosynthetic pathway, namely steroidogenic enzymes gene expression; and 3) to identify the presence of testicular melatonin receptors subtypes (mel1a, mel1b) in both LD and 16SD hamster testes.

Because interactions between the testicular serotonergic and CRH systems leading to the regulation of androgen production have been previously described (30–32), we proposed to examine whether the melatonergic system interacts with the serotonergic and/or CRH systems in hamster Leydig cells and evaluate their participation in the control of the testicular function.

Materials and Methods

Animals

Male Syrian hamsters (*M. auratus*) were raised in our animal care unit (Charles River descendants, Animal Care Laboratory, Instituto de Biología y Medicina Experimental, Buenos Aires). Hamsters were kept in rooms at 23 ± 2 C and maintained from birth in LD (14 h light, 10 h dark; lights on 0700–2100 h) photoperiod. Animals had free access to water and Purina formula chow. For the present study, several groups of 12 adult hamsters (90–100 d) were kept under LD conditions for 16 wk or transferred to a SD photoperiod (6 h light, 18 h dark; lights on 0900–1500 h) for 16 wk. It is important to mention that hamsters from our colony reach the maximal gonadal regression after 16 wk of SD photoperiod [see additional information in Frungieri *et al.* (29)]. Then hamsters were killed by asphyxia with carbon dioxide (CO_2) according to protocols for animal use, approved by the Institutional Animal Care and Use Committee (Instituto de Biología y Medicina Experimental-Consejo Nacional de Investigaciones Científicas y Técnicas) following National Institutes of Health guidelines. At the time of killing, testicular weight and testosterone circulating levels were determined as follows (means ± SEM): testicular weight = 1.75 ± 0.16 g and 0.16 ± 0.09 g (n = 24), testosterone levels = 20.49 ± 0.94 pmol/ml, and 3.26 ± 0.69 pmol/ml (n = 24) for LD and 16SD hamsters, respectively.

For RT-PCR and Western blotting studies, testes were rapidly removed and frozen at −80 C until assays were performed.

In other groups of animals, testes were dissected and used for Leydig cells purification. Then Leydig cells were employed for *in vitro* incubations and subsequent determination of mRNA (by RT-PCR) and protein (Western blotting) expression for measurement of intracellular CRH concentration by enzyme immunoassay (EIA) or quantification of cAMP and androgens levels in the incubation media by RIA.

Hamster Leydig cells purification and in vitro incubations

In all experiments, Leydig cells were isolated from a pool of 24 testes obtained from 12 adult hamsters (90–100 d of age) maintained in LD photoperiod for 16 wk or exposed to a 16SD. Leydig cells were isolated under sterile conditions using a discontinuous Percoll density gradient as previously described by Frungieri *et al.* (32). 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 cells preparations was 97.5–98.5%. To evaluate enrichment in Leydig cells, the activity of 3β-hydroxysteroid dehydrogenase was measured as previously described Levy *et al.* (33). Cell preparations were 85–90% enriched with hamster Leydig cells. Petri dishes with 1.5 ml medium 199 containing 2.5×10^5 cells (for RT-PCR, *in vitro* cAMP and androgen production) or 3.0×10^6 cells (for determination of CRH concentration) were incubated at 37 C under a humid atmosphere of 5% CO_2 in presence of 0.1 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor (Sigma Chemical, St. Louis, MO) and in presence or absence of the following chemicals: 100 mIU/ml human chorionic gonadotropin (hCG) (Ayerst, Princeton, NJ; specific activity, 59 IU/mg), 10 pM to 1 μM melatonin (Sigma), 1 μM luzindole (Sigma), 1 μM CRH (Sigma), 1 μM CRH receptor antagonist (α-helical CRH-[9–41]; Peninsula Laboratories, Belmont, CA), 1 μM (±)1-[2,5-dimethoxy-4-iodophyryl]-2-amino propane hydrochloride (Biochemicals, Inc., Wayland, MA), 1 μM ketanserin tartrate (Biochemicals), 1 μM metoclopramide hydrochloride (Biochemicals), 1 μM 4-iodo-*N*-[2-[4-(methoxyphenyl)-1-piperazinyl] ethyl]-*N*-2-pyridinyl-benzamide hydrochloride (*p*-MPPI; Biochemicals), and 1 μM (±) 8-hydroxy-2(D1-*N*-propylamino) tetralin hydrobromide (Biochemicals).

Luzindole stock solution was prepared in ethanol and consequently, for those experiments in which luzindole effect was tested, control incubations received the same vehicle (ethanol) as treated cells. Other chemicals listed above were dissolved in medium 199. After 30 min (for semiquantitative RT-PCR assays) or 3 h incubation (for determination of cAMP, androgens, and CRH levels), cells in the incubation media were transferred to tubes and centrifuged at 1200 × *g* for 10 min. No evidence of morphological changes was detected when microscopic studies of cellular morphology were performed in hamster Leydig cells after incubation with the drugs previously mentioned. Cells were used for RNA extraction and RT-PCR or for CRH quantification by EIA. Media were

frozen at −20 C until androgens concentrations and cAMP levels were determined by RIA.

RT-PCR analysis

RNA was extracted from total testicular tissue or isolated Leydig cells from adult hamsters kept under LD conditions for 16 wk or exposed to a 16SD photoperiod using the Purescript kit (Biozym, Hessisch Oldenburg, Germany). Then reverse transcription reaction using oligo dT_{15} primers followed by PCR amplification was performed (34). Commercial cDNAs from reverse transcribed human testicular mRNA (pooled from 19 men; Clontech Inc., Palo Alto, CA; one sample from Invitrogen, Karlsruhe, Germany) were also used for PCR amplification. Information about oligonucleotide primers employed and cDNAs isolated are given in Table 1. When information about exon structure was available at GenBank, oligonucleotide primers were designed as homologous to regions of different exons: steroidogenic acute regulatory (StAR) protein, P450 side-chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), 5α-reductase isoform 1 (5α-R1), α-tubulin, CRH, CRH subtype 1 receptor (CRH-R1) genes.

PCR products were separated on 2% agarose gels and visualized with ethidium bromide. The identity of PCR products was checked by sequencing, using a fluorescence-based dideoxy-sequencing reaction and an automated sequence analysis on an ABI model 373A DNA sequencer (Agowa GmbH, Berlin, Germany).

Commercial cDNA from reverse transcribed human brain mRNA (Clontech), hamster brain tissue, rat pituitary tissue, and rat GH adenoma cell line GH3 (35) were also employed as positive controls.

Androgens assay

Determinations of testosterone and androstane-3α,17β-diol (3α-Diol) levels in the incubation media were carried out by RIA without extraction using antibodies obtained from Immunotech Diagnostic (Montreal, Canada). A specific antibody to 3α-diol-15-CMO-BSA showing 5% cross-reaction with testosterone was used according to the method described by Frungieri *et al.* (36). Testosterone was measured using an antibody to testosterone-7α-butyrate-BSA that is known to have 35% cross-reactivity with dihydrotestosterone (DHT). The minimum detectable assay concentrations were 0.215 pmol/ml for testosterone and 0.105 pmol/ml for 3α-Diol. Intraassay coefficient of variation was less than 12% for testosterone and less than 15% for 3α-Diol, and interassay coefficient of variation was less than 15% for each of these two steroids.

In vitro testosterone and 3α-Diol production from Leydig cells have been expressed in terms of picomoles per 10^6 Leydig cells.

cAMP assay

Determinations of cAMP levels in the incubation media were carried out by RIA according to the method described by Del Punta *et al.* (37) and validated by Frungieri *et al.* (32). 2′-*O*-Monosuccinyladenosine-3′,5′-cyclic monophosphate tyrosyl methyl ester (TME-cAMP) was purchased from Sigma. TME-cAMP was radiolabeled with $Na^{125}I$ (DuPont NEN Life Science Products, Boston, MA) in our laboratory by the method of chloramine T (specific activity, 600 Ci/mmol) described by Birnbaumer (38). Specific antibody for cAMP was a gift from Dr. A. F. Parlow and the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD).

Samples and standards were acetylated before the assay to increase sensitivity of detection (39). Under these conditions, the standard curve was linear between 0.125 and 20 pmol/ml. Intra- and interassay coefficients of variation were less than 8% and less than 10%, respectively.

In vitro cAMP production from Leydig cells has been expressed as picomoles per 10^6 Leydig cells.

Immunoblotting

Cells and tissues were homogenized in 62.5 mM Tris-HCl buffer (pH 6.8) containing 10% sucrose and 2% sodium dodecyl sulfate by sonication. Samples were heated (95 C for 5 min) under reducing conditions (10% mercaptoethanol), loaded on tricine-sodium dodecyl sulfate-polyacrylamide gels (12%), electrophoretically separated, and blotted onto nitrocellulose (34). Blots were incubated with rabbit antihuman mel1a receptor antibody (CIDtech Research Inc., Mississauga, Canada, 1:100, overnight) in presence or absence of a specific blocking peptide (CIDtech Research, 1:200, overnight), and subsequently with peroxidase-labeled secondary antibody (donkey antirabbit IgG, 1:1000, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Signals were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

CRH assay

Determinations of intracellular CRH levels were carried out by a commercial EIA (Peninsula Laboratories) after concentration and extraction through C18 Sep-columns (Peninsula Laboratories). Approximately 3.0×10^6 Leydig cells were incubated in presence or absence of 1 μM melatonin for 3 h, centrifuged at $1,200 \times g$ for 10 min, suspended in 10 mM PBS (pH 7.4) containing 2.6 mM EDTA and 10 μg/ml aprotinin, sonicated, and centrifuged at $105,000 \times g$ for 1 h. Supernatants were injected into a 200-mg C18 column and eluted with 60% acetonitrile in 1% trifluoroacetic acid. Eluted fractions were evaporated to dryness in

TABLE 1. Oligonucleotide primers employed for PCR amplification of cDNAs obtained after reverse transcription (RT) from total testicular tissue or isolated Leydig cells of adult hamsters

Target		Sense primers (5′–3′)	Antisense primers (5′–3′)	Expected cDNA length (bp)	Annealing temperature (C)
StAR		GAGTGGAACCCCAATGTC	GCACCATGCAAGTGGGAC	243	56
P450scc		GAGTCCCAGCGGTTCAT	CCTCCTGCCAGCATCTC	291	54
3β-HSD		TCAATGTGAAAGGTACCC	ATCATAGCTTTGGTGAGG	499	51
17β-HSD		CAATGGGACAATGGGCAG	GTGTCATCTTGACTACGG	359	54
5α-R1		CAGGAGCTGCCCTCGATG	CCTGGTTTTCTCAGATTCC	361	56
3α-HSD		AGGCCATGGAGAAGTGTAA	CACGCTGCAGCTGGTAG	344	54
α-Tubulin	First set	GGCAAGGAGATCATTGAC	TCTCAGGGAAGCAGTGAT	398	54
	Second nested set	GTCTCCAGGGCTTCTTG	GTCTACCATGAAGGCACA	223	54
mel1a	First set	GATGGAATCTGGGATATC	CCACGAACAGCCACTCTG	561	55
	Second nested set	CTGCTACATTTGTCACAG	ACCACCACTGCTATCGTG	201	55
mel1b	First set	GCCTCATCTGGCTCCTCA	GCAGCTGTTGAAGTAAGC	422	55
	Second nested set	TATTCCTGCACCTTCATC	CGGGCCTGGAGCACCA	131	55
CRH	First set	AGAAACTCAGAGCCCAAG	CATCAGTTTCCTGTTGCTG	720	54
	Second hemi-nested set	GAATACTTCCTCCGCCTG	CATCAGTTTCCTGTTGCTG	362	54
CRH-R1	First set	ATCCACTGGAACCTCATCTC	GAAAGAGTTGAAGTAGATGAA	590	58
	Second hemi-nested set	GTTGGTGACAGCTGCCTA	GAAAGAGTTGAAGTAGATGAA	483	56

GenBank accession numbers: StAR, U66490; P450scc, AF323965; 3β-HSD, L38710; 17β-HSD, AY426534; 5α-R1, NM_175283, J05035, and BT006834; 3α-HSD, NM_003739 and D17310; α-tubulin, M12252; mel1a, AF061158 and NM_005958; mel1b, U57555, NM_005959, and AF141863; CRH, NM_031019 and NM_000756; CRH-R1, AY034599 and X72304.

a centrifugal concentrator and then dried extracted were stored at −20 C until CRH quantification. The minimum detectable concentration in the EIA was 168.2 fmol/ml. Intra- and interassay coefficients of variation were less than 8% and less than 11%, respectively. Intracellular CRH concentrations were expressed as femtomoles per 10^6 Leydig cells.

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Student-Newman-Keuls test or Kruskal-Wallis test for multiple comparisons. Data are expressed as the mean ± SEM from three independent experiments (three to six replicates per experiment) performed on different cell preparations. For semiquantitative RT-PCR studies, bands were quantified by densitometry and normalized to α-tubulin housekeeping gene using IMAGE (Scion Corp., Frederick, MD).

Results

Inhibitory effect of melatonin on in vitro production of androgens from freshly isolated hamster Leydig cells

After a 3-h incubation, hCG-stimulated (100 mIU/ml) *in vitro* production of testosterone from LD-Leydig cells was significantly inhibited by melatonin within a range of 10 pM and 1 μM (Fig. 1A). Whereas, in 16SD-Leydig cells, testosterone production in presence of hCG into the incubation media remained unaffected by melatonin (Fig. 1B), hCG-stimulated 3α-Diol production was inhibited by concentrations of melatonin between 100 pM and 1 μM (Fig. 1C).

Basal testosterone production in LD-Leydig cells showed a dose-dependent inhibition by melatonin (Fig. 1A). This hormone did not alter the production of testosterone and 3α-Diol from 16SD-Leydig cells in absence of hCG in the incubation media (Fig. 1, B and C).

Inhibitory effect of melatonin on gene expression of StAR and steroidogenic enzymes in freshly isolated hamster Leydig cells

The addition of hCG (100 mIU/ml) to both LD and 16SD Leydig cells resulted in an increased expression of StAR, P450scc, 3β-HSD, and 17β-HSD (Fig. 2).

In hCG-stimulated LD and 16SD Leydig cells, addition of 1 μM melatonin significantly reduced mRNA levels of StAR, P450scc, 3β-HSD, and 17β-HSD (Fig. 2). Novel sequence information about 17β-HSD obtained from the analysis of hamster Leydig cells (representing five independently derived identical sequences) was submitted to GenBank (accession no. AY426534). This partial sequence shows 91.3% homology with human and 88.9% homology with rat and mouse 17β-HSD at the nucleotide level.

hCG (100 mIU/ml) induced 5α-R1 expression in LD Leydig cells (Fig. 2, *left panel*), but it did not affect 5α-R1 expression in 16SD Leydig cells (Fig. 2, *right panel*). Moreover, melatonin markedly inhibited the expression of 5α-R1 in hCG-stimulated LD Leydig cells (Fig. 2, *left panel*), but it significantly induced 5α-R1 expression in hCG-stimulated 16SD Leydig cells (Fig. 2, *right panel*).

3α-HSD showed a weak expression in LD Leydig cells (Fig. 2, *left panel*). Nevertheless, in 16SD Leydig cells, 3α-HSD expression was clearly detected in basal conditions and, moreover, induced by hCG (Fig. 2, *right panel*). In addition, expression of 3α-HSD in hCG-stimulated 16SD Leydig cells was significantly reduced when melatonin was added to the incubation media (Fig. 2, *right panel*).

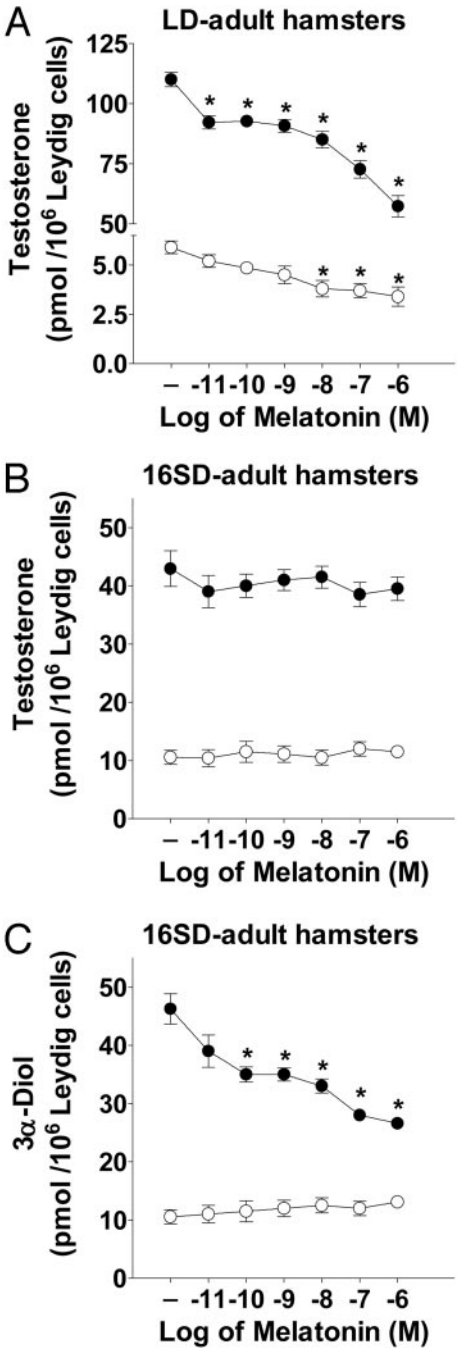

FIG. 1. Inhibitory effect of melatonin on *in vitro* production of androgens from freshly isolated hamster Leydig cells. Effect of different concentrations (10 pM to 1 μM) of melatonin on basal (*open circles*) and maximally hCG-stimulated (*closed circles*, 100 mIU/ml) testosterone (A and B) and 3α-Diol (C) production in Leydig cells isolated from adult hamsters kept in a LD (14 h light, 10 h dark) photoperiod (A) and in Leydig cells isolated from adult hamsters exposed to 16SD (6 h light, 18 h dark) (B and C). Incubation time was 3 h. In line plot graphics, the mean ± SEM from three independent experiments (five to six replicates per experiment) performed on different cell preparations are shown. For each experiment, Leydig cells were isolated from a pool of 24 testes obtained from 12 adult hamsters. Data were analyzed using ANOVA followed by the Student-Newman-Keuls test. Where *SEM lines* are not present, SEM was encompassed by the area of the *symbol*. *, $P < 0.05$, compared with the control group.

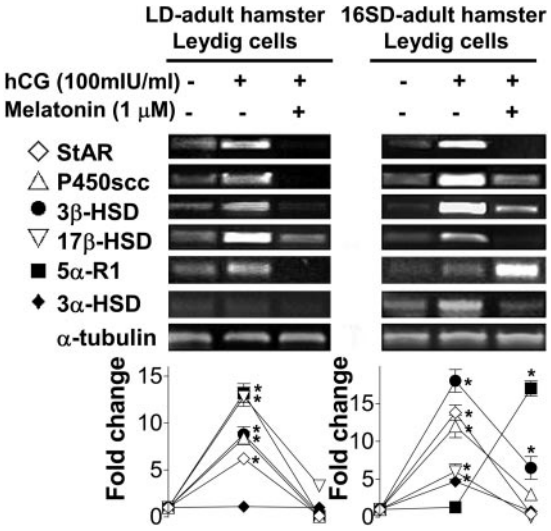

FIG. 2. Inhibitory effect of melatonin on gene expression of StAR and steroidogenic enzymes in freshly isolated hamster Leydig cells. Ethidium bromide-stained agarose gels showing cDNA fragments that, after sequencing, were shown to correspond to the testicular StAR protein, P450scc, 3β-HSD, 17β-HSD, 5α-R1, 3α-HSD, and α-tubulin, in Leydig cells isolated from adult hamsters kept in LD (14 h light, 10 h dark) photoperiod (*left panel*) or exposed to 16SD (6 h light, 18 h dark) (*right panel*). Incubation time was 30 min. These representative ethidium bromide-stained agarose gels show results obtained from one of the three experiments that yielded comparable results. In all experiments, PCR bands were quantified by densitometry. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to α-tubulin gene. Line plot graphics show the mean ± SEM from three independent experiments (three replicates per experiment) performed on different cell preparations. For each experiment, Leydig cells were isolated from a pool of 24 testes obtained from 12 adult hamsters. Data were analyzed using ANOVA followed by the Student-Newman-Keuls test. Where *SEM lines* are not present, SEM was encompassed by the area of the *symbol*. *, $P < 0.05$, compared with the control group.

The inhibitory action of melatonin on in vitro production of cAMP and androgens from freshly isolated hamster Leydig cells is reverted by luzindole, a melatonin receptor antagonist

In LD Leydig cells treated with 1 μM melatonin, basal and hCG-stimulated testosterone productions were decreased in 42.5% (picomoles per 10^6 Leydig cells, untreated: 5.97 ± 0.71 *vs.* melatonin treated: 3.43 ± 0.49) and 48.5% (picomoles per 10^6 Leydig cells, untreated: 106.73 ± 7.82 *vs.* melatonin treated: 55.03 ± 1.78), respectively ($P < 0.05$, Fig. 3A). Both basal and maximal hCG-stimulated cAMP productions from LD Leydig cells in presence of melatonin into the incubation media, were significantly diminished in 45.6% (picomoles per 10^6 Leydig cells, untreated: 16.13 ± 1.23 *vs.* melatonin treated: 8.77 ± 0.74) and 44.6% (picomoles per 10^6 Leydig cells, untreated: 31.95 ± 1.61 *vs.* melatonin treated: 17.70 ± 1.29), respectively ($P < 0.05$, Fig. 3B).

Luzindole (1 μM), a mel1a/1b receptor antagonist, reverted the inhibitory action of melatonin on testosterone and cAMP production in LD Leydig cells (Fig. 3, A and B, respectively). Nevertheless, in absence of melatonin in the incubation media, luzindole did not affect testosterone and cAMP production (Fig. 3, A and B, respectively).

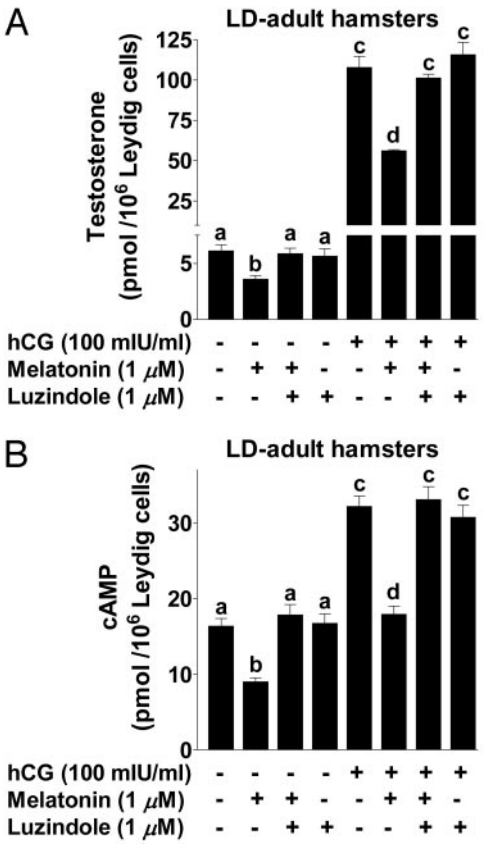

FIG. 3. Luzindole, a melatonin receptor antagonist, reverts the inhibitory action of melatonin on *in vitro* production of testosterone and cAMP in Leydig cells isolated from hamsters kept in LD photoperiod. Effects of melatonin (1 μM) and luzindole (1 μM) on basal and maximally hCG-stimulated (100 mIU/ml) testosterone (A) and cAMP (B) production in Leydig cells isolated from adult hamsters kept in a LD (14 h light, 10 h dark) photoperiod. Incubation time was 3 h. Bar plot graphics show the mean ± SEM from three independent experiments (five to six replicates per experiment) performed on different cell preparations. For each experiment, Leydig cells were isolated from a pool of 24 testes obtained from 12 adult hamsters. Data were analyzed using ANOVA followed by the Student-Newman-Keuls test. Where *SEM lines* are not present, SEM was encompassed by the area of the *bar*. All groups were compared; *different letters above the bars* denote a statistically significant difference between the groups ($P < 0.05$).

In 16SD Leydig cells treated with melatonin, basal and hCGstimulated testosterone production remained unchanged (Fig. 4A). Moreover, melatonin did not affect the levels of 3α-Diol and cAMP secreted to the media in basal conditions (Fig. 4, B and C, respectively). In contrast, under maximal hCG stimulation, melatonin decreased 3α-Diol and cAMP productions from 16SD Leydig cells in 64.5% (picomoles per 10^6 Leydig cells, untreated: 46.26 ± 2.61 *vs.* melatonin treated: 26.60 ± 0.99) and 42.5% (picomoles per 10^6 Leydig cells, untreated: 163.10 ± 1.37 *vs.* melatonin treated: 58.32 ± 2.09), respectively ($P < 0.05$, Fig. 4, B and C, respectively). The inhibitory action of melatonin on hCG-stimulated cAMP and 3α-Diol production in 16SD Leydig cells was blocked by 1 μM luzindole (Fig. 4, B and C, respectively). In absence of melatonin into the incubation media, luzindole did not affect androgen and cAMP productions (Fig. 4, A–C).

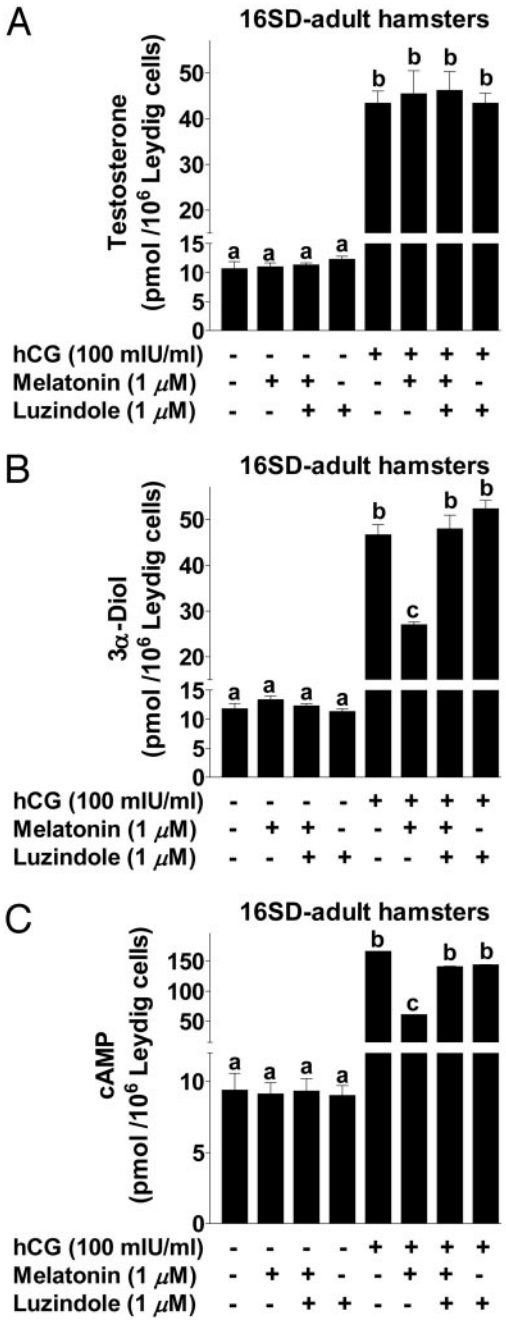

FIG. 4. Luzindole, a melatonin receptor antagonist, reverts the inhibitory action of melatonin on *in vitro* production of 3α-Diol and cAMP in Leydig cells isolated from hamsters exposed to 16SD. Effects of melatonin (1 μM) and luzindole (1 μM) on basal and maximally hCG-stimulated (100 mIU/ml) testosterone (A), 3α-Diol (B), and cAMP (C) production in Leydig cells isolated from adult hamsters exposed to 16SD (8 h light, 16 h dark). Incubation time was 3 h. Bar plot graphics show the mean ± SEM from three independent experiments (five to six replicates per experiment) performed on different cell preparations. For each experiment, Leydig cells were isolated from a pool of 24 testes obtained from 12 adult hamsters. Data were analyzed using ANOVA followed by the Student-Newman-Keuls test. Where *SEM lines* are not present, SEM was encompassed by the area of the *bar*. All groups were compared; *different letters above the bars* denote a statistically significant difference between the groups ($P < 0.05$).

Identification of mel1a in testes by RT-PCR and immunohistochemistry

Using RT-PCR, we amplified cDNA fragments (201 bp) that, after sequencing, were shown to correspond to mel1a receptors from the rat GH adenoma cell line GH3 (positive control), a commercial human testicular cDNA, LD adult hamster testes, 16SD adult hamster testes, Leydig cells isolated from adult animals maintained under LD conditions, and Leydig cells obtained from adult hamsters exposed to 16SD (Fig. 5A).

Mel1b receptors were expressed in all positive control samples employed, namely human cDNAs from brain, adult hamster brain, and, after a second PCR using nested primers, also in GH3 cells (Fig. 5B). However, we did not amplify cDNAs corresponding to mel1b receptors either in the first or second PCR using nested primers in human cDNAs from testes, LD hamster testes, 16SD hamster testes, LD hamster Leydig cells, or 16SD hamster Leydig cells (Fig. 5B).

When Western blotting analyses using an anti-mel1a antibody were performed, expected immunoreactive bands at approximately 37 kDa were seen in rat adult pituitary (positive control) and purified LD as well as 16SD hamster Leydig cells (Fig. 5C). The identity of these 37-kDa bands was indicated by preadsorption of the antibody with a specific blocking peptide (Fig. 5C).

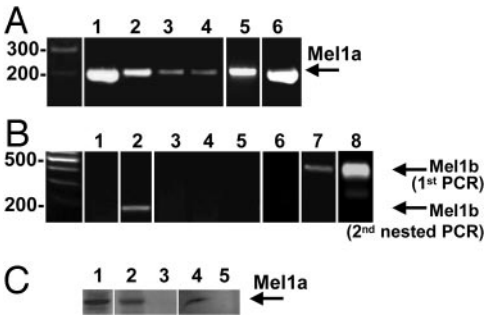

FIG. 5. RT-PCR and Western blot identification of mel1a in testes. Gene expression analysis of mel1a (A) and mel1b (B) receptor subtypes in testes. A, Ethidium bromide-stained agarose gels showing 201-bp cDNA fragments that, after sequencing, were shown to correspond to the Syrian hamster and human mel1a receptor subtype in the suprachiasmatic nuclei. B, Ethidium bromide-stained agarose gels showing 422-bp and 131-bp (after second nested PCR) cDNA fragments 100% identical with previously identified mel1b receptor subtype in human brain and retina and in the rat suprachiasmatic nuclei. A and B, Lane 1, normal human testes (pooled normal testicular cDNA); lane 2, rat GH adenoma cell line GH3; lane 3, testes from adult hamsters kept in a LD (14 h light, 10 h dark) photoperiod; lane 4, testes from adult hamsters exposed to 16SD (6 h light, 18 h dark); lane 5, Leydig cells isolated from adult hamsters kept in LD photoperiod; lane 6, Leydig cells isolated from adult hamsters exposed to 16SD; lane 7, normal human brain (commercial cDNA); and lane 8, adult hamster brain. C, Western blot analysis of rat pituitary (lane 1), Leydig cells isolated from adult hamsters kept in LD photoperiod (lanes 2 and 3), and Leydig cells isolated from adult hamsters exposed to16SD (lanes 4 and 5) probed with anti-mel1a without preadsorption (lanes 1, 2, and 4) or after preadsorption (lanes 3 and 5) with a specific blocking peptide. Anti-mel1a dilution 1:100, blocking peptide dilution 1:200, enhanced chemiluminescence method. Fifteen micrograms of protein from rat pituitary homogenate (lane 1) and 100 μg protein from Leydig cells homogenates (lanes 2–5) were loaded on the gel.

The inhibitory action of melatonin on maximally hCG-stimulated in vitro production of cAMP and androgens from freshly isolated hamster Leydig cells is reverted by the competitive CRH receptor antagonist α-helical CRH-[9–41] but not affected by p-*MPPI [a 5-hydroxytryptamine ($5HT)_{1A}$ receptor blocker] or ketanserin (a $5HT_{2A/1C}$ receptor blocker)*

Both 1 μM CRH and 1 μM melatonin significantly inhibited hCG-stimulated androgen and cAMP productions in both LD Leydig cells (Fig. 6, A and B) and 16SD Leydig cells (Fig. 6, C and D).

α-Helical CRH-[9–41], a competitive CRH receptor antagonist, reverted the inhibitory action of CRH on hCG-induced production of testosterone and cAMP from LD hamster Leydig cells (Fig. 6, A and B). Moreover, α-helical CRH-[9–41] also blocked the modulatory action of CRH on hCG-induced production of 3α-Diol and cAMP from 16SD hamster Leydig cells (Fig. 6, C and D). The inhibitory effect of melatonin on hCG-stimulated testosterone and cAMP production from LD hamster Leydig cells (Fig. 6, A and B) as well as the production of 3α-Diol and cAMP from 16SD hamster Leydig cells (Fig. 6, C and D) was also reverted in presence of α-helical CRH-[9–41] in the incubation media. Nevertheless, incubation of hamster Leydig cells with α-helical CRH-[9–41] in absence of CRH and melatonin did not affect androgens and cAMP production (Fig. 6, A–D).

We have already shown that hamster Leydig cells contain the serotoninergic receptors $5HT_{1A}$ and $5HT_2$ (32). The inhibitory action of serotonin and (±) 8-hydroxy-2(D1-*N*-propylamino) tetralin hydrobromide (a $5HT_{1A}$ receptor agonist) on hCG-stimulated testosterone production from hamster Leydig cells is respectively partially and totally reverted by *p*-MPPI, a specific antagonist of $5HT_{1A}$ receptors (32). Moreover, ketanserin ($5HT_{2A/1C}$ receptor antagonist) partially reverts the inhibitory effect of serotonin and totally blocks the negative action of (±)1-[2,5-dimethoxy-4-iodophyryl]-2-amino propane hydrochloride (a $5HT_{2/1C}$ receptor agonist) on androgen production from hamster Leydig cells (32). In the present study, we analyzed whether the addition of *p*-MPPI and ketanserin into the incubation media of hamster Leydig cells alters the negative modulation of melatonin on androgen production. Both *p*-MPPI and ketanserin failed to revert the inhibitory action of melatonin on androgen production under hCG-stimulated conditions (data not shown).

Identification of CRH and CRH-R1 in testes: stimulatory effect of melatonin on mRNA levels and intracellular concentration of CRH in hamster Leydig cells

We detected mRNA expression of CRH and CRH-R1 in LD adult hamster testes, 16SD adult hamster testes, and Leydig cells isolated from adult animals maintained under LD conditions or exposed to a 16SD (Fig. 7A). Moreover, CRH and CRH-R1 were also expressed in commercial human testicular cDNAs (Fig. 7A).

Melatonin significantly induced mRNA expression of CRH in basal and hCG maximally stimulated LD hamster Leydig cells (Fig. 7B).

Under hCG-stimulated conditions, melatonin also upregulated CRH expression in 16SD Leydig cells (Fig. 7C). Nevertheless, in absence of hCG in the media, mRNA CRH levels remained unaffected by melatonin in 16SD hamster Leydig cells (Fig. 7C).

Moreover, melatonin increased intracellular CRH concen-

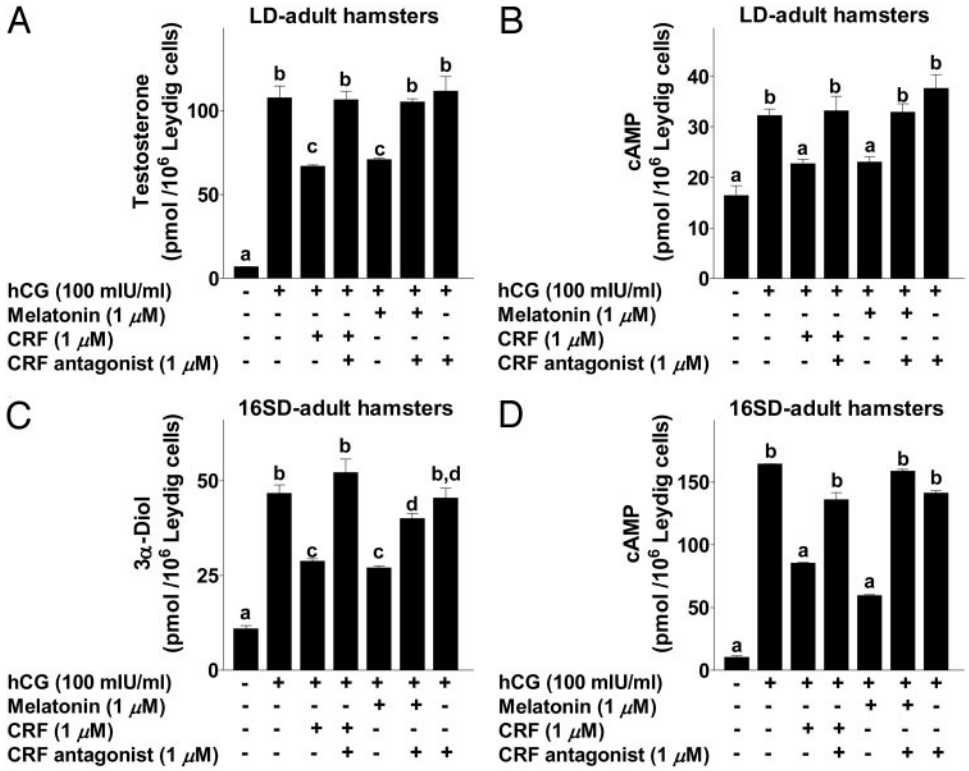

FIG. 6. The competitive CRH receptor antagonist α-helical CRH-[9–41] reverts the inhibitory effect of CRH and melatonin on maximally hCG-stimulated *in vitro* production of cAMP and androgens from freshly isolated hamster Leydig cells. Effect of 1 μM α-helical CRH-[9–41] on maximally hCG-stimulated (100 mIU/ml) testosterone (A), 3α-Diol (C), and cAMP (B and D) production from LD hamster (A and B, *upper panels*) and 16SD hamster (C and D, *bottom panels*) Leydig cells in presence of 1 μM CRH or 1 μM melatonin. Incubation time was 3 h. Bar plot graphics show the mean ± SEM from three independent experiments (five to six replicates per experiment) performed on different cell preparations. For each experiment, Leydig cells were isolated from a pool of 24 testes obtained from 12 adult hamsters. Data were analyzed using ANOVA followed by the Student-Newman-Keuls test. Where *SEM lines* are not present, SEM was encompassed by the area of the *bar*. All groups were compared; *different letters above the bars* denote a statistically significant difference between the groups ($P < 0.05$).

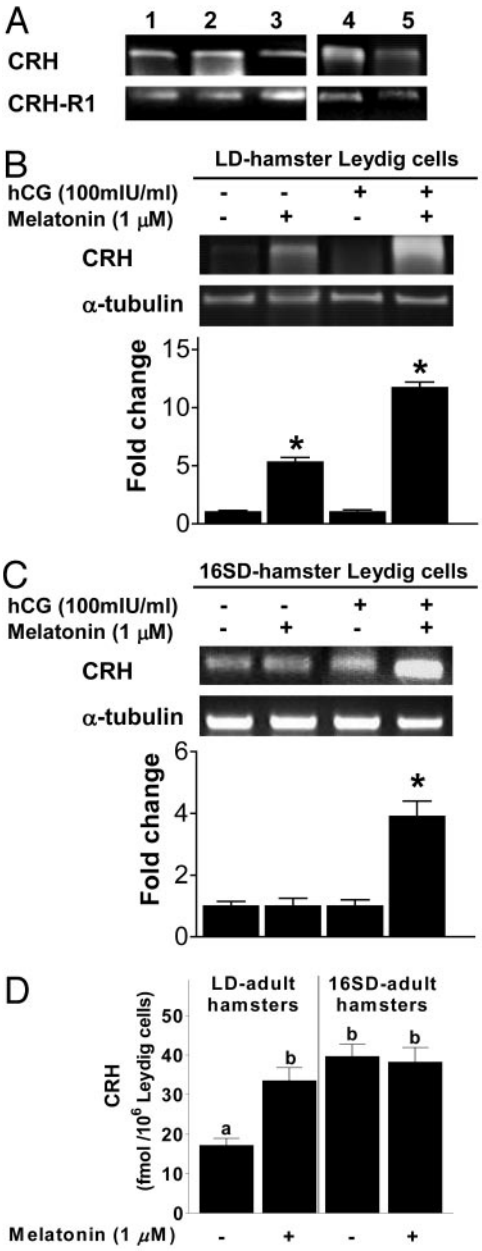

FIG. 7. RT-PCR identification of CRH and CRH-R1 in testes. Stimulatory effect of melatonin on mRNA levels and intracellular concentration of CRH in hamster Leydig cells. A, Gene expression analysis of CRH and CRH-R1 in testes. Ethidium bromide-stained agarose gel showing cDNA fragments that, after sequencing, were shown to correspond to the brain CRH and CRH-R1. Lane 1, Testes from adult hamsters kept in a LD (14 h light,10 h dark) photoperiod; lane 2, testes from adult hamsters exposed to 16SD (6 h light, 18 h dark); lane 3, normal human testes (pooled normal testicular cDNA); lane 4, Leydig cells isolated from adult hamsters kept in LD photoperiod; and lane 5, Leydig cells isolated from adult hamsters exposed to 16SD. B and C, Effect of 1 μM melatonin on basal and hCG-stimulated (100 mIU/ml) gene expression of CRH in LD hamster Leydig cells (B) and 16SD hamster Leydig cells (C). Incubation time was 30 min. These representative ethidium bromide-stained agarose gels show results obtained from one of the three experiments that yielded comparable results. In all experiments, PCR bands were quantified by densitometry. Results are expressed as fold change relative to the control (basal conditions), which was assigned a value of 1, and normalized to α-tubulin gene. Bar plot graphics show the mean ± SEM from three independent experiments (three replicates per experiment) performed on different cell preparations. For each experiment, Leydig cells were isolated from a pool of 24 testes obtained from 12 adult hamsters. Data were analyzed using ANOVA followed by the Student-Newman-Keuls test. Where *SEM lines* are not present, SEM was encompassed by the area of the *bar*. *, $P < 0.05$, compared with the control group. D, Effect of 1 μM melatonin on intracellular CRH concentration in basal LD and 16SD hamster Leydig cells (D). Incubation time was 3 h. Bar plot graphics show the mean ± SEM from three independent experiments (three replicates per experiment) performed on different cell preparations. For each experiment, Leydig cells were isolated from a pool of 24 testes obtained from 12 adult hamsters. Data were analyzed using ANOVA followed by the Student-Newman-Keuls test. Where *SEM lines* are not present, SEM was encompassed by the area of the *bar*. All groups were compared; *different letters above the bars* denote a statistically significant difference between the groups ($P < 0.05$).

tration in basal LD Leydig cells but showed no effect in basal 16SD Leydig cells (Fig. 7D).

Discussion

Our studies provide evidence for the existence of a melatonergic system in the testis. This testicular melatonergic system, working in concert with the primary effect of melatonin on the hypothalamic-pituitary axis, may thus act as local modulator of steroidogenesis in the seasonal breeder Syrian hamster. The previously unknown signaling pathway associated to the effect of melatonin on testicular activity involves down-regulation of StAR and key steroidogenic enzymes (P450scc, 3β-HSD, and 17β-HSD) expression leading to inhibition of the hCG-stimulated cAMP and androgen production. Furthermore, our results indicate that acting through specific mel1a receptors located in Leydig cells and interacting with the testicular CRH system, melatonin may represent an as-yet-unrecognized local inhibitory control of the testicular function.

Melatonin synthesis in the pineal gland involves the conversion of serotonin into melatonin by modulation of the activity of its biosynthetic enzymes: serotonin *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase (40). Newly synthesized melatonin passively diffuses from pinealocytes into the blood almost entirely at night (15). Particularly for Syrian hamsters, circulating melatonin shows a clear circadian rhythm reaching peak levels (~130 pM) several hours after lights off. Then circulating melatonin concentration begins to fall and reaches daytime levels after lights on (~43 pM) (15, 41).

It has been already determined that pineal-derived melatonin in circulation is taken up by peripheral tissues including the testes (13–15, 42, 43). In addition to this pineal source, the ability of the testes to locally synthesize melatonin has also been demonstrated in a nonphotoperiodic mammal (rat) and a bird (quail) (16–19). The important role played by melatonin in the regulation of the reproductive activity in seasonal breeders has been known for a long time (43). Concomitantly with the already described action of melatonin on the hypothalamus and the pituitary, and in accordance with initial reports from Valenti *et al.* (22) for rat testis, we detected that physiological concentrations of melatonin exert a direct inhibitory effect on hCG-stimulated cAMP and androgen production from purified LD and 16SD hamster Leydig cells.

Therefore, these results point out the potential relevance of melatonin in the local modulation of androgen production. Whereas melatonin reduced basal and hCG-stimulated testosterone production from LD hamster Leydig cells, it did not alter testosterone secretion from 16SD hamster Leydig cells. However, in 16SD hamster Leydig cells, melatonin significantly diminished hCG-stimulated 3α-Diol production but showed no effect on basal 3α-Diol secretion into the incubation media. Previous observations made by our group and other authors indicated that for hamsters, testicular and circulating levels of androgens (testosterone, DHT, 3α-Diol) are markedly reduced during the regression period (26–29). Nevertheless, inactive adult hamster testes release more 5α-reduced compounds (DHT + 3α-Diol) than active adult hamster testes, 3α-Diol being the main androgen produced from regressed testes under *in vitro* conditions (44). Employing semiquantitative RT-PCR we found that melatonin reduces the expression of StAR protein and important steroidogenic enzymes: P450scc, 3β-HSD, and 17β-HSD in both LD and 16SD hamster Leydig cells.

Our results are supported by previous publications reporting a melatonergic inhibition of StAR expression in MA-10 mouse Leydig tumor cells (45), and 17–20 desmolase enzymatic activity in rat Leydig cells (46). Moreover, adult rats injected with melatonin show a significant decrease in their testicular activity of 3β-HSD and 17β-HSD (47). In LD Leydig cells melatonin clearly inhibits mRNA expression of 5α-R1, an enzymatic isoform that plays a crucial role for the testicular conversion of testosterone into the active and non-aromatizable testosterone metabolite DHT (48). Nevertheless, 5α-R1 is significantly induced in melatonin-treated 16SD Leydig cells.

In this context, we previously described that 5α-reductase activity is markedly increased in 16SD hamster testes when compared with LD hamster testes (44), and 16SD hamster Leydig cells produce more DHT than LD hamster Leydig cells (44). Thus, in SD hamster testes, melatonin stimulates the conversion of testosterone into 5α-reduced androgens via 5α-R1 induction. On the other hand, in LD Leydig cells, 3α-HSD (an enzyme that catalyzes the interconversion between DHT and 3α-Diol) shows a weak expression after 40 PCR cycles but, in 16SD Leydig cells, 3α-HSD is easily detectable in both basal and hCG-stimulated conditions. Ji *et al.* (49) recently demonstrated that 3α-HSD expression is increased by DHT treatment in a prostate cancer cell line. Furthermore, we detected that 3α-HSD expression is significantly reduced in the presence of melatonin into the incubation media of hCG-treated 16SD Leydig cells. Consequently, in SD hamster testes, DHT and 3α-Diol productions are controlled by melatonin via induction of 5α-R1 and inhibition of 3α-HSD.

The present results indicate that melatonin exerts an inhibitory action on hCG-stimulated androgen production in hamster testes. Moreover, in 16SD hamster Leydig cells melatonin shows an additional role inducing the conversion of non-5α-reduced into 5α-reduced androgens and subsequently modulating the production of the main androgen 3α-Diol.

Testosterone production from Leydig cells is stimulated by not only LH, which is the primary messenger and activates the cAMP pathway, but also by several endocrine and paracrine factors, which can act through non-cAMP-dependent pathways including GnRH (50). Valenti *et al.* (51) previously reported that melatonin also participates in the regulation of GnRH-induced testosterone secretion in adult rat Leydig cells cultured *in vitro*.

Other well-known functions of melatonin could additionally participate in the regulation of testicular activity. For instance, numerous *in vitro* and *in vivo* studies documented the ability of both physiological and pharmacological concentrations of melatonin to protect against free radical destruction and, consequently, to modulate cellular proliferation and differentiation (52, 53).

To our knowledge, characterization of melatonin receptors in testes has been exclusively assayed by 2-[^{125}I]iodomelatonin-binding studies (23–25, 46). Pharmacological assays and binding capability to radioligand studies performed were unable to discriminate among different subtypes of melatonin receptors (54, 55). In the present study, we initially employed luzindole, a high-affinity melatonin receptor antagonist that binds to both mel1a and mel1b sites, albeit with different affinities (5). Luzindole reverted the inhibitory action of melatonin on cAMP and androgen production from LD and 16SD hamster Leydig cells. Further experiments using RT-PCR and Western blot techniques allowed us to characterize the presence of mel1a but not mel1b sites in both LD and 16SD hamster testes. Mel1a receptor subtype is expressed in the suprachiasmatic nucleus of the hypothalamus and pars tuberalis of the pituitary (6, 7), but it has been also detected in several peripheral reproductive tissues including placenta, prostate, epididymis, and ovary (56–59). Because the expression of mel1b receptors was not detected in hamster Leydig cell membranes, we could assume that the melatonergic modulation of steroidogenesis reverted by the melatonin receptor antagonist luzindole takes place through the binding of melatonin to mel1a receptors.

In the central nervous system, *in vivo* and/or *in vitro* studies have described cross-interactions between the melatonergic and serotonergic systems (60–66), which could take place at receptor and/or second messenger levels. In the testis, our group as well as other authors has described the existence of interactions between the inhibitory serotonergic and CRH systems involved in the modulation of androgen production in rat (31) and Syrian hamster (32). Because serotonin is the limiting substrate in the biosynthesis of melatonin (1) and, in an attempt to establish interactions between the melatonergic system and other well-described testicular regulatory systems, we analyzed the effect of CRH, $5HT_{1A}$ and $5HT_{2A}$ receptor antagonists on the modulatory action of melatonin at gonadal level. The $5HT_{1A}$ antagonist, *p*-MPPI, as well as the $5HT_{2A/1C}$ antagonist, ketanserin, failed to revert the inhibitory melatonergic regulation on hCG-stimulated androgen production, suggesting that testicular $5HT_{1A}$ and $5HT_{2A}$ receptors do not interact with the testicular melatonin system. Unexpectedly, the competitive CRH receptor antagonist, α-helical CRH (9–41), blocked the inhibitory effect of melatonin on hCG-stimulated cAMP and androgen production in both LD and 16SD hamster Leydig cells, suggesting that melatonin effect on steroidogenesis could take place through the local CRH system.

It has been described that in mouse Leydig cells, CRH directly stimulated basal testosterone production but did not influence the maximum hCG-induced testosterone production (67). Controversial findings about CRH effect on rat Leydig cells have been reported. Fabbri *et al.* (30) and Dufau *et al.* (31) described CRH as a negative modulator of hCG-stimulated testicular steroidogenesis in rats. Nevertheless, no influence of CRH on the basal and hCG-stimulated testosterone production from rat Leydig cells was observed by Heinrich *et al.* (67). In this work, we described an inhibitory effect of CRH on gonadotropin-induced cAMP and androgen production in both LD and 16SD hamster Leydig cells. Moreover, in hamster Leydig cells, we found expression of CRH-R1 and the presence of intracellular CRH at mRNA and protein levels. These observations agree with previous reports indicating CRH secretion from rat Leydig cells (30, 31) and the existence of CRH-R1 in mouse and rat testes (67, 68).

When LD Leydig cells were incubated in presence of melatonin, we detected the up-regulation of mRNA CRH expression in both basal and hCG-stimulated conditions. Nevertheless, in 16SD Leydig cells, melatonin induced mRNA CRH expression exclusively when hCG was added into the incubation media. Subsequent determination of CRH concentration in hamster Leydig cells by a commercial EIA verified the semiquantitative RT-PCR results achieved under basal conditions. Unfortunately, because hCG induces the secretion of CRH to the media (31), intracellular peptide levels in hCG-stimulated Leydig cells were out of the detection limit of our EIA.

Under basal conditions, CRH concentration in 16SD hamster Leydig cells was unaffected by melatonin but doubled if compared with untreated LD hamster Leydig cells and similar to those detected in melatonin-treated LD hamster Leydig cells. These results let us speculate that Leydig cells of regressed adult hamsters exposed to light deprivation and extended night melatonin secretion from pineal gland into circulation (40, 41) are already challenged to produce a higher amount of CRH than those synthesized from control LD Leydig cells.

Taken together, these data indicate that the effect of melatonin on steroidogenesis involves the interaction between the melatonergic system and the local CRH system. Thus, melatonin stimulates CRH production from Leydig cells and its subsequent inhibitory action on hCG-stimulated androgen production. New studies are in progress to gain more insight into the mechanisms related to the interaction between the testicular melatonergic and CRH systems leading to regulation of steroidogenesis, *e.g.* modulation of receptors number, changes in the level and/or activity of signaling pathways components, induction/repression of specific genes expression.

In seasonal breeders, testicular activity results from the gonadotropic effect of the pineal-derived melatonin secreted during darkness mainly by acting at the level of the brain and pituitary. The nature of the photoperiodic message that reaches the retina (*i.e.* pro- or antigonadotropic) depends on the species (LD, SD, or nonseasonal breeders), daylight variations and their consequences on the duration and magnitude of nightly elevated melatonin, and/or the period of sensitivity of the reproductive system to melatonin (13). In addition to this central melatonergic regulatory mechanism, the present study describes a potential local effect of melatonin on the testis that could work in concert enhancing/antagonizing the primary effect of this hormone on testicular activity through the hypothalamic-pituitary axis. In the Syrian hamster, specific mel1a receptors were located in Leydig cells and down-regulation of the gene expression of StAR protein and key steroidogenic enzymes P450scc, 3β-HSD, and 17β-HSD by melatonin was shown. Moreover, interactions between the testicular melatonergic and CRH systems were described.

In conclusion, our current findings have demonstrated the existence of a melatonergic system in the Syrian hamster testis and have established the biological basis and possible physiological implications of this system as local modulator of the testicular hCG-stimulated cAMP and androgen production. Nevertheless, previous reports have indicated that melatonin directs its effects on the testes through the hypothalamic-pituitary axis and not directly at the gonadal level (2, 4, 69, 70). Thus, more studies are required before the biological relevance of our results and, consequently, the role of local action of melatonin in the hamster testes can be placed in its proper perspective. We have also identified the expression of all components of the melatonergic system (mel1a, CRH, CRH-R1) in cDNAs from normal human testes but whether the inhibitory action of melatonin in the testis of the LD seasonal breeder Syrian hamster can be extended to nonseasonal reproductive mammalian species including man remains to be clarified.

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