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Expression of melatonin (MT1, MT2) and melatonin-related receptors in the adult rat testes and during development

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Summary

It is well known that melatonin provokes reproductive alterations in response to changes in hours of daylight in seasonally breeding mammals, exerting a regulatory role at different levels of the hypothalamic–pituitary–gonadal axis. Although it has also been demonstrated that melatonin may affect testicular activity in vertebrates, until now, very few data support the hypothesis of a local action of melatonin in the male gonads. The aim of this study was to investigate whether MT1, MT2 melatonin receptors and the H9 melatonin-related receptor, are expressed in the adult rat testes and during development. A semi-quantitative RT-PCR method was used to analyse the expression of MT1, MT2 and H9 receptors mRNAs in several rat tissues, mainly focusing on testes during development and adult life. Our results provide molecular evidences of the presence of both MT1 and, for the first time, MT2 melatonin receptors as well as of the H9 melatonin-related receptor in the examined tissues, including adult testes. During development MT1 and MT2 transcripts are expressed at lower levels in testes of rats from 1 day to 1 week of age, lightly increased at 2 weeks of age and remained permanently expressed throughout development until 6 months. These data strongly support the hypothesis that melatonin acts directly in male vertebrate gonads suggesting that rat testes may be a suitable model to verify the role of indolamine in vertebrate testicular activity.

Keywords: Development, Melatonin receptors, Rat, Reproduction, Testes

Introduction

Melatonin, an indolamine secreted by the pineal gland during the hours of darkness, plays a central role in a variety of important physiological processes, including reproduction (Morgan *et al.*, 1994). In particular, melatonin influences the timing of mammalian circadian rhythms and regulates the reproductive changes that occur in response to hours of daylight in seasonally breeding mammals (for reviews, Reiter, 1991a–c; Bartness *et al.*, 1993).

It is well known that melatonin elicits its effects via specific receptors which are coupled to G protein and are characterized by a seven transmembrane-spanning domain (Dubocovich *et al.*, 1997). To date, three high affinity melatonin receptor subtypes have been identified by molecular cloning studies: MT1 (previously termed mel 1a) and MT2 (mel 1b) melatonin receptors (Reppert *et al.*, 1995, 1996), while an additional subtype, mel 1c receptor, that is not expressed in mammals, has been found in *Xenopus*, chickens and zebrafish (Wiechmann *et al.*, 1999). In addition, there is evidence for a nanomolar melatonin binding site in the brain and kidneys of hamsters

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(Duncan *et al.*, 1988; Pickering & Niles, 1990; Paul *et al.*, 1999); the quinine reductase enzymes called MT3.

Melatonin receptors have been detected in several areas of central nervous system, including suprachiasmatic nuclei, hippocampus, cerebellar cortex, prefrontal cortex, basal ganglia, substantia nigra, ventral tegmental area, nucleus accumbens and retinal horizontal, amacrine and ganglion cells (Pandi-Perumal *et al.*, 2008).

Apart from the well characterized role of melatonin in the hypothalamus and pituitary, melatonin receptors have been also identified in several peripheral tissues (Pandi-Perumal *et al.*, 2008). Among these, an action at testicular level has recently been suggested. In fact, it has been demonstrated that melatonin inhibits testosterone production by Leydig cells of mammals (Olivares *et al.*, 1989; Niedziela *et al.*, 1995; Valenti *et al.*, 1995, 1999; Kus *et al.*, 2002) both in *in vivo* and in *in vitro* experiments. Moreover, melatonin binding sites in the gonads of several vertebrate species have been demonstrated (Vera *et al.*, 1997; Shiu *et al.*, 2000; Clemens *et al.*, 2001; Kus *et al.*, 2002) and melatonin receptors coupled via a pertussis toxin-sensitive G-protein are present in adult rat Leydig cells (Valenti *et al.*, 1997).

In order to acquire more information concerning the molecular evidence on melatonin receptor(s) in the rat, we investigated the expression of melatonergic receptors in testes and in different tissues. Here we report the cloning of MT1 and, for the first time, MT2 melatonin receptors, other than a melatonin-related orphan H9 receptor (also known as GPR50) (Gubitz & Reppert, 1999) from adult rat testes. Finally we analysed the expression of MT1 and MT2 mRNAs during development.

Materials and methods

Animals and tissue collection

Sprague–Dawley rats (*Rattus norvegicus*) were housed under definite conditions (12D:12L) and they were fed standard food and provided with water ad libitum.

Animals at several stages of development, 1-day-, 3-day-, 1-week- and 2-week-old (infantile); 3-week- and 1-month-old (prepubertal); and 3-month-, 6-month-old (adult; three animal/each time) were killed by decapitation under ketamine anesthesia (100 mg/kg i.p.) in accordance with local and national guidelines covering experimental animals. Testes were dissected from rats at each stages, while from adult animals were dissected muscle, adrenal glands, spleen, exorbital glands, brain, lung and testes.

Specimens were collected at noon, according to Sallinen *et al.* (2005), which showed no significant difference in the expression of MT1 and MT2 receptor mRNAs between midnight and noon. All tissues were quickly frozen by immersion in liquid nitrogen and stored at –80 °C until RNA extraction. In addition, some pieces of these tissues were fixed in Bouin's fluid for histological analysis.

RNA and first strand cDNA synthesis

Total RNA was isolated from different rat tissues according to Chomczynski & Sacchi (1987). The tissues were homogenized in 10 ml lysis buffer (guanidine thiocyanate 4 M, sodium citrate 25 mM pH 7.0, 0.5% sarcosyl and β_2-mercaptoethanol 0.1 M) followed by extraction with phenol acid:chloroform:isoamyl alcohol (50:49:1) and by precipitation with isopropanol. The resulting RNA pellet was dissolved in water.

Three microgrammes of total RNA were reverse-transcribed into cDNA using 40 ng random hexameric primers and 100 U Superscript III RT enzyme (Invitrogen) according to manufacturer's instruction.

PCR cloning and sequencing

Based on the published sequence of Rattus norvegicus melatonin receptors (MT1–MT2; EMBL data bank accession number AF130341–XM001074702) and melatonin-related receptor (H9; EMBL data bank accession number U52218) mRNAs, primers were designed to amplify cDNA in different tissues of Rattus norvegicus. For MT1 the primers were as follows: MT1 forward = 5′-CTACATTTGCCACAGTCTC-3′; RMEL1 reverse = 5′-CATATCCTTAAGTAGCAGAA AG-3′. For MT2 the primers were as follows: MT2 forward = 5′-CATGCTCCCCTCTACATCAG-3′ MT2 reverse = 5′-CAGGCGTAGCTTTCTCTCAG-3′. For H9 the primers were as follows: H9 forward = 5′-TC CAGTACAATGCGGATCTTC-3′; H9 reverse = 5′-CAG CAAACTGGTTGTCAGGAT-3′. PCR reactions (25 μl volume) were performed in the presence of 3 μl of cDNA, 1.5 U *Taq* polymerase (Invitrogen) and its buffer 1×, $MgCl_2$ 1.5 mM, dNTP 0.2 mM and 5 pmol of each primer. PCR amplification was carried out for 43 cycles (MT1–MT2) or 30 cycles (H9) with denaturing at 94 °C for 30 s, annealing at 56 °C (MT1) or 58 °C (MT2–H9) for 45 s and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. Amplification products were electrophoresed on 1.2% agarose. The expected DNA fragments (253 bp for MT1, 267 bp for MT2 and 301 bp for H9) were purified by QIAGEN gel extraction kit (QIAGEN) and cloned into the pGemT easy vector according to the manufacturer's instruction. Sequence determination was performed on both strands with the dideoxynucleotide chain termination method (Sanger *et al.*, 1974). The deduced aminoacid sequences were compared with the EMBL Genbank database.

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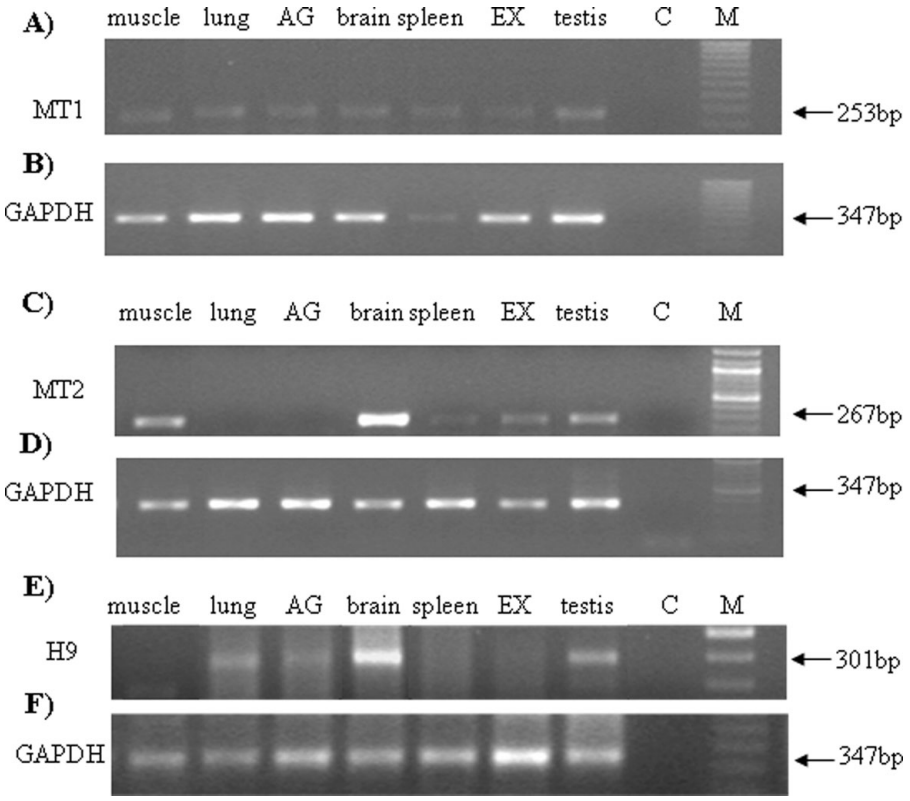

Figure 1 Agarose gel electrophoresis of representative RT-PCR products. (*A*) MT1 mRNA expression in several rat tissues; (*C*) MT2 mRNA expression in several rat tissues; (*E*) H9 mRNA expression in several rat tissues. AG = adrenal gland; EX = exorbital gland. (*B*, *D*, *F*) GAPDH expression on the same samples used as control. C = control PCR; M = molecular marker.

PCR and quantification

The expression of MT1 mRNA in *Rattus norvegicus* testes at different stages of development, as well as MT1, MT2 and H9 mRNAs in several tissues, were evaluated using a semiquantitative RT-PCR, as described above. An appropriate region (347-bp fragment) of *Rattus norvegicus* GAPDH transcript (EMBL databank accession number NM_017008), was amplified with specific oligonucleotide primers (GAPDH forward: 5′-GCATCCTGCACCACCAACT-3′, GAPDH reverse: 5′-GCCTGCTTCACCACCTTCTT-3′) and used as control. GAPDH amplification was carried out for 30 cycles with denaturing at 94 °C for 30 s, annealing at 58 °C for 45 s and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. The expected RT-PCR products were separated by agarose gel (1.2%) electrophoresis and the quantization was performed using GELDOC 1.00-UV fluorescent gel documentation system (BioRAD).

Statistical analysis

Data obtained from three separate sets of RT-PCR experiments were analysed using one-way analysis of variance (ANOVA). If the effects were significant, the Duncan's test was used for post-ANOVA multiple comparisons ($p < 0.05$). All data are presented as the mean ± standard error mean (SEM).

Results

Expression of MT1, MT2 and H9 mRNA in several rat tissues

Total RNA from several rat tissues (muscle, adrenal glands, spleen, exorbital glands, brain, lung and testes) was subjected to RT-PCR assay using specific primers for the membrane melatonin receptors (MT1 and MT2) and melatonin related receptor (H9).

Agarose gel electrophoresis of RT-PCR products using oligonucleotides for MT1 showed a single cDNA band of the expected size (253 bp) in all the analysed tissues (Fig. 1*A*). Differently, using oligonucleotides for MT2, a single cDNA band of the expected size (267 bp) was detected in muscle, brain, spleen, exorbital glands and testes, while no specific band was obtained in adrenal glands and lung (Fig. 1*C*).

Analysis of RT-PCR products using primers for H9, detected a specific band in brain, testes, adrenal glands

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Table 1 Performance of MT1, MT2 and H9 transcripts in several rat tissues.

	Muscle	Lung	Adrenal glands	Brain	Spleen	Exorbital glands	Testes
MT1	+	+	+	+	+	+	+
MT2	+	−	−	+	+	+	+
H9	−	+	+	+	−	−	+

Expression of melatonin receptors (MT1, MT2 and H9) in different tissues.

and lung. This product had the predicted size (301 bp). No specific bands were obtained in the other tissues (Fig. 1*E*).

For each analysis, the amount of cDNA in each line was controlled using specific primers for the GAPDH (Fig. 1*B*, *D*, *F*).

In Table 1 is reported the different expression of MT1, MT2 and H9 transcripts in the several tissues analysed. All the transcripts are widely distributed in the whole brain as well as in the majority of peripheral rat tissues.

An *in situ* hybridization analysis was carried out to localize MT1, MT2 and H9 transcripts at testicular level; due to the low expression of the three transcripts in the testes, no signals have been detected (data not shown).

Expression of MT1 and MT2 mRNA in rat testes during development

In order to evaluate a possible role of melatonin in rat testes during *post natal* development, the expression of melatonin receptor (MT1 and MT2) transcripts was analysed by RT-PCR. The assessment demonstrated the persistent expression of both the mRNAs in rat testes at all the examined time points, from 1 day to 6 months of age (Figs. 2*A*, 3*A*). In detail, MT1 and MT2 transcripts were expressed at lower levels in testes of rats from 1 day to 1 week of age, than they lightly increased at 2 weeks of age and remained permanently expressed throughout development until 6 months. The amount of cDNA in each line was controlled using specific primers for GAPDH (Figs. 2*B*, 3*B*). In Figures 2*C* and 3*C* the relative ratios of the densitometry of *Rattus norvegicus* MT1 and MT2 cDNA respectively versus GAPDH cDNA are reported ($p < 0.05$).

Discussion

The involvement of melatonin in the reproductive process is well documented (Reiter, 1980, 1981; Arendt, 1986; Hazlerigg *et al.*, 2001; Bittman *et al.*, 1985; Cassone *et al.*, 1993). Melatonin provokes reproductive alterations in response to changes in hours of daylight in seasonally breeding mammals (Reiter, 1991a–c; Bartness *et al.*, 1993). It has been shown that melatonin can exert its regulatory role at various levels of the hypothalamic–pituitary–gonadal axis (Stankov & Reiter, 1990; Aleandri *et al.*, 1996; Vanecek & Watanabe, 1998). The presence of melatonin receptors in the brain's suprachiasmatic nuclei and in the pituitary pars tuberalis indicates the hypothalamic–pituitary axis as the main site through which melatonin may modulate reproductive functions in vertebrates (Anton-Tay *et al.*, 1968; Weaver, 1999). However, it has been determined that pineal-derived circulating melatonin is taken up by peripheral tissues including the testes (Wurtman & Axelrod, 1966; Cardinali *et al.*, 1979; Reiter, 1981, 1991b). In addition, the ability of the testes to locally synthesize melatonin in a non-photoperiodic mammal (rat) and a bird (quail) has also been demonstrated (Tijmes *et al.*, 1996; Kato *et al.*, 1999; Fu *et al.*, 2001; Stefulj *et al.*, 2001).

Many authors have demonstrated the existence of a mutual relationship between the pineal gland and the testes (for review see Reiter, 1980, 1981). *In vitro* experiments in hamster (Niedziela *et al.*, 1995) and rat (Valenti *et al.*, 1995) indicated that melatonin inhibits testosterone secretion by Leydig cells, while Wu *et al.* (2001) observed that melatonin suppresses steroidogenesis through specific binding sites in hCG/cAMP analogue-treated MA-10 mouse Leydig cells.

Moreover, the characterization of melatonin receptors in testes has been mainly assayed by radioreceptor assay using 2-[^{125}I]-iodomelatonin-binding analysis (Vera *et al.*, 1997; Shiu *et al.*, 2000; Clemens *et al.*, 2001); recently Frungieri *et al.* (2005), using RT-PCR and western blot techniques, have demonstrated the presence of MT1 but not MT2 sites in hamster testes. In addition, Reppert *et al.* (1996) have cloned an orphan G protein-coupled receptor, designed H9 and also known as GPR50 (Gubitz & Reppert, 1999), from a human pituitary cDNA library. The H9 cDNA encodes a protein that is 45% identical at the amino acid level to human MT1 and MT2 melatonin receptors. It has also been reported an extensive and detailed analysis of melatonin-related receptor mRNA expression in the mouse central nervous system and various peripheral tissues including testes; the results suggested for this type of receptor a conserved function in neuroendocrine regulation and a potential role in coordinating physiological response in the CNS and peripheral tissues (Drew *et al.*, 2001). In this respect, it is also to be highlighted that rat testis development is regulated by maternal melatonin as well as by the beginning of the own melatonin rhythm of the offspring (Diaz *et al.*, 1999).

The present study provides evidence for the existence of both MT1 and MT2 melatonin receptor transcripts other than the H9 melatonin-related receptor transcript, in adult rat testes and during development.

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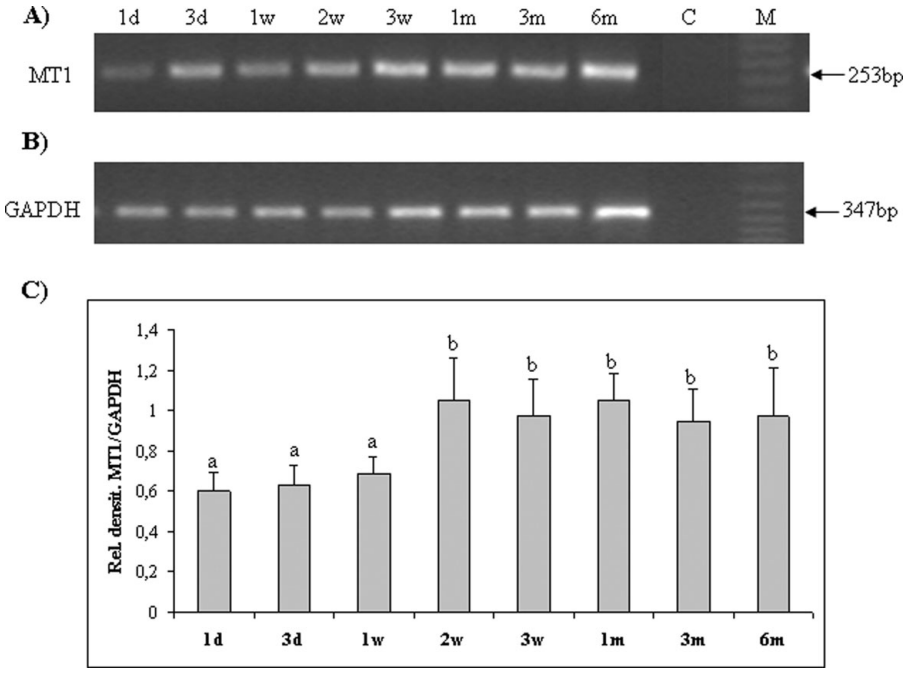

Figure 2 Agarose gel electrophoresis of representative RT-PCR products. (*A*) MT1 mRNA expression in rat testicular samples from 1-day-old, 3-day-old (d), 1-week-old, 2-week-old, 3-week-old (w), 1-month-old, 3-month-old, 6-month-old (m) old rats. (*B*) GAPDH expression on the same samples used as control. C = control PCR; M = molecular marker. (*C*) Relative densitometry of the MT1 and GAPDH band measured with Gel Doc. a vs b = $p < 0.05$.

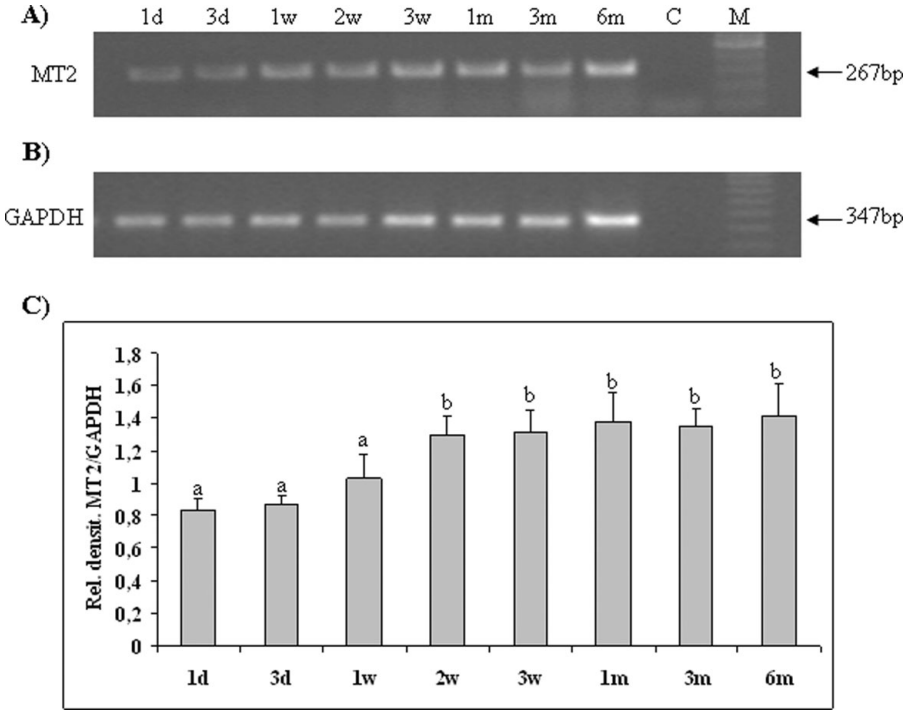

Figure 3 Agarose gel electrophoresis of representative RT-PCR products. (*A*) MT2 mRNA expression in rat testicular samples from 1-day-old, 3-day-old (d), 1-week-old, 2-week-old, 3-week-old (w), 1-month-old, 3-month-old, 6-month-old (m) old rats. (*B*) GAPDH expression on the same samples used as control. C = control PCR; M = molecular marker. (*C*) Relative densitometry of the MT2 and GAPDH band measured with Gel Doc. a vs b = $p < 0.05$.

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As shown in Table 1, our results indicate that MT1, MT2 and H9 transcripts are widely distributed in the whole brain as well as in peripheral rat tissues (muscle, adrenal glands, spleen, exorbital glands, lung and testes). Interestingly, MT1 is mostly expressed in the majority of peripheral tissues while MT2 is expressed in the above mentioned tissues with the exception of the adrenal glands and lung (Fig. 1). MT1, MT2 and H9 receptors are coexpressed in the brain, which represents the main target of melatonin action and, surprisingly, in the testes of the rat (Table 1).

The *in situ* hybridization performed in rat testes failed to show the localization of MT1, MT2 and H9 mRNAs probably because of the low expression of their mRNAs in the majority of the tissues examined (data not shown).

Lastly, RT-PCR analysis showed that the level of MT1 and MT2 transcripts increased in the testes of rats at 2 weeks of age and remained permanently expressed until six months. These results, in accordance with those previously reported by Diaz *et al.* (1999), support the idea that melatonin exerts a role during rat development; in particular, the increasing levels of receptors mRNAs at the onset of puberty suggest that the expression of these receptors in the pups is mainly associated to their own melatonin rhythm, fully established in this period.

Until now, the only molecular evidence of melatonin receptor subtypes in vertebrate testes is MT1 described by Frungieri *et al.* (2005) in hamsters. The presence of MT1, MT2 and H9 transcripts in the rat testes strongly supports a role of the indolamine in the male gonads.

It is worth remembering that the physiological role of melatonin in the testes is still not fully understood. Probably the indoleamine represents an as-yet-unrecognized local inhibitory control of the testicular function. An inhibitory role of melatonin in vertebrate testicular activity is supported by many studies. Ultrastructural changes in Leydig cells have been demonstrated in rat after pinealectomy (Kus *et al.*, 2000) and in mice after melatonin treatment for 22 consecutive days (Redins *et al.*, 2002). A prolonged exposure to indolamine reduces the number and affinity of melatonin receptor binding sites on rat Leydig cell membranes and causes hypersensitization to LH challenge, resulting in higher cAMP and testosterone secretion (Valenti *et al.*, 2001). In addition, our recent studies, using the testes of the frog *Rana esculenta* as a model, support the hypothesis of an inhibitory role exerted by melatonin in vertebrate testes. In fact, we highlighted that melatonin inhibits both the cellular proliferation of primary spermatogonia (d'Istria *et al.*, 2003) and the proliferation and/or differentiation of mast cells (Izzo *et al.*, 2004) induced *in vivo* and *in vitro* by 17β-estradiol. In addition, our observations indicate that melatonin might act on Leydig cells, as after *in vivo* or *in vitro* melatonin treatments many Leydig cells display degenerative morphological changes (d'Istria *et al.*, 2004).

This study provides molecular evidence of the presence of both MT1 and, for the first time, MT2 melatonin receptors other than of H9 melatonin-related receptor in adult rat testes and during development. These data strongly support the hypothesis that melatonin acts directly in male vertebrate gonads. However, further studies are required to assess the biological relevance of melatonin in testicular regulation. Consequently, a suitable model to verify the role of indolamine in vertebrate testicular activity may be provided by rat testes.

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