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New insights into melatonin/CRH signaling in hamster Leydig cells

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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.

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

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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