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Pleiotropic actions of melatonin in testicular peritubular myoid cells of immature Syrian hamsters

Eugenia Riviere [a,1], Soledad P. Rossi [a,b,1], Yamil E. Tavalieri [c], Mónica M. Muñoz de Toro [c], Ricardo S. Calandra [a], Artur Mayerhofer [d], María E. Matzkin [a,b,2], Mónica B. Frungieri [a,e,*,2]

[a] Instituto de Biología y Medicina Experimental, IBYME-CONICET, Ciudad de Buenos Aires C1428ADN, Argentina
[b] Cátedra de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Ciudad de Buenos Aires C1121ABG, Argentina
[c] Instituto de Salud y Ambiente del Litoral (ISAL, UNL-CONICET), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe 3000, Argentina
[d] Cell Biology, Anatomy III, Faculty of Medicine, Biomedical Center Munich (BMC), Ludwig-Maximilian-University (LMU), 82152 Martinsried, Germany.
[e] Cátedra de Química, Ciclo Básico Común, Ciudad de Buenos Aires C1405CAE, Argentina

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ABSTRACT

Background: Peritubular myoid cells are emerging as key regulators of testicular function in adulthood. However, little is known about the role of testicular peritubular myoid cells (TPMCs) in the development of the male gonad. We found that, compared to testes of young adult hamsters, gonads of 21 day-old animals show increased melatonin concentration, seminiferous tubular wall thickening and a heterogeneous packaging of its collagen fibers thus raising the question whether melatonin may be involved in the regulation of TPMCs.
Methods: We established primary cultures of TPMCs from immature hamsters (ihaTPMCs), which we found express melatonergic receptors.
Results: Exogeneous melatonin decreased the levels of inflammatory markers (NLRP3 inflammasome, IL1β) but increased the expression of cyclooxygenase 2 (COX2, key enzyme mediating prostaglandin synthesis) and of the glial cell line-derived neurotrophic factor (GDNF) in ihaTPMCs. Melatonin also stimulated ihaTPMCs proliferation and the expression of extracellular matrix proteins such as collagen type I and IV. Furthermore, collagen gel contraction assays revealed an enhanced ability of ihaTPMCs to contract in the presence of melatonin.
Conclusion: Melatonin regulates immune and inflammatory functions as well as contractile phenotype of the peritubular wall in the hamster testis.
General significance: If transferable to the *in vivo* situation, melatonin-dependent induction of ihaTPMCs to produce factors known to exert paracrine effects in other somatic cell populations of the gonad suggests that the influence of melatonin may go beyond the peritubular wall and indicates its contribution to testicular development and the establishment of a normal and sustainable spermatogenesis.

1. Introduction

Melatonin is a neurohormone secreted by the pineal gland. Particularly, in seasonal breeders that successfully mate and reproduce only during certain times of the year, photoperiod influences the activity of the hypothalamic-pituitary-testicular axis through changes in melatonin secretion by the pineal gland [1].

In addition to its action on the hypothalamic-pituitary-testicular axis, pineal gland-derived melatonin is released into bloodstream reaching the male gonad where it exerts direct effects [1]. Local intratesticular synthesis of this indolamine complements pineal gland-derived melatonin [1]. In this context, our research group has previously quantified melatonin concentration in testes of hamsters and humans [2,3].

Several actions of this indolamine on testicular somatic cells including Leydig cells, Sertoli cells and certain immune cells have previously been described by our group and others [1–4]. Melatonin negatively modulates steroidogenesis of Leydig cells [2,4], influences energy metabolism of Sertoli cells [5], promotes anti-proliferative

* Corresponding author at: Institute of Biology and Experimental Medicine (IBYME-CONICET), Vuelta de Obligado 2490, Ciudad de Buenos Aires 1428, Argentina.
E-mail address: mfrungieri@dna.uba.ar (M.B. Frungieri).
[1] Equal contribution.
[2] Equal contribution.

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actions on testicular macrophages and exerts regulatory roles in the inflammatory and oxidative states in testicular mast cells and Sertoli cells [3,6,7].

Beneficial effects of melatonin have been reported on the inflammatory-oxidative status in rodent testicular aging [8,9]. Furthermore, in young and middle-aged men with idiopathic infertility receiving a daily oral dose of this indolamine, an attenuation of testicular inflammation and oxidative stress was found [10].

Testicular peritubular myoid cells (TPMCs) are the least studied testicular cells. These cells with known contractile activity are the primary cellular component of the seminiferous tubules wall. Functions of TPMCs are related to the structural support of the seminiferous tubules, transport of immobile sperm and their secretory activity [11–13]. In addition, TPMCs, alongside Sertoli cells, are functional components of the spermatogonial stem cells (SSCs) niche.

The role of TPMCs during development is not well examined but a previous study suggests that, in addition to Sertoli cells, TPMCs also participate in the regulation of seminiferous cords elongation and the postnatal testicular growth [14].

The Golden (Syrian) hamster is a thoroughly studied seasonal breeder in which the influence of melatonin on gonadal activity is well-known [1]. We initially established that, compared to testes of young adult hamsters, gonads of 21 day-old animals show increased melatonin concentration. A thickening of the wall of the seminiferous tubules and a differential collagen fibers organization were observed as well. Hence we speculated that melatonin may be involved in the regulation of TPMCs during development. To test this hypothesis, in the present work we established a cell culture method for immature hamster TPMCs and investigated whether melatonin is able to impact their immunological properties, contractile activity, as well as the expression of extracellular matrix (ECM) components and of the glial cell line-derived neurotrophic factor (GDNF, known to maintain SSCs self-renewal).

2. Materials and methods

2.1. Animals

Syrian Golden hamsters (*Mesocricetus auratus*), raised in our unit care [Charles River descendants, Animal Care Lab., Instituto de Biología y Medicina Experimental (IBYME), Buenos Aires, Argentina], were kept from birth to the weaning age of 21 days in rooms at 23 ± 2 °C under a long day photoperiod (14 h light, 10 h darkness; lights on 7:00–21:00 h). All animal experiments obeyed the Animal Research Reporting of *In Vivo* Experiments (ARRIVE) guidelines. Hamsters had free access to water and Purina formula chow. After weaning, hamsters were euthanized 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 (CICUAL, IBYME-CONICET), Buenos Aires, Argentina (Protocol Number CE-037/2019), following the National Research Council's guide for the care and use of laboratory animals, USA. Testes were weighed and subsequently dissected for isolation and purification of TPMCs, Sertoli cells, germ cells and Leydig cells. Additionally, immature (21 day-old) and adult (90 day-old) hamsters were sacrificed and testes used in biometric measurements, histological assays and determination of melatonin levels.

2.2. Biometric measurements and histological assays

At the time of sacrifice, testes from 21 and 90 day-old animals were dissected, freed from adherent tissues, and weighed on an analytical scale (Mettler Instrument AG, Schwerzenbach, Switzerland). The volume of each removed testis was estimated directly by water displacement (Archimedes principle) [15]. Testicular density was calculated by dividing the testicular weight by the testicular volume.

For histological and morphometric examinations, hamster testes were fixed in 10% paraformaldehyde (PFA) solution, dehydrated, embedded in paraffin wax and sections of 5 μm were obtained from three different levels. Subsequently, testicular sections were deparaffinized, hydrated and stained with Sirius Red (Direct Red 80, Sigma-Aldrich, St. Louis, MO, USA) in picric acid solution (picrosirius red) [9,16]. Picrosirius red-stained sections were examined by both bright field microscopy and polarized light microscopy [9,16].

The thickness of the tubular wall was determined on 40–50 tubules randomly selected from picrosirius red-stained specimens using a bright field microscope with an ocular micrometer, a magnification of 10,000 x and under oil immersion as was described [9,10]. The measurement of the tubular thickness was made on a computer using the image analysis software ImageJ (NIH, Bethesda, MA, USA, http://imagej.nih.gov/ij/).

2.3. Melatonin immunoassay

Melatonin concentration and content in testes of immature and adult hamsters were determined with a commercially available enzyme-linked immunoassay kit (IBL International, Hamburg, Germany) following the manufacturer's instructions as previously described [2,3,9].

Briefly, testes were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, pH 7.4) supplemented with 1 mM sodium orthovanadate, 1 mM sodium fluoride and a commercial mixture of protease inhibitors (Roche Applied Science GmbH, Mannheim, Germany). Standards, controls 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, Waltham, MA, USA). Melatonin concentration and content are expressed as femtomole (fmol) per g tissue and fmol per testis, respectively.

2.4. Purification of hamster Leydig cells and germ cells

Leydig cells and germ cells from immature hamsters (ihaLCs and ihaGCs, respectively) were purified from a testicular cell suspension obtained through digestion with 0.2 mg/ml collagenase type I (Sigma-Aldrich), filtration using a 100 μm Nylon cell strainer and separation in a discontinuous Percoll density gradient as previously described [17]. ihaLCs and ihaGCs migrated to the 53% and 37% Percoll fractions, respectively. These Percoll fractions were recovered and used for cell viability analyses using 0.4% Trypan-blue. Then, ihaLCs and ihaGCs were fixed with 4% PFA for 10 min and subsequently used in immunocytochemical analyses.

2.5. Purification of hamster testicular peritubular myoid cells and Sertoli cells

TPMCs and Sertoli cells from immature hamsters (ihaTPMCs and ihaSCs, respectively) were purified using a protocol adapted from [18]. Decapsulated testes were incubated in a water bath at 37 °C for 5 min in the presence of 2.5 mg/ml trypsin solution in order to separate the seminiferous tubules. Reaction was stopped by adding 2.5 mg/ml trypsin inhibitor solution for 5 min. Tubules were intensely washed 9 times with 30 ml Hanks' solution (Ca^{2+}/Mg^{2+} free, Hepes 10 mM) and allowed to settle for 10 min. A second enzymatic digestion was done in a water bath at 34 °C in the presence of 0.2 mg/ml collagenase type I solution (Sigma–Aldrich) until the tubules appeared shortened in length under a light microscope. At the end of incubation, collagenase activity was stopped by adding 30 ml Hanks' solution and a decantation step of 10 min ensued. Supernatant containing ihaTPMCs was centrifuged and resuspended in RPMI1640 medium (Sigma–Aldrich).

Simultaneously, decantation pellet was washed with 40 ml Hanks' solution and allowed to settle for 30 min. Afterwards, a third enzymatic digestion was performed in a water bath at 34 °C in the presence of 0.2 mg/ml collagenase type I solution (Sigma–Aldrich) for 8 min.

Reaction was stopped by adding 40 ml Hanks' solution and a second decantation step of 30 min ensued. Pellet was resuspended in 10 ml RPMI1640 medium and filtered through a membrane filter with a 100 μm pore diameter. Eluate containing ihaSCs was centrifuged and resuspended in RPMI1640 medium.

An aliquot of both cell suspensions containing ihaTPMCs and ihaSCs, respectively, were incubated for 5 min with 0.4% Trypan-blue and used for cell counting and viability assay in a light microscope. Approximately, 1×10^6 ihaTPMCs and 1×10^6 ihaSCs were seeded in petri dishes with 1 ml RPMI1640 medium and 10% fetal calf serum (FCS) under a humid atmosphere at 34 °C in a mixture of 5% CO2–95% air for 48 h.

At that point, cells were washed with Hanks' solution. ihaTPMCs were used for *in vitro* incubations. In addition, ihaTPMCs and ihaSCs were fixed with 4% PFA for 10 min and used in immunocytochemical analyses.

2.6. Immunocytochemical characterization of cultured cells

In ihaTPMCs, ihaSCs, ihaGCs and ihaLCs fixed with 4% PFA, the endogenous peroxidase reactivity was quenched by a 20 min pretreatment with 10% methanol and 6.3% H_2O_2 in 0.01 M phosphate-buffered saline (PBS, pH 7.4). Afterwards, cells were permeabilized by 5 min incubation with 0.05% saponin and nonspecific proteins were blocked with 5% horse normal serum [for immunodetection of α-smooth muscle actin (α-SMA)], 5% goat normal serum [for immunodetection of anti-Müllerian hormone (AMH), SOX9 and steroidogenic acute regulatory protein (StAR)] or 5% donkey normal serum (for immunodetection of VASA) prepared in PBS for 30 min. After several wash steps, fixed hamster cells were incubated for 24 h in a humidified chamber at 4 °C with the following antibodies: monoclonal mouse anti-α-SMA (1:100, Sigma-Aldrich), polyclonal rabbit anti-AMH (1:150, see further details in [19]), polyclonal rabbit anti-SOX9 (1:25, Santa Cruz Biotechnology, Santa Cruz, CA, EEUU), polyclonal goat anti-VASA (1:100, Santa Cruz Biotechnology), and polyclonal rabbit anti-StAR (1:100, see further details in [20]) diluted in incubation buffer (2% horse normal serum for α-SMA, 2% goat normal serum in PBS for AMH, SOX9 and StAR, or 2% donkey normal serum in PBS for VASA). On the second day, cells were washed and incubated for 2 h at room temperature with the following biotinylated secondary antisera: horse anti-mouse IgG (1:500, Vector Laboratories Inc., Burlingame, CA, USA) for α-SMA, goat anti-rabbit IgG (1:500, Vector Laboratories Inc., housekeeping) for AMH, SOX9 and StAR, and donkey anti-goat IgG (1:500, Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for VASA. Immunoreactions were detected using the avidin-biotin-peroxidase system (Vector Laboratories Inc.). Negative controls included omission of the primary antibody or incubations with non-immune serum.

2.7. "In vitro" incubations of immature hamster testicular peritubular myoid cells

Immature hamster testicular peritubular myoid cells (ihaTPMCs) were incubated in the presence or absence of 1 μM and 0.1 nM melatonin (Sigma-Aldrich) for 3, 5 and/or 16 h. Melatonin was dissolved in RPMI1640 medium, which was then used as vehicle for control conditions.

After incubations, ihaTPMCs were used for RNA extraction followed by RT-qPCR and for protein extraction followed by immunoblotting, or alternatively fixed with 4% PFA for immunofluorescence assays.

2.8. RT-PCR and RT-qPCR analyses

Total RNA was prepared from hamster testes and ihaTPMCs applying TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturers' instructions. Reverse transcription (RT) reactions were performed using 500 ng total RNA and dN6 random primers. PCR assays were done using oligonucleotide primers for melatonin type 1 receptor (MT1) (5′- GTGGTGGACATTCTGGGCA and 5′- TGGGCATGATGGCGACTAG) and melatonin type 2 receptor (MT2) (5′- TATTCCTGCACCTTCATC and 5′- CGGGCCTGGAGCACCA). PCR conditions were 95 °C for 5 min, followed by cycles of 94 °C for 1 min, 55–60 °C (annealing temperature) for 1 min and 72 °C for 1 min, and a final incubation at 72 °C for 5 min. 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 (Applied Biosystems, Foster City, CA, USA).

qPCR assays were performed as previously described [2] using oligonucleotide primers for the inflammasome nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3 (NLRP3) (5′-CTGAAAGCTCTCCACCTC and 5′-CGCTTTTCCATCTTCTTCA), cyclooxygenase 2 (COX2) (5′- CTGGCGCTCAGCCATACAG and 5′-ACACTCATACATACACCTCGGT), GDNF (5′-GCAGACCCATCGCCTTTGAT and 5′-ATCCACACCTTTTAGCGGAATG), biglycan (5′-TCAAGCTCCTCCAGGTGGT and 5′- GGTCAGTGACGCAGCGGAA), decorin (5′-GCTCTCCTACATCCGCATTG and 5′- TCAATCCCAACTTAGCCAAA), collagen type I alpha 2 chain (COL1A2) (5′-AACAGCGTTGCATACCTGGA and 5′-GAATTCTTGGTCAGCGCCAC), collagen type 4 alpha 1 chain (COL4A1) (5′-ACAATGTGTGCAACTTCGCC and 5′- CGAAGGAGTAGCCGATCCAC), α-SMA (5′- ACCCAGTGTGGAGCAGCCC and 5′- TTGTCACACACCAAGGCAGT), calponin (5′- GAAGACGAAAGGAAACAAGGT and 5′-GCTTGGGGTCGTAGAGGTG), and myosin heavy chain 11 (MYH-11) (5′-GAAGACGAAAGGAAACAAGGT and 5′-GCTTGGGGTCGTAGAGGTG).

GAPDH was chosen as the housekeeping gene (5′-TGCACCACCAACTGCTTAGC and 5′-GGCATGGACTGTGGTCATGAG). Reactions were conducted using SYBR Green PCR Master Mix (Roche Applied Science GmbH) and the CFX96-Touch Real-Time PCR Detection System (BIORAD, Hercules, CA, USA). Reaction conditions were as follows: 10 min at 95 °C (one cycle), followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 60 °C.

Following the mathematical model of [21], the fold change of mRNA gene expression was determined for each sample by calculating $(\text{E target})^{\Delta Ct(\text{target})}/(\text{E housekeeping})^{\Delta Ct(\text{houskeeping})}$, where E is the efficiency of the primer set, Ct 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 microlitres of cDNA per reaction vs Ct value over at least four orders of magnitude ($E = 10^{-(1/\text{slope})}$).

2.9. Immunoblotting

ihaTPMCs were homogenized in RIPA buffer supplemented with 1 mM sodium orthovanadate, 1 mM sodium fluoride and a commercial mixture of protease inhibitors (Roche Applied Science GmbH). After 30 min in ice, homogenates were centrifuged at 13,000 xg for 10 min. Supernatants were collected and protein concentrations were measured by the method of [22]. Samples were heated at 95 °C for 5 min under reducing conditions (10% 2-mercaptoethanol), loaded onto 15% tricine-SDS-polyacrylamide gels, electrophoretically separated and blotted onto nitrocellulose membrane.

Blots were performed as previously described [10] using the following commercial antibodies and sera: mouse monoclonal anti-interleukin 1β (IL1β) antibody (1:500, Santa Cruz Biotechnology); rabbit polyclonal anti-COX2 serum (1:250, Cayman Chemical, Ann Arbor, MI, EEUU); mouse monoclonal anti-GDNF antibody (1:200, Santa Cruz Biotechnology); mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (1:200, Santa Cruz Biotechnology); rabbit polyclonal anti-collagen type IV alpha 2 chain (COL4A2) serum (1:200, Santa Cruz Biotechnology), mouse monoclonal anti-α-SMA antibody (1:500, Sigma-Aldrich); rabbit monoclonal anti-calponin antibody (1:500, Epitomics, Burlingame, CA, EEUU); and mouse monoclonal anti-β-actin antibody (1:1000, Santa Cruz Biotechnology).

Subsequently, blots were incubated with peroxidase-conjugated secondary antibodies: goat anti-rabbit IgG serum (1:2500; BIORAD) for calponin, COX2 and COL4A2; and goat anti-mouse IgG serum (1:3000; BIORAD) for α-SMA, lL1β, GDNF, PCNA and β-actin.

Signals were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and visualized using a chemiluminescence imaging equipment (G-Box, Syngene™, Cambridge, United Kingdom). Bands were quantified by densitometry and normalized to β-actin using SCION IMAGE (SCION Corporation, Frederick, MD, USA).

2.10. Immunofluorescence assays

ihaTPMCs fixed in 4% PFA were washed in PBS and incubated for 30 min in 2% normal goat serum prepared in PBS, followed by an overnight incubation in a humidified chamber at 4 °C with a rabbit polyclonal anti-COL4A2 serum (1:50, Santa Cruz Biotechnology) diluted in 2% normal goat serum prepared in PBS. The following day, cells were washed with PBS and incubated for 1 h at room temperature with mouse anti-rabbit-IgG FITC antiserum (1:500, Santa Cruz Biotechnology). Cells were washed and nuclei were counterstained with 0.5 μg/ml DAPI (SIGMA-Aldrich) for 5 min. Cover slips were mounted using ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA). Examination was performed by fluorescence confocal microscopy (Spinning Disk - TIRF Olympus DSU IX83, Tokyo, Japan). For control purposes, either the first antibody was omitted or incubation was carried out with 2% normal goat serum.

2.11. Proliferation assays

Approximately 4×10^5 ihaTPMCs were plated on 96-well plates and incubated in the presence or absence of melatonin (1 μM and 0.1 nM) for 24 h. Cell viability was determined using the CellTiter 96 AQueous One Solution cell proliferation assay (Promega Corporation, Madison, WI, USA) as described by [3].

Levels of PCNA protein expression were visualized by immunoblotting and quantified as previously described in section 2.9.

2.12. Collagen gel contraction assays

The method was performed as described by [23]. Collagen was purified from rats' tails following a protocol described by [24]. Approximately 4×10^6 ihaTPMCs were seeded onto gel lattices. FCS 10% was used as a positive control, since it is probed to be a potent stimulator of cell contraction. Medium without FCS supplement served as basal conditions. FCS 10% and melatonin (1 μM and 0.1 nM) were added and collagen lattices were released from the surrounding brim of the wells. Immediately, pictures of the free-floating collagen gels were taken at 0, 24, 48 and 72 h. Gel area sizes were analyzed with ImageJ and normalized to the area of the well. The degree of contraction was evaluated by determining the area of the gel matrix before and after treatment. Results are expressed as the percentage change of matrix area before and after treatment.

2.13. Statistical analyses

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

3. Results

3.1. Quantification of melatonin concentration and biometric parameters in testes of immature and adult hamsters

As expected, testicular weight in young adult (90 day-old) hamsters

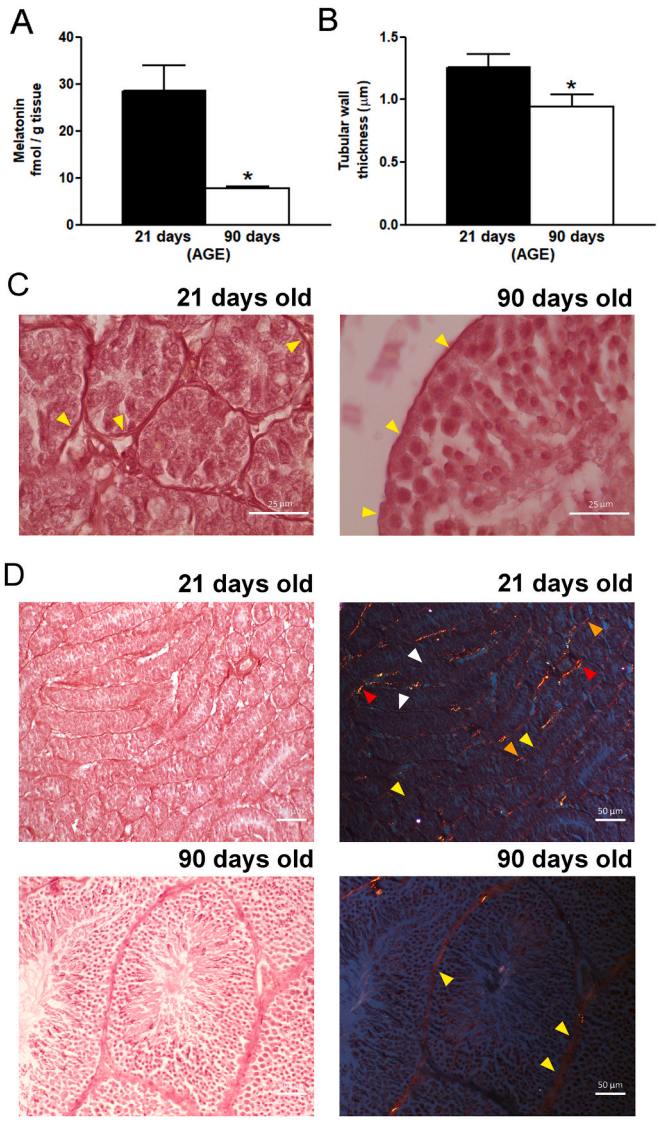

Fig. 1. Melatonin concentration as well as thickness and features of the tubular wall in testes of immature and adult Syrian hamsters.
(A) Testicular melatonin concentration was determined using a commercial ELISA kit in immature (21 day-old) and adult (90 day-old) Syrian hamsters kept under a long day photoperiod (LD: 14 h light per day). Bar plot graph represents the mean ± S.E.M. (n = nine animals per group). *$p < 0.05$. Student's *t*-test.
(B) and (C) In picrosirius red-stained testicular sections from immature (21 day-old) and adult (90 day-old) Syrian hamsters kept under a LD photoperiod, the thickness of the tubular wall was determined in a bright field microscope with an ocular micrometer under oil immersion. Bar: 25 μm.
Bar plot graph represents the mean ± S.E.M. (n = nine animals per group). *$p < 0.05$. Student's t-test (B). Arrowheads highlight the thickness of the tubular wall (C).
(D) In picrosirius red-stained testicular sections from immature (21 day-old) and adult (90 day-old) Syrian hamsters kept under a LD photoperiod, the wall of the seminiferous tubules was examined by bright field microscopy (left panels, bar: 50 μm) and polarized light microscopy (right panels, bar: 50 μm). Representative sections are shown. Red arrowheads feature collagen fibers with high birefringence, orange arrowheads highpoint collagen fibers with medium birefringence, yellow arrowheads point out collagen fibers with low birefringence, and white arrowheads show collagen fibers without birefringence.

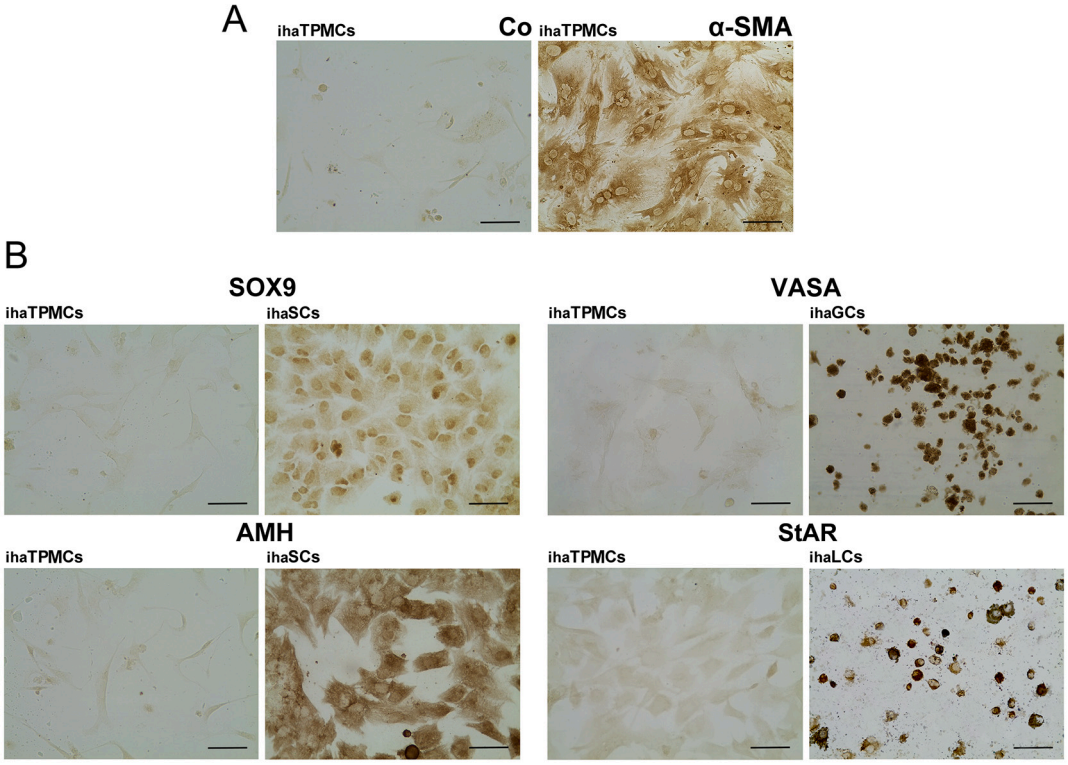

Fig. 2. Characterization of primary cultures of testicular peritubular myoid cells from immature hamsters.
(A) Positive immunostaining for α-smooth muscle actin (α-SMA) was found in primary cultures of testicular peritubular myoid cells from immature hamsters (ihaTPMCs). No reaction was observed when ihaTPMCs were incubated only with normal non-immune serum and the biotin conjugated antibody (Co, control). Bar, 50 μm.
(B) Positive immunostaining was seen for SOX9 and anti-Müllerian hormone (AMH) in Sertoli cells from immature hamsters (ihaSCs), for VASA in germ cells from immature hamsters (ihaGCs), and for StAR in Leydig cells from immature hamsters (ihaLCs). However, ihaTPMCs depicted negative immunostaining for markers of Sertoli cells (SOX9 and AMH), germ cells (VASA) and Leydig cells (StAR). Bar, 50 μm.

was 25-fold higher than in immature (21 day-old) animals (g, mean ± S.E.M., testes of immature hamsters: 0.07 ± 0.01 vs. testes of young adult hamsters: 1.78 ± 0.15, $n = 9$, $p < 0.05$).

No significant differences were seen in tissue density between testis of immature hamsters and gonads of young adult animals (g/cm^3, mean ± S.E.M., testes of immature hamsters: 1.08 ± 0.05 vs. testes of young adult hamsters: 1.06 ± 0.01, $n = 9$, $p > 0.05$).

Melatonin concentration was significantly increased in testes of immature hamsters compared to testes of young adult animals (Fig. 1A). In contrast, testicular melatonin content was higher in testes of adult hamsters than in testes of immature animals (fmol / testis, mean ± S.E.M., testes of immature hamsters: 1.97 ± 0.37 vs. testes of young adult hamsters: 13.75 ± 0.82, n = 9, $p < 0.05$).

Picrosirius red is a histology stain used to identify total collagen, one of the main components of the ECM. Picrosirius red-stained sections were used to evaluate thickness and the organization of collagen fibers in the wall of seminiferous tubules (Fig. 1B, C and D). Thickness of the tubular wall was greater in testes of immature hamsters compared to those values detected in gonads of young adult animals (Fig. 1B and C).

Whereas under standard bright field microscopy, sections stained with picrosirius red dye showed the presence of red collagen fibers in the wall of the seminiferous tubules of immature and young adult hamsters (Fig. 1D, left panels), under polarized light microscopy yellow-red birefringence collagen fibers were seen in the tubular wall of all testicular sections examined (Fig. 1D, right panels).

The wall of the seminiferous tubules in immature hamsters showed heterogeneous birefringence. Zones of the tubular wall with high, medium, low or complete absence of birefringence can be identified (Fig. 1D, right panel). In contrast, collagen fibers in the tubular wall of adult hamsters present a homogeneous and weak birefringence (Fig. 1D, right panel).

3.2. Characterization of primary cultures of immature hamster testicular peritubular myoid cells

Following the protocol for isolation of rat TPMCs reported by [18] with some modifications due to the different consistency of rat and hamster testes, we set up a protocol for the isolation and purification of ihaTPMCs from immature (21 day-old) hamsters. ihaTPMCs were initially identified upon immunocytochemical analyses using an antibody that specifically recognizes α-SMA (Fig. 2A, right panel). No reaction was observed when ihaTPMCs were incubated only with normal non-immune serum and the biotin conjugated antibody (Fig. 2A, left panel, Co: control).

Potential contaminations of ihaTPMCs primary cultures with other testicular cell populations were evaluated using antibodies against Sertoli cells markers (SOX9, AMH), a Leydig cells marker (StAR) and a germ cells marker (VASA). Expression of SOX9, AMH, StAR and VASA was undetectable in ihaTPMCs primary cultures (Fig. 2B). As expected, immature hamster Sertoli cells (ihaSCs) were positively stained with SOX9 and AMH antibodies, immature hamster Leydig cells (ihaLCs) showed immunostaining for StAR and immature hamster germ cells (ihaGCs) exhibited staining for VASA (Fig. 2B).

3.3. Immature hamster testicular peritubular myoid cells express melatonergic receptors

RT-PCR studies detected the expression of the melatonergic receptors MT1 and MT2 in testis of immature hamsters (ihatestis) and in primary cultures of ihaTPMCs (Fig. 3).

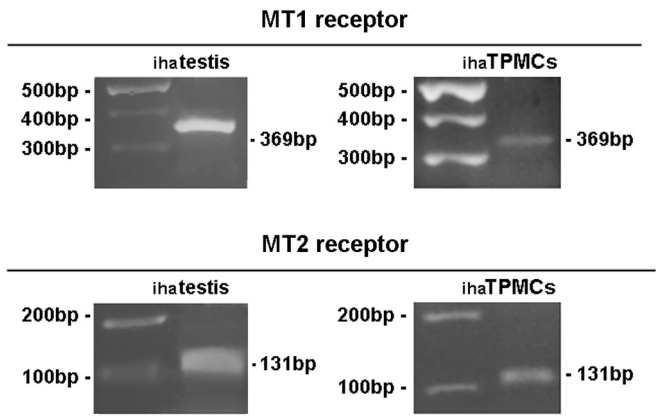

Fig. 3. Expression of melatonergic receptors in testicular peritubular myoid cells from immature hamsters.
mRNA expression of the melatonin type 1 receptor (MT1, 369 bp) and the melatonin type 2 receptor (MT2, 131 bp) was detected by RT-PCR in testis of immature hamsters (ihatestis) and in testicular peritubular myoid cells from immature hamsters (ihaTPMCs).

3.4. Melatonin decreases the expression of the inflammasome NLRP3 and the proinflammatory cytokine IL1β, but stimulates the expression of COX2 in immature hamster testicular peritubular myoid cells

To examine the role of melatonin in the regulation of the inflammatory response in ihaTPMCs, the expression levels of the inflammasome NLPR3, the proinflammatory cytokine IL1β and COX2, the key enzyme mediating prostaglandin synthesis, were investigated.

After 3 h incubations, 1 μM melatonin concentration decreased the mRNA levels of NLRP3 but increased mRNA levels of COX2 in ihaTPMCs (Fig. 4A). mRNA levels of NLRP3 and COX2 were unaffected when ihaTPMCs were incubated in the presence of 0.1 nM melatonin (Fig. 4A). After 5 h incubations, both melatonin concentrations (1 μM and 0.1 nM) diminished the protein levels of IL1β but elevated the protein levels of COX2 (Fig. 4B). After 16 h incubations, 1 μM melatonin decreased the protein levels of IL1β (Fig. 4B). The protein levels of COX2 were higher in ihaTPMCs incubated for 16 h in the presence of melatonin (1 μM and 0.1 nM) than in ihaTPMCs kept under basal conditions (Fig. 4B).

3.5. Melatonin increases the expression of GDNF in immature hamster testicular peritubular myoid cells

Possible effects of melatonin on the expression levels of GDNF in ihaTPMCs were evaluated by RT-qPCR and immunoblot assays.

The mRNA levels of GDNF were significantly increased in ihaTPMCs incubated for 3 h in the presence of a melatonin concentration of 1 μM

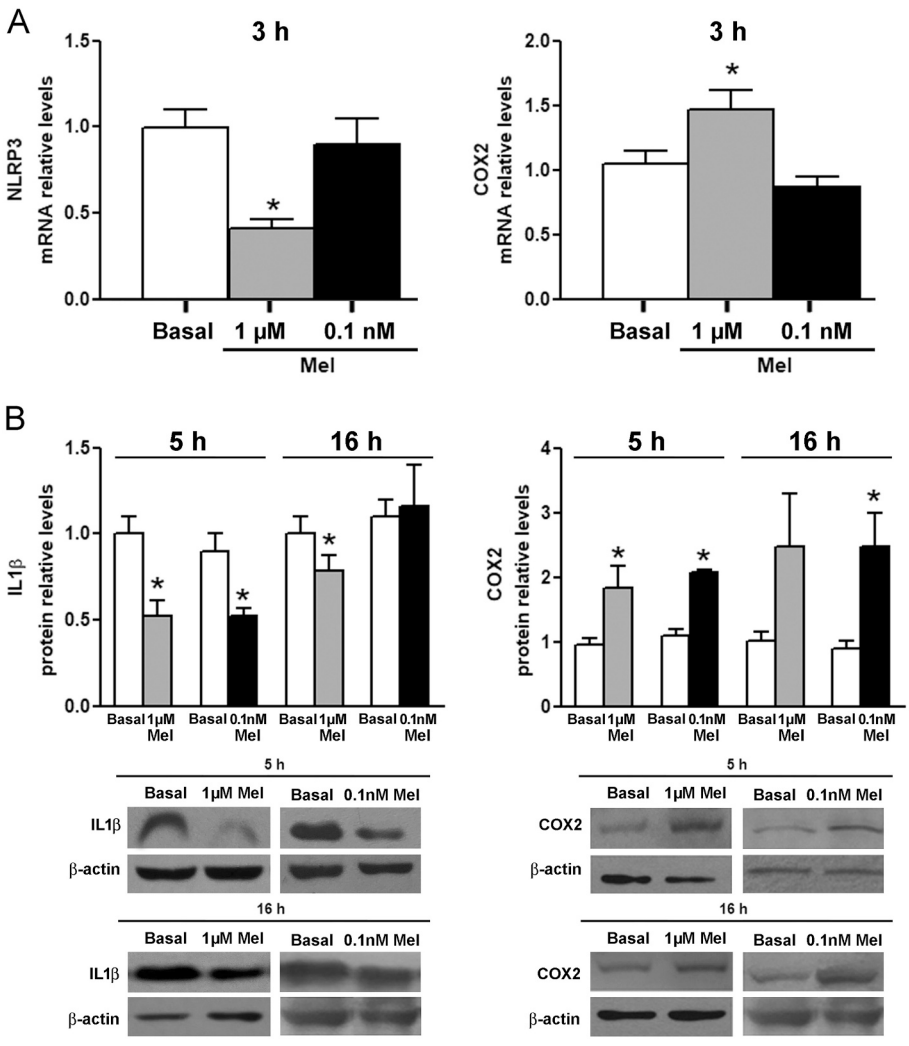

Fig. 4. Role of melatonin in the expression of the inflammasome NLRP3, IL1β and COX2 in testicular peritubular myoid cells from immature hamsters.
(A) Testicular peritubular myoid cells from immature hamsters (ihaTPMCs) were incubated in RPMI1640 medium in the presence or absence of melatonin (Mel, 1 μM and 0.1 nM) for 3 h. Leucine rich repeat and pyrin domain containing 3 (NLRP3) and cyclo-oxygenase 2 (COX2) mRNA levels were determined by RT-qPCR using GAPDH as the housekeeping gene. Bar plot graphs represent the mean ± S.E.M. of the mRNA levels obtained from one of three-four independent experiments that showed similar results (see Supplementary Table 1). Results were analyzed using the mathematical model of Pfaffl. $^*p < 0.05$. Student-Newman–Keuls test.
(B) ihaTPMCs were incubated in RPMI1640 medium in the presence or absence of melatonin (Mel, 1 μM and 0.1 nM) for 5 and 16 h. Interleukin 1β (IL1β) and COX2 protein levels were determined by immunoblotting. Bar plot graphs represent the mean ± S.E.M. and show the quantification by densitometry of the bands obtained from one of two-three experiments performed in different cell preparations that showed similar results (see Supplementary Table 1). Results are expressed as fold change relative to the control (basal conditions) and normalized to β-actin. $^*p < 0.05$. Student's *t*-test. Immunoblots shown are representative.

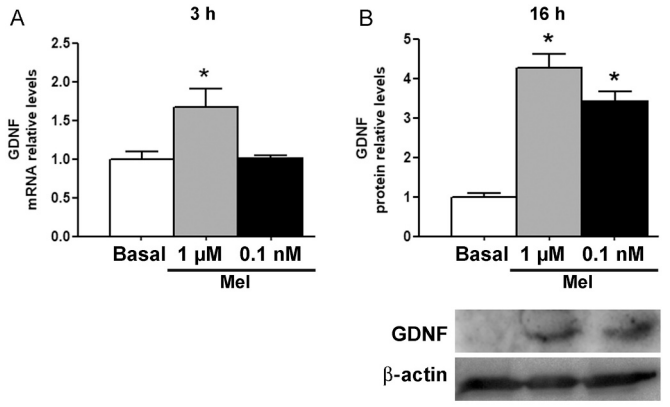

Fig. 5. Role of melatonin in GDNF expression in testicular peritubular myoid cells from immature hamsters.

(A) Testicular peritubular myoid cells from immature hamsters (ihaTPMCs) were incubated in RPMI1640 medium in the presence or absence of melatonin (Mel, 1 μM and 0.1 nM) for 3 h. Glial cell line-derived neurotrophic factor (GDNF) mRNA levels were determined by RT-qPCR using GAPDH as the housekeeping gene. Bar plot graph represents the mean ± S.E.M. of the mRNA levels obtained from one of four independent experiments that showed similar results (see Supplementary Table 2). Results were analyzed using the mathematical model of Pfaffl. $*p < 0.05$. Student-Newman–Keuls test.

(B) ihaTPMCs were incubated in RPMI1640 medium in the presence or absence of melatonin (Mel, 1 μM and 0.1 nM) for 5 and 16 h. GDNF protein levels were determined by immunoblotting. Bar plot graph represents the mean ± S.E.M. and show the quantification by densitometry of the bands obtained from one of two experiments performed in different cell preparations that showed similar results (see Supplementary Table 2). Results are expressed as fold change relative to the control (basal conditions) and normalized to β-actin. $*p < 0.05$. Student-Newman–Keuls test. Immunoblots shown are representative.

(Fig. 5A) but unaffected when ihaTPMCs were incubated in the presence of 0.1 nM melatonin (Fig. 5A).

After 16 h incubations in the presence of both doses of melatonin (1 μM and 0.1 nM), protein GDNF levels significantly increased in ihaTPMCs (Fig. 5B).

3.6. Melatonin stimulates proliferation and the expression of extracellular matrix components in immature hamster testicular peritubular myoid cells

After 24 h incubations, a concentration of 1 μM melatonin significantly stimulated metabolic activity, and therefore increased cell number of ihaTPMCs (Fig. 6A). The expression of PCNA in ihaTPMCs was also markedly increased by 1 μM melatonin (Fig. 6B). However, a dose of 0.1 nM melatonin did not affect ihaTPMCs proliferation (Figs. 6A and 6B).

After a short time incubation (3 h), melatonin did not alter the expression of the ECM components biglycan and decorin, but elevated the mRNA levels of collagen (COL1A2 and COL4A1) in ihaTPMCs (Fig. 6C). Furthermore, immunoblotting showed a significant increase in the levels of COL4A2 after 16 h incubations with 1 μM melatonin (Fig. 6D)

Immunoblotting also revealed that the induction of COL4A2 expression after 16 h incubations in the presence of 0.1 nM melatonin was not statistically significant (Fig. 6D). Nevertheless, immunofluorescence assays suggested that both melatonin concentrations (1 μM and 0.1 nM) would induce COL4A2 expression in ihaTPMCs (Fig. 7).

3.7. Role of melatonin in contractile ability in immature hamster testicular peritubular myoid cells

In order to evaluate a possible role of melatonin in the contractile function of ihaTPMCs, the expression levels of contractility proteins were examined. After 3 h incubations in the presence of 1 μM melatonin, mRNA levels of α-SMA, calponin and MYH-11 were significantly increased (Fig. 8A). Instead, they were unaffected when ihaTPMCs were incubated with 0.1 nM melatonin (Fig. 8A).

After 5 and 16 h incubations, melatonin (1 μM and 0.1 nM) elevated protein levels of calponin and α-SMA around two-three folds (Fig. 8B).

A collagen gel contraction assay was used to assess contractility in ihaTPMCs. To induce maximal contraction, a nonselective agent (FCS 10%) was used (Fig. 9). Results obtained in collagen gel contractility assays indicate that after 48 and 72 h incubations, a melatonin concentration of 1 μM significantly reduces the collagen percentage well area (Fig. 9) indicating cell contraction. Furthermore, a reduction in gel size was also seen after 72 h incubations of ihaTPMCs with a melatonin dose of 0.1 nM (Fig. 9).

4. Discussion

This study provides evidence for a novel cell population target for melatonin in the testis, namely peritubular myoid cells. Studies in TPMCs isolated from immature hamsters show a plethora of actions related to the regulation of the immunological and inflammatory status, secretory activity and contractile phenotype of these cells. Furthermore, high testicular concentrations of melatonin as well as thickness and a differential collagen fibers organization of the peritubular wall in 21 day-old hamsters imply *in vivo* relevance of the cellular studies.

We studied TPMCs from immature hamsters instead of adult animals based on four premises. First, previous reports indicate that the cytodifferentiation of TPMCs and maturation of the myoid cell contractile apparatus are dated around the end of the third week of age in rodents [25–27]. Second, testicular development includes an initial time period in which somatic and germ cells undergo proliferation and differentiation that will result in the first wave of spermatogenesis and will determine the continuous future production of sustainable sperm in mature testis [28]. Third, our current determinations indicate that testicular melatonin concentration in immature hamsters is 4-fold higher than in adult animals and finally, four, our present findings show that ihaTPMCs express MT1 and MT2 receptors and consequently, highlight that these cells of the tubular compartment are target of melatonin action. In this context, melatonergic receptors have previously been described by our group and other authors in several cell populations of the testis including Leydig cells, Sertoli cells, and immune cells (T helper cells, lymphocytes, granulocytes, mast cells and monocytes/macrophages) [1].

In the wild, hamster mating season begins in spring and continues through the summer [29]. Thus, in nature, early cohorts of immature pups will be exposed to the long days of summer, whereas later cohorts of immature hamsters will be under the shorten days of the autumn. Interestingly, in the Syrian hamster, photoperiod does not influence the timing of the onset of puberty. Thus, even though Syrian hamsters would be artificially born and raised on short days to mimic the restrictive photoperiodic conditions of winter, the reproductive system would still develop normally for the first 5–8 weeks of life [30]. Bearing this mind, in this work, hamsters were born and kept under a long day photoperiod until day 21 when they were sacrificed and testes were removed to purify TPMCs and investigate the potential role of melatonin in testicular development.

Melatonin, as a photoperiod transducer hormone synthesized in the pineal gland, passively diffuses from pinealocytes into the blood almost entirely at night [2]. In addition to the pineal-derived melatonin released into circulation that reaches the testis [1], the ability of the male gonad to locally synthesize melatonin has also been demonstrated in several mammals and birds [31–34]. Particularly, Leydig cells and testicular mast cells have been reported as site in the testis in which melatonin synthesis takes place [1]. Now, we detected the expression of the two key enzymes involved in the synthesis of melatonin, arylalkylamine *N*-acetyltransferase (AANAT) and hydroxyindole-*O*-

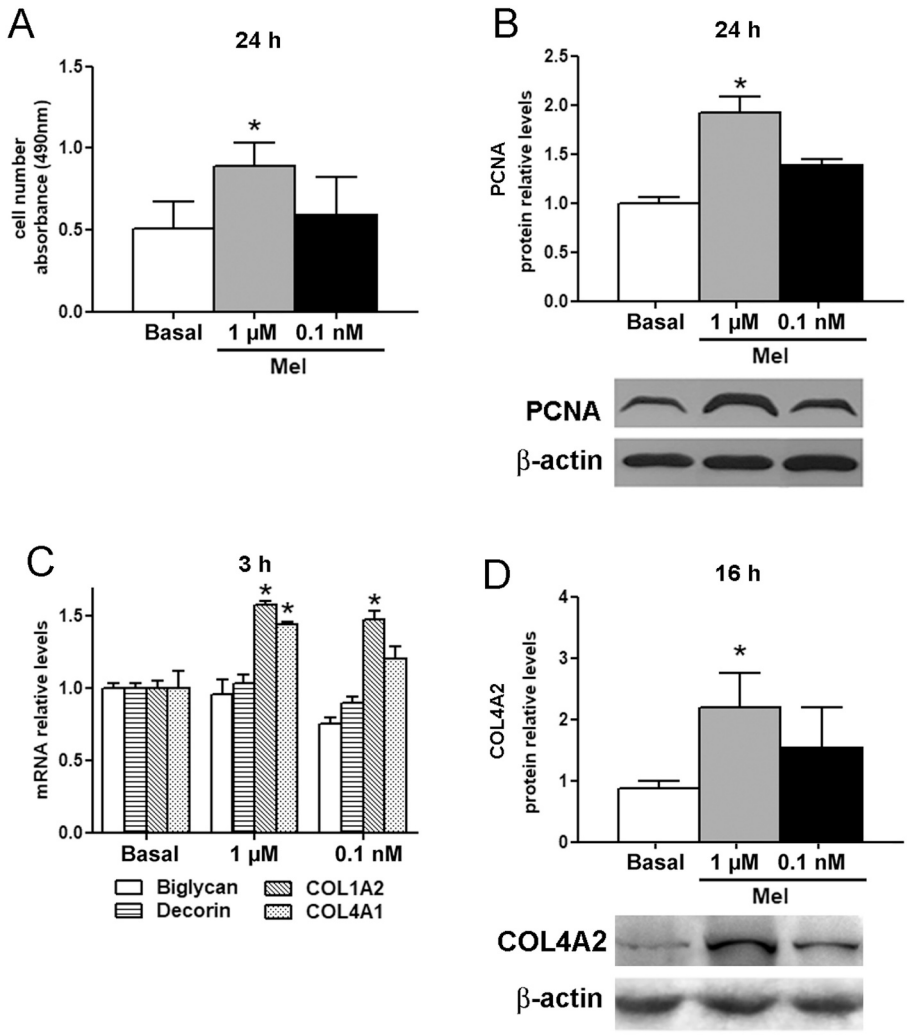

Fig. 6. Role of melatonin in cell proliferation and the expression of ECM components in testicular peritubular myoid cells from immature hamsters

(A) Testicular peritubular myoid cells from immature hamsters (ihaTPMCs) were incubated in RPMI1640 medium in the presence or absence of melatonin (Mel, 1 μM and 0.1 nM) for 24 h. Cell viability was determined using a colorimetric cell viability assay. Values obtained from the different experimental groups were expressed as cell number absorbance (490 nm) and averaged (mean ± S.E.M, $n = 8$). *$p < 0.05$. Student-Newman–Keuls test.

(B) and (D) ihaTPMCs were incubated in RPMI1640 medium in the presence or absence of melatonin (Mel, 1 μM and 0.1 nM) for 16 (D) and 24 h (B). Proliferating cell nuclear antigen (PCNA) (B) and collagen type IV alpha 2 chain (COL4A2) (D) protein levels were determined by immunoblotting. Bar plot graphs represent the mean ± S.E.M. and show the quantification by densitometry of the bands obtained from one of three experiments performed in different cell preparations that showed similar results (see Supplementary Table 3). Results are expressed as fold change relative to the control (basal conditions) and normalized to β-actin. *$p < 0.05$. Student-Newman–Keuls test. Immunoblots shown are representative.

(C) ihaTPMCs were incubated in RPMI1640 medium in the presence or absence of melatonin (Mel, 1 μM and 0.1 nM) for 3 h. Biglycan, decorin, collagen type I alpha 2 chain (COL1A2) and collagen type IV alpha 1 chain (COL4A1) mRNA levels were determined by RT-qPCR using GAPDH as the housekeeping gene. Bar plot graph represents the mean ± S.E.M. of the mRNA levels obtained from one of three-four independent experiments that showed similar results (see Supplementary Table 3). Results were analyzed using the mathematical model of Pfaffl. *$p < 0.05$. Student-Newman–Keuls test.

methyltransferase (HIOMT), in testis of immature hamsters as well as in primary cultures of ihaTPMCs (Supplementary Fig. 1).

As previously mentioned, we found that in testes of immature hamsters melatonin concentration is higher than in testes of adult animals. These variations in testicular melatonin concentration during sexual development could derive from alterations in its local synthesis and/or from changes in its pineal gland synthesis, release to circulation and uptake by the testis. On the contrary, melatonin content is lower in testes of immature animals than in testes of adult hamsters. It is important to mention that testicular weight in immature hamsters represents only 4% of the gonad weight in adult animals. These changes of the testicular weight during sexual development explain why testicular melatonin content is significantly higher in testes of adult animals than in testes of immature hamsters in spite of testicular melatonin concentration being markedly increased in gonads of 21 day-old hamsters.

Considering the circulating and testicular melatonin concentrations described in hamsters in previous reports and in this study [2,9], and bearing in mind the testicular tissue density, melatonin doses ranging from 0.1 to 10.0 nM can be considered physiological concentrations. Therefore, in the present work, primary cultures of ihaTPMCs were incubated with or without a 0.1 nM physiological dose of melatonin.

We also tested the effects of a supraphysiological dose of melatonin on ihaTPMCs. Melatonin is commonly used for the treatment of sleep disorders in a dose range from 3.0 to 10 mg per day. Because bioavailability in humans after a daily oral administration of melatonin has previously been documented to be between 3 and 33% of the initial dose [35–38], circulating levels of melatonin after its absorption, distribution, metabolism and excretion would range from 0.075–2.80 μM. Therefore, an average 1 μM supraphysiological concentration of melatonin has been chosen to resemble the potential impact of daily oral supplementation with this indolamine that might produce a pharmacologic effect in TPMCs and, consequently, alter testicular functionality.

Immunocytochemical analysis revealed that almost 100% of cells in our primary cultures of ihaTPMCs exhibited positive staining for α-SMA, a specific marker which has already been reported to be expressed in the developing TPMCs in the first few days after birth [27]. Absence of expression of certain proteins (i.e SOX9 and AMH markers of Sertoli cells, VASA marker of germ cells and StAR marker of Leydig cells) indicates that primary cultures of ihaTPMCs are not contaminated with other cell populations of the testis.

In addition, PCR and immunoblotting data further revealed that ihaTPMCs resemble TPMCs from other species in terms of their capacity to express GDNF, immunological and inflammatory factors (COX2, IL1β, NLRP3), other contractile markers (calponin, MYH-11), and their ability to produce ECM components (biglycan, decorin, collagen) [39–45].

Previously, our group established that the roles of PMCs in the testis go beyond sperm transport and include, among others, immunological contributions which might be related to sterile inflammation in male subfertility and infertility [23,46–50]. Taking into account this background and the large body of evidence about antiinflammatory actions of melatonin in different tissues and cells [51], primary cultures of ihaTPMCs were incubated in the presence of absence of melatonin and

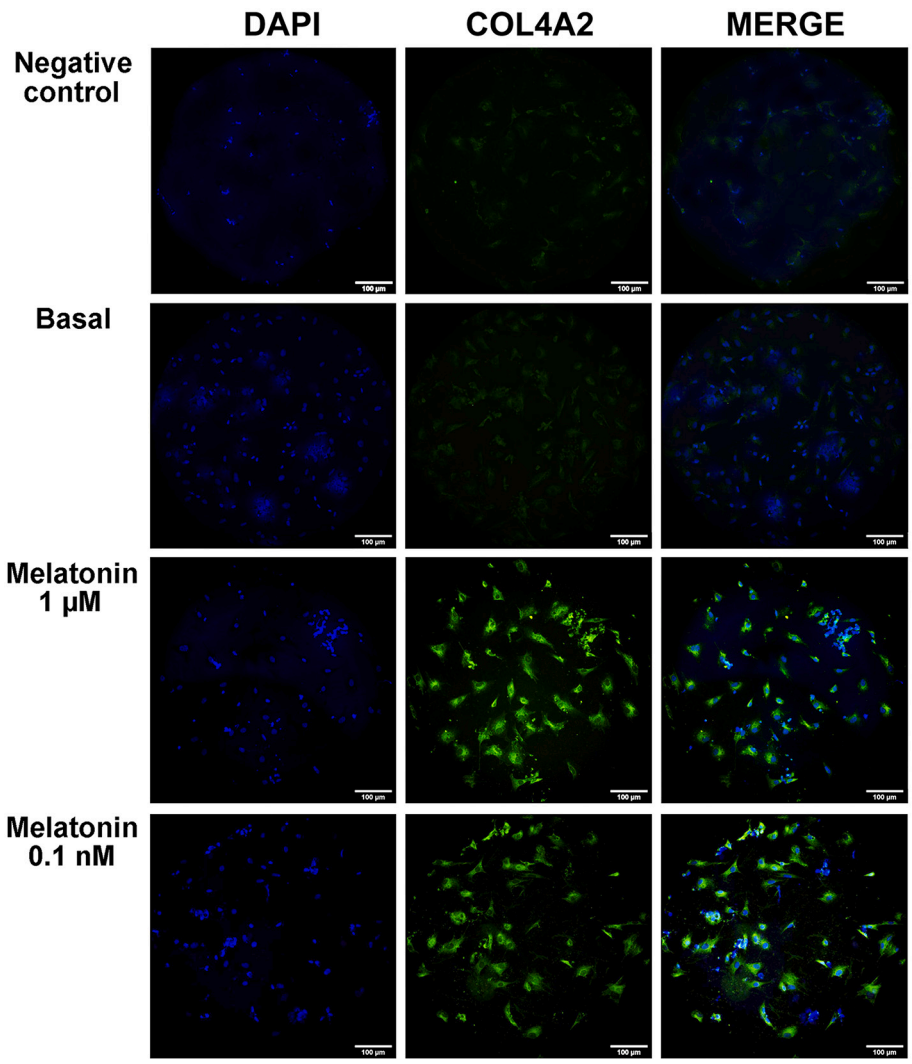

Fig. 7. Role of melatonin in COL4A2 expression in testicular peritubular myoid cells from immature hamsters.
Testicular peritubular myoid cells from immature hamsters (ihaTPMCs) were incubated in RPMI1640 medium in the presence or absence of melatonin (Mel, 1 µM and 0.1 nM) for 16 h. Collagen type IV alpha 2 chain (COL4A2) immunostaining (green) was detected by immunofluorescence.
Weak reaction was observed when ihaTPMCs incubated in the absence of melatonin were immunostained with the specific COL4A2 antibody (Basal) or when, alternatively, the first antibody was omitted and cells were incubated only with normal non-immune serum and the conjugated antibody (Negative control). Bar, 100 µm.
Nuclei were counterstained with DAPI (blue) and the merged image is shown (right panel). Bar, 100 µm.

the expression of inflammatory compounds was assayed. This indolamine decreased the expression of the proinflammatory markers inflammasome NLRP3 and cytokine IL1β. It is important to mention that the NLRP3 inflammasome is a multimeric protein complex that initiates an inflammatory response and triggers the release of proinflammatory cytokines [52]. In fact, secretion of the cytokine IL1β depends on the activation of the NLRP3 inflammasome pathway [53,54]. In the testis, we recently reported a significant decrease in the local gonadal concentration of melatonin in aged Syrian hamsters that is accompanied by increased local inflammatory processes [9]. We also established that melatonin levels in testes from men with altered spermatogenesis of unknown aetiology inversely correlates with specific markers of inflammation [3], and that melatonin daily oral supplementation in those patients with idiopathic infertility attenuates testicular inflammation [10].

Melatonin also exhibits antiinflammatory actions on human testicular macrophages decreasing the expression of TNFα and IL1β in this immune cell population [3].

Although prostaglandins are compounds known as mediators of inflammation, unexpectedly, we found that in ihaTPMCs melatonin upregulated the expression of COX2, the inducible isoform of the key enzyme responsible for production of prostaglandins and other eicosanoids [55]. In the last decades, in addition to their inflammatory actions, prostaglandins have also been associated to the regulation of a diversity of physiological systems. Regarding the rodent testis, it has been described that certain prostaglandins act as physiological mediators implicated in the modulation of steroidogenic function in Leydig cells [56–58], energy metabolism in Sertoli cells [58,59], and contractile ability in human TPMCs [11,12,23,43,60,61]. Therefore, we could speculate that those prostaglandins presumably secreted from ihaTPMCs in response to melatonin act as autocrine and/or paracrine regulatory factors more than as inflammatory triggers in the testis.

We also found that melatonin stimulates the mRNA and protein expression of GDNF in ihaTPMCs. GDNF is mostly secreted by Sertoli cells [62] and it is already known that melatonin regulates GDNF production in this cell population of the seminiferous tubules [63]. However, GDNF has also been described in TPMCs [12,23,64].

In testes of immature mammals, GDNF stimulates Sertoli cell proliferation [65], keeps spermatogonia undifferentiated and is an essential factor in the survival and renewal of SSCs [66]. In mice showing deficiencies in GDNF expression in TPMCs, a complete disruption of spermatogenesis was described suggesting that GDNF secreted by TPMCs would be essential for the preservation of a normal spermatogenesis [67,68]. Therefore, melatonin modulation of GDNF production and secretion in both somatic cell populations of the testis, Sertoli cells and TPMCs, could play a central role in the homeostatic control of the prepubertal gonad.

In this work, we detected that a supraphysiological concentration of melatonin stimulated ihaTPMCs proliferation but a physiological dose had no effect suggesting that this indolamine would not be relevant in

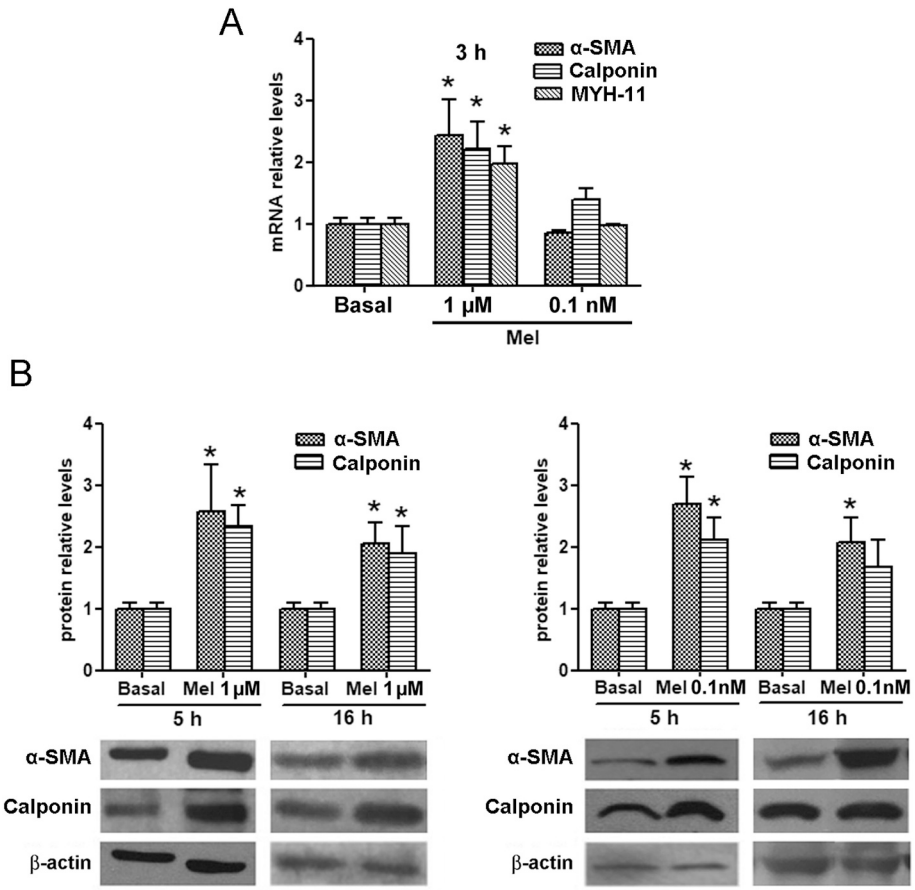

Fig. 8. Role of melatonin in the expression of contractile smooth muscle cells markers in testicular peritubular myoid cells from immature hamsters.
(A) Testicular peritubular myoid cells from immature hamsters (ihaTPMCs) were incubated in RPMI1640 medium in the presence or absence of melatonin (Mel, 1 μM and 0.1 nM) for 3 h. α-smooth muscle actin (α-SMA), calponin and myosin heavy chain 11 (MYH-11) mRNA levels were determined by RT-qPCR using GAPDH as the housekeeping gene. Bar plot graph represents the mean ± S.E.M. of the mRNA levels obtained from one of three-four independent experiments that showed similar results (see Supplementary Table 4). Results were analyzed using the mathematical model of Pfaffl. $*p < 0.05$. Student-Newman–Keuls test.
(B) ihaTPMCs were incubated in RPMI1640 medium in the presence or absence of melatonin (Mel, 1 μM, left panel and 0.1 nM, right panel) for 5 and 16 h. α-SMA and calponin protein levels were determined by immunoblotting. Bar plot graphs represent the mean ± S.E.M. and show the quantification by densitometry of the bands obtained from one of three experiments performed in different cell preparations that showed similar results (see Supplementary Table 4). Results are expressed as fold change relative to the control (basal conditions) and normalized to β-actin. $*p < 0.05$. Student's *t*-test. Immunoblots shown are representative.

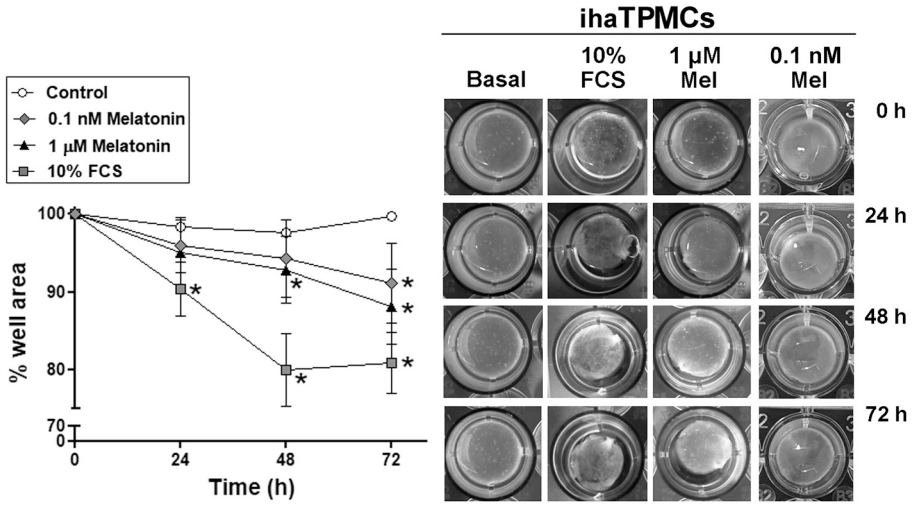

Fig. 9. Role of melatonin in contractility properties in testicular peritubular myoid cells from immature hamsters.
Testicular peritubular myoid cells from immature hamsters (ihaTPMCs) were incubated in RPMI1640 medium in the presence or absence of melatonin (Mel, 1 μM and 0.1 nM) for 24, 48 and 72 h.
10% FCS was used as positive control of cell contraction and medium without FCS served as basal (control) conditions. Representative collagen gel lattices are shown. Plot graph represents the mean ± S.E.M. of the results obtained from one of two-three independent assays (see Supplementary Table 5). Results are expressed as the change of matrix gel area in percentage compared with the corresponding control matrices (without stimulus). $*p < 0.05$. Student's t-test.

ihaTPMCs proliferation. However, melatonin supplementation is commonly used in the pediatric population for treatment of occasional sleeplessness and/or migraine [69,70], therefore pediatricians should take into consideration the potential implications of melatonin administration during childhood on TPMCs proliferation and, consequently, on testicular growth and physiology.

Although TPMCs proliferation shows certain relevance for the process of testicular tubular wall thickening [71,72], it has been postulated that, in fact, the ECM is the one that acts as a true driver of this process [73]. We observed that the thickness of the tubular wall is higher in testes of immature hamsters than in gonads of adult animals suggesting quantitative and/or qualitative changes in ECM during sexual development. Testicular ECM is composed of glycoproteins and polysaccharides. Collagen, decorin and biglycan are among the main fibers of testicular ECM [45,74–76]. We found that melatonin does not affect decorin and biglycan expression but significantly induces collagen type I and collagen type IV expression in ihaTPMCs. The six alpha chains of type IV collagen are organized into three different networks: alpha1/alpha2, alpha3/alpha4/alpha5, and alpha1/alpha2/alpha5/alpha6. Particularly, the alpha1/alpha2 network is expressed from birth in the

testis while the alpha1/alpha2/alpha5/alpha6 network is expressed by 5–6 weeks of age, and the alpha3/alpha4/alpha5 network by 2 months of age [75]. Thus, only the alpha1/alpha2 network seems to be essential for the onset of spermatogenesis. In this context, we found that melatonin stimulates the expression of both, COL4A1 and COL4A2 in ihaTPMCs which might partially explain the thickening of the tubular wall in immature testes.

In addition to the major thickness of the tubular wall in testes of immature hamsters compared to gonads of young adult animals, the tubular wall of immature hamsters showed heterogeneous birefringence when, trying to identify total collagen, testicular sections were stained with picrosirius red and examined under polarized light microscopy. In 21 day-old animals, it was possible to distinguish yellow-red birefringence collagen fibers with high, medium and low birefringence, and also zones of the tubular wall without birefringence. In our analysis, sometimes, even a single tubule showed parts of the wall with high birefringence, parts with medium-low birefringence and other parts without birefringence. In contrast, collagen fibers in the tubular wall of adult hamsters presented a more homogeneous birefringence but also a weaker one. Such discrepancies between immature and adult testes could be explained by the fact that while the testicular development in 21 day-old animals is ongoing, peritubular myoid cells in testes of adult hamsters are fully functional and essential to propel the tubular content to the rete testis. Therefore, at these stages of sexual development, a differential balance between the packaging and relaxation of the collagen fibers of the tubular wall might be required.

Previously, we described an aged-related thickening of the tubular wall in hamsters [9]. Interestingly, when aged hamsters were transferred from a long day to a short day photoperiod for 16 weeks resembling the restrictive light conditions of winter, both testicular melatonin concentration and the tubular wall thickness increased [9]. These results are in agreement with the significant increase in collagen expression observed in the current study in primary cultures of ihaTPMCs incubated in the presence of a physiological concentration of melatonin.

Numerous factors have been postulated to be involved in the regulation of the contractile function of PMCs, among them, prostaglandins [12,23,61,77], neurotransmitters [77,78] and sex hormones [79,80]. However, until now, no effect of melatonin on PMC contractility has been described. This study reveals a stimulatory effect of melatonin on the contractile ability of ihaTPMCs. Furthermore, expression of certain markers of contractile phenotype (α-SMA, calponin, MYH-11), evaluated by RT-qPCR and immunoblotting, was upregulated in primary cultures of ihaTPMCs treated with melatonin.

The contractility abilities of TPMCs seem to be interlinked with fertility. In men with impaired spermatogenesis, testicular melatonin concentration is diminished [10] and smooth muscle cells markers in TPMCs are often lost or reduced [61]. Consequently, high levels of melatonin and positive modulation of ihaTPMCs contraction by this indolamine at the time that both somatic and germs cells undergo stages of developmental change that will result in the first wave of spermatogenesis, could be crucial for a future continuous sperm production.

5. Conclusion

Our study established a novel potential target of melatonin in the testis, the TPMCs. The fact that melatonin inhibits immunological and inflammatory properties but stimulates contractile functions in ihaTPMCs let us to postulate a regulatory role of this indolamine in the developing testis. Because this study has been focalized to describe the role of melatonin in immature TMPCs, future investigations employing novel mechanistic approaches will be required to verify or discard this hypothesis. For example, immature rodents could be intraperitoneally injected with different doses of melatonin to study future sperm quality and quantity or, alternatively, knock in/knock out rodents could be used to evaluate the consequences of specific activation/inhibition of melatonin receptors in TMPCs.

TPMCs have a unique anatomical position. They are separated from Sertoli cells and spermatogonia only by the basal lamina. This anatomical proximity suggests multiple interactions between TPMCs-Sertoli cells-SCCs via secreted factors. Therefore, the secretion of certain factors in the immature testis in response to melatonin, i.e. GDNF and prostaglandins, which can act as autocrine and/or paracrine regulators, provides the basis for upcoming research targeting melatonin as a central player warranting a normal and sustainable production of sperm.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2022.130187.

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Author contributions

E.R, S.P.R., Y.E.T and M.E.M.: methodology, data curation, writing-original draft, writing-review.

M.M.M.deT., R.S.C. and A.M.: writing original- draft and writing-review.

S.P.R.,M.E.M. and M.B.F: conceptualization, methodology, data curation, formal analysis, funding acquisition, project administration, writing-original draft and writing-review.

Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

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