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RESEARCH PAPER

The end of a myth: cloning and characterization of the ovine melatonin MT_2 receptor

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Background and purpose: For many years, it was suspected that sheep expressed only one melatonin receptor (closely resembling MT_1 from other mammal species). Here we report the cloning of another melatonin receptor, MT_2, from sheep.
Experimental approach: Using a thermo-resistant reverse transcriptase and polymerase chain reaction primer set homologous to the bovine MT_2 mRNA sequence, we have cloned and characterized MT_2 receptors from sheep retina.
Key results: The ovine MT_2 receptor presents 96%, 72% and 67% identity with cattle, human and rat respectively. This MT_2 receptor stably expressed in CHO-K1 cells showed high-affinity 2[^{125}I]-iodomelatonin binding (K_D = 0.04 nM). The rank order of inhibition of 2[^{125}I]-iodomelatonin binding by melatonin, 4-phenyl-2-propionamidotetralin and luzindole was similar to that exhibited by MT_2 receptors of other species (melatonin > 4-phenyl-2-propionamidotetralin > luzindole). However, its pharmacological profile was closer to that of rat, rather than human MT_2 receptors. Functionally, the ovine MT_2 receptors were coupled to G_i proteins leading to inhibition of adenylyl cyclase, as the other melatonin receptors. In sheep brain, MT_2 mRNA was expressed in pars tuberalis, choroid plexus and retina, and moderately in mammillary bodies. Real-time polymerase chain reaction showed that in sheep pars tuberalis, premammillary hypothalamus and mammillary bodies, the temporal pattern of expression of MT_1 and MT_2 mRNA was not parallel in the three tissues.
Conclusion and implications: Co-expression of MT_1 and MT_2 receptors in all analysed sheep brain tissues suggests that MT_2 receptors may participate in melatonin regulation of seasonal anovulatory activity in ewes by modulating MT_1 receptor action.
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Keywords: melatonin; sheep; MT_2 receptor; cloning; pharmacology

Abbreviations: BSA, bovine serum albumin; CHO, Chinese hamster ovary cell line; ERK, extracellular signal-regulated kinase; GPCR, G protein coupled receptor; HA, haemagglutinin epitope; 4P-PDOT, 4-phenyl-2-propionamidotetralin; RACE, rapid amplification of cDNA 3′ and 5′ ends; RT-PCR, reverse transcriptase-polymerase chain reaction; ZT, zeitgeber time

Introduction

Melatonin is the pineal hormone that is secreted exclusively at night by the pineal gland (Arendt, 2005) and is implicated in a number of physiological functions. These include, among others, mood, sleep, circadian rhythms, the immune system and reproduction. In seasonally breeding animals, there is unequivocal evidence that melatonin, through its daily duration of secretion, is the primary transducer of photoperiodic information to the neuroendocrine axis (Malpaux, 2006). However, the mechanisms of action are poorly known and particularly the type of receptor mediating this effect has not yet been identified.

So far, three high-affinity melatonin receptor subtypes have been cloned. They have been classified as MT_1, MT_2 (previously known as Mel1a and Mel1b respectively) and Mel1c (Reppert *et al.*, 1996; Boutin *et al.*, 2005). All these subtypes display similar high binding affinity for melatonin (subnanomolar range) and the same rank of order for the binding of common ligands (Dubocovich, 1995; Dubocovich and Markowska, 2005). Structurally, high-affinity melatonin receptor subtypes define a distinct receptor family within the superfamily of G protein-coupled receptors (GPCRs), as they

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have been shown to be functionally coupled to both *Pertussis* toxin-sensitive and *Pertussis* toxin-insensitive G proteins (Morgan *et al.*, 1990; Drew *et al.*, 2002). A fourth melatonin binding site, MT_3, displaying lower affinity for melatonin (in the nanomolar range), was purified from human tissues using a biochemical approach and identified as the enzyme, quinone reductase 2 (Nosjean *et al.*, 2000). In mammals, MT_1 receptors are expressed in all species studied to date. By contrast, the situation is more complex concerning MT_2 receptors that have been found to be expressed in many mammalian species (human, rat, mice, see Reppert *et al.*, 1996 for details) but not in two species: several genera of hamsters (*Phodopus sungorus, Phodopus campbelli* and *Mesocricetus auratus*) (Weaver *et al.*, 1996) and sheep (Drew *et al.*, 1998; Barrett *et al.*, 2003; Migaud *et al.*, 2005), in which melatonin is a major regulator of seasonal physiology. In the Siberian hamster, the MT_2 receptor gene cannot encode a functional receptor, due to nonsense mutations in the coding region of the receptor cDNA (Weaver *et al.*, 1996). In sheep, only MT_1 receptors have been identified, cloned and characterized (Mailliet *et al.*, 2004) and have thus been thought to be involved in mediating all the effects of melatonin on seasonal reproduction. Indeed, MT_1 receptor mRNA is detected in the premammillary hypothalamus, the target structure of melatonin for its reproductive effects (Migaud *et al.*, 2005). In addition, a correlation has been observed between the frequency of a mutated allele of the gene of the MT_1 receptor and the intensity of seasonal anovulatory activity in ewes (Pelletier *et al.*, 2000). Interest in the MT_2 receptor was recently revived by two observations. First, Xiao and colleagues (2007) have reported a partial sequence for the ovine *MTNR1B* gene encoding the MT_2 receptor, indicating that this subtype might also be expressed in sheep, but no proof of the existence of a functional MT_2 receptor in this species was brought in this particular work. Second, recent studies suggested that MT_2 receptors may be preferentially engaged into heterodimers in cells co-expressing both MT_1 and MT_2 receptors (Ayoub *et al.*, 2004). Although the importance of this heterodimerization has to be established in native mammalian tissues, this reinforces the need to analyse jointly where MT_1 and MT_2 receptors are expressed and how their expression is regulated.

In order to check whether the absence of the ovine MT_2 receptor mRNA was due to technical cloning difficulties, we tested numerous reverse transcriptase-polymerase chain reaction (RT-PCR) conditions. Using a thermo-resistant reverse transcriptase, we succeed in cloning the complete cDNA of the MT_2 receptor from sheep retina. After stable expression of this receptor in CHO-K1 cells, we have pharmacologically and functionally characterized the ovine MT_2 receptor, using as reference compounds, luzindole and 4-phenyl-2-propionamidotetraline (4P-PDOT) (Jockers *et al.*, 2008). Interestingly, these two antagonists distinguished between ovine MT_1 and MT_2 receptors. Real-time PCR also allowed the comparison of the expression of MT_1 and MT_2 receptors in different sheep brain tissues. Obviously, the demonstration that sheep possesses functional MT_2 receptors changes our understanding of melatonin physiology in these species. As sheep is the preferred species for *in vivo* studies on melatonin (as it is less distantly related to human in terms of diurnal/nocturnal behaviour than rodents), this discovery is of great importance for the understanding of melatonin actions.

Methods

Animals and tissue preparation

Animals were killed between 06:00 and 12:00 h (late night and morning) by licensed butchers in an official slaughterhouse under the authorization No A37801 for Animal Experimentation and Surgery from the French Ministry of Agriculture. Tissue samples (retina, mammillary bodies, hippocampus, premammillary hypothalamus, caudate nucleus, choroid plexus, pineal gland and pars tuberalis) were collected from 14 adult Ile-de-France ewes, immediately frozen in liquid nitrogen and kept at –80°C for RNA extraction. The interval between death and freezing of the brain samples was less than 10 min.

Cloning of ovine MT_2 receptor cDNA

Sheep retina DNA-free total RNA (4 μg) was converted into cDNA with oligodT in accordance with ThermoScript cDNA synthesis protocol from Invitrogen. Oligonucleotide primers for *MTNR1B* receptor gene were designed from bovine *MTNR1B* receptor cDNA and gene previously cloned (XM_607095 and contig Ensembl ENSBTAG00000001270; Genbank). The sequences of the primers of the first primer set were as follows: sense primer, 5′-ataaagaggacagggctgaggc-3′ (5′UTR bovine *MTNR1B*; bases 265–287 of contig Ensembl ENSBTAG00000001270) and antisense primer 5′-tcatttcctgagtgcgtggc-3′ (end of coding region of bovine *MTNR1B*; bases 15583–15603 of contig Ensembl ENSBTAG00000001270) to produce a band of 1667 base pairs. PCR reaction was performed in 100 μL containing 10 mM dNTPs, 2 mM $MgCl_2$, 0.8 μM primers, 2 μL cDNA, 20 μL of solution Q and 1U DNA polymerase (Core kit, Qiagen, Courtaboeuf, France) with a 35-cycle programme of 94°C for 40 s, 55°C for 40 s and 72°C for 2 min, a hot start at 94°C for 3 min and a final extension at 72°C for 5 min. The amplified DNA fragment was subcloned into pcR4TOPO and then into pcDNA3.1D-V5HisTOPO vectors in accordance with Invitrogen protocols. The 3 haemagglutinin (HA) flag epitope (sequence YPYDVPDYAYPYDVPDYAYPYYDVPDYAD) was introduced by PCR reaction into the ovine MT_2 receptor/pcDNA3.1D-V5HisTOPO expression construct at the N-terminus between the first methionine residue and the second residue of the ovine MT_2 receptor. The nucleotide sequence analysis on both DNA strands was determined by the dideoxy chain termination method using the BigDye Terminator Cycle Sequencing Kit in a Model 3730 Sequencing System (Applied Biosystems, Foster City, CA). The nomenclature of the receptors follows the recommendations of BJP's Guide to Receptors and Channels (Alexander *et al.*, 2008).

Rapid amplification of cDNA 5′ and 3′ ends (5′- and 3′-RACE)

The corresponding first-strand cDNAs were prepared from 4 μg of total RNA, using the ThermoScript reverse

transcriptase, 5′-CDS® primer (modified oligo-dT primer) and BD™ SMART IIA® primer of the BD™ SMART® RACE cDNA amplification kit (Clontech, Mountain View, CA). Dilutions of each 5′ and 3′ RACE-ready cDNAs were used in PCR amplification reactions with the SMART® RACE kit universal primer mix and either gene-specific antisense exon 2 primers (5′-ccagactcaccaagaacaggttacc-3′; position 737 of 761, EU679365) to amplify 5′ ends of ovine MT_2 mRNA. Alternatively, an MT_2-specific sense primer (5′-ggcaaccgcaagctccggaacgc-3′; position 713 of 735, EU679365) was also used in combination with the universal primer mix to amplify 3′ ends of ovine MT_2 mRNA. The 5′ and 3′ RACE cDNAs syntheses and PCR amplifications were performed according to the manufacturer's instruction (Clontech, Mountain View, CA). The 3′ and 5′ RACE PCR products were purified from the gel and inserted into a pCR4-TOPO vector (Invitrogen, Carlsbad, CA). To avoid possible sequencing errors due to RACE artefacts, the sequence analyses were performed on five (5′ RACE) and four (3′ RACE) independent clones derived from each RACE. DNA sequencing was performed on both strands using an automated DNA sequence analyser 3730.

Establishment of stable CHO-K1/3HA-oMT$_2$ cell line

CHO-K1 cells, obtained from the American Type Culture Collection, were transfected with 3HA-oMT2/pcDNA3.1D-V5HisTOPO plasmid using lipofectAMINE (Invitrogen, Carlsbad, CA). CHO-K1 cells stably expressing 3HA-oMT$_2$ were selected using geneticin (0.8 mg·mL^{-1}). CHO-K1/3HA-oMT$_2$ cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 2 mM glutamine, 500 units·mL^{-1} penicillin/streptomycin and 400 μg·mL^{-1} geneticin.

Indirect immunofluorescence and confocal microscopy

CHO-K1 cells stably expressing ovine MT_2 receptors were seeded at 1×10^5 cells per well of the eight-well Lab-Tek chamber slide (Nunc, Napervile, IL) in 0.4 mL medium. Cells were fixed by treatment with 4% formaldehyde in phosphate buffered saline (PBS) for 15 min then blocked with 0.2% bovine serum albumin (BSA) and glycine (100 mM) in PBS for 30 min at 25°C. For visualization of HA epitope-tagged ovine MT_2 receptors, cell surface receptors were stained using a 1:500 dilution of monoclonal anti-HA IgG for 1 h at 37°C in PBS containing 0.2% BSA. After washing in PBS supplemented with 10% goat serum and 0.2% BSA, cells were incubated in a humidified chamber for 45 min at 37°C with secondary antibody (1:100 Alexa fluor 488-conjugated goat anti-mouse IgG; Sigma). Cells were then washed twice with PBS/0.2% BSA, and coverslips were applied using Vectashield® Mounting medium containing 4′, 6′-diamidino-2-phenylindole to stain nuclei (Vector Lab, Burlingame, CA). Confocal microscopy was performed using a Zeiss LSM510 laser scanning microscope and a Zeiss 63 × 1.4 numerical aperture water immersion lens with dual line switching excitation (488 nm for Alexa fluor 488) and emission (515–540 nM) filter sets. Immunofluorescence images were captured with a Photometrics 16-bit cooled digital camera.

Membrane preparations

Human and rat receptors were used as previously cloned and described in our laboratory (Audinot *et al.*, 2003; 2008 respectively). CHO-K1 cell lines stably expressing rat, human or ovine MT_2 receptors or the ovine MT_1 receptor were grown to confluence, harvested in phosphate buffer containing 2 mM EDTA and centrifuged at 1000× *g* for 5 min (4°C). The resulting pellet was suspended in 5 mM Tris/HCl, pH 7.4, containing 2 mM EDTA, and homogenized using a Kineatica Polytron (30 s, in ice 13 000 rpm). The homogenate was then centrifuged (20 000× *g*, 30 min, 4°C), and the resulting pellet was suspended in 75 mM Tris/HCl, pH 7.4, containing 2 mM EDTA and 12.5 mM $MgCl_2$. Determination of protein content was performed according to Lowry using the Biorad kit (Bio-Rad SA, Ivry-sur-Seine, France). Aliquots of membrane preparations were stored in binding buffer (50 mM Tris/HCl, pH 7.4 containing 5 mM $MgCl_2$ and 1 mM EDTA) at –80°C until use.

2-[^{125}I]-melatonin binding assay

Membranes were incubated for 2 h at 37°C in binding buffer in a final volume of 250 μL containing 2-[^{125}I]-melatonin (20 pM) for competition experiments. The results were expressed as *K*i, taking into account the concentration of radioligand used in each experiments. Non-specific binding was defined with 1 μM 2-iodomelatonin. Reaction was stopped by rapid filtration through GF/B Unifilters, followed by three successive washes with ice-cold buffer. Data were analysed by using the program PRISM (GraphPad Software Inc., San Diego, CA). For saturation assays, the density of binding sites B_{max} and the dissociation constant of the radioligand (K_D) values were calculated according to the method of Scatchard. For competition experiments, inhibition constants (K_i) were calculated according to the Cheng–Prusoff equation: $K_i = IC_{50}/[1 + (L/K_D)]$, where IC_{50} is the measured inhibitory concentration 50%, L, the concentration of 2-[^{125}I]-iodomelatonin and K_D, the dissociation constant (Cheng and Prussoff, 1973).

[^{35}S]-GTPγS binding assay

Membranes and drugs were diluted in binding buffer (20 mM HEPES, Ph 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 3 μM GDP, 20 mg·mL^{-1} saponin). For agonist tests, incubation was started by the addition of 0.2 nM [^{35}S]-GTPγS to membranes and ligands, and carried on for 60 min at room temperature in a final volume of 250 μL. To test for antagonist activity, membranes were pre-incubated for 30 min with 3 nM melatonin and concentration of the tested compound. Reaction was started by the addition of 0.2 nM [^{35}S]-GTPγS and followed by 60 min incubations. Non-specific binding was assessed using unlabelled GTPγS (10 μM). All reactions were stopped by rapid filtration through GF/B unifilters pre-soaked with distilled water, followed by three successive washes with ice-cold buffer. Data were analysed by using the program PRISM to yield EC_{50} and E_{max} values for agonists. Antagonist potencies were expressed as K_B with $K_B = IC_{50}/1 + ([ago]/EC_{50}ago)$, where IC_{50} is the inhibitory concentration of antagonist that gives 50% inhibition of [^{35}S]-GTPγS binding in the presence of a fixed

concentration of agonist [(ago)] and EC_{50}ago is the EC_{50} of the agonist when tested alone.

cAMP assay

CHO-K1/3HA-oMT_2 cells were maintained in culture at less than 80% confluence. They were detached using Cell Dissociation Buffer (SIGMA), washed in 1× PBS (Invitrogen) and resuspended in HAM-F12 medium (Invitrogen) + IBMX (500 μM). Production of cAMP was assessed in triplicates, in black half-well 96-well plates (CORNING), using the cAMP dynamic2 kit (CISBIO), as described by the manufacturer. In brief, 30 000 cells were incubated with 5 μM forskolin and varying concentrations of melatonin in 50 μL of HAM-F12 medium for 30 min at 20°C. Then, 25 μL of cAMP-d2 followed by 25 μL europium cryptate anti-cAMP antibody (diluted as described in the manufacturer's protocol) was added in each well. The signal was quantified after 60 min of incubation using an EnVision time resolved-fluorescence resonance energy transfer reader (Perkin Elmer). Graphic representations and data analysis were generated with PRISM 4.03 (Graphpad).

Cellular dielectric spectroscopy

CHO-K1 cells expressing ovine, human or rat MT_2 receptors were plated at the density of 80 000 cells per well onto MDS Analytical 96-well assay plates with embedded electrodes, and were incubated at 37°C, CO_2 6% for 24 h, in the presence of 100 ng·mL^{-1} *Pertussis* toxin, as specified. Prior to the measurement, cells were washed three times with Hank's balanced salt solution, 0.1% BSA, 20 mM HEPES, pH 7.4 and were left to equilibrate at 28°C for 30 min. The impedance measurement was performed on a CellKey system (MDS Analytica, Concord, Canada), where the signal was recorded for 5 min before online addition of melatonin and 15 min thereafter. The cells in each well were stimulated once with a single concentration of compounds. The resulting data are expressed as the maximal signal corrected for the baseline, and represented as a percentage.

RT-PCR analysis

Total RNAs were extracted from tissues using Trizol protocol (Invitrogen, Carlsbad, CA), purified by phenol/chloroform and ethanol precipitation. Total RNA Samples were then heated at 96°C for 6 min and digested with 2 U of DNase I (DNA-free kit, Ambion, Austin, TX) at 37°C for 1 h. DNase I was removed using DNase I inactivation reagent (DNA-free kit, Ambion, Austin, TX) following the manufacturer's recommendations. Specific sense and antisense 22-25mer oligonucleotides were directed towards selected regions of exon 2 of the gene coding *MTNR1A* receptor (Genbank: U14109). The sequences 5′-gaattgcccatcaaccgctattgc-3′ (bases 445–469) and 5′-acagaagacgactacgagcatcg-3′ (bases 691–714) correspond to the upper-strand and lower-strand primers respectively. Oligonucleotide primers for *MTNR1B* receptor gene encoding MT_2 receptor were designed from exon 2 of the ovine receptor gene previously cloned (Genbank: EU679365). The sequences of the primers were as follows: sense primer, 5′-ggtaacctgttcttggtgagtctgg-3′ (bases 1164–1184) and antisense primer 5′-gcagataatctcccacctgatgcc-3′ (bases 1545–1567) to produce a band at 800 base pairs. The ovine GAPDH (Genbank: AF030943) served as a control for the quality of cDNA and forward and reverse primers were 5′-gtgatgctggtgctgagtac-3′ (bases 127–146) and 5′gtagaagagtgagtgtcgc-3′ (bases 745–727) respectively. Total RNAs were primed with oligodT (successively 70°C 5 min, 0°C 5 min and 25°C 5 min) and converted into cDNA using a reverse-transcriptase (ImProm RT System, Promega, Charbonnières-les-Bains, France) for 60 min at 42°C. After enzyme inactivation (15 min at 70°C), cDNAs were subjected to PCR amplification. PCRs were run in 100 μL containing 10 mM dNTPs, 2 mM $MgCl_2$, 0.8 μM primers, 2 μL cDNA and 1U DNA polymerase (PCR green GoTaq Master Mix, Promega, Charbonnières-les-Bains, France). PCRs were preceded by 2 min pre-denaturation step at 95°C, then run for 30 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min followed by a 5 min extension period. A PCR control was performed by replacing the cDNA sample with water. mRNA not reverse-transcribed and ovine genomic DNA were used as a negative and positive control respectively. Ten microliters of PCR were loaded in parallel with the molecular weight marker (Gel Pilot 1 kb Plus Ladder, Qiagen, Courtaboeuf, France) on a 2% agarose gel containing ethidium bromide. To confirm the identity of the sequences, the amplified cDNA fragments were sequenced.

Real-time PCR

Thirty Ile de France sires were placed in a controlled photoperiodic environment under 12:12 light : dark cycle. In order to collect the biological samples during the same day, animals were separated in two groups and placed in opposite photoperiodic conditions with lights on between 09:00 and 21:00 h for half of the animals (n = 15) and lights on between 18:00–06:00 for the other half (n = 15). After 3 weeks of adaptation in these controlled photoperiodic regimen, animals were killed and three tissues, pars tuberalis, premammillary hypothalamus and mammillary bodies were collected at six time points of the day–night cycle. These points are expressed using ZT 0 (zeitgeber time 0, time of lights on) as a reference: ZT 1.5, 6, 10.5, 13.5, 18 and 22.5 (n = 5 structures per condition). Pars tuberalis and premammillary hypothalamus express MT_1 receptors and are involved in seasonal control of physiological functions, in contrast to the mammillary bodies. When tissue collection occurred during the night, dim red light was used in order to prevent a potential effect of the light. Structures were then immediately frozen in liquid nitrogen and stocked at –80°C until RNA extraction. After tissue homogenization in QIAzol Lysis Reagent from the RNeasy Lipid Tissus MiniKit (Qiagen, France), 1 pg of luciferase mRNA (Promega, France) was added in each sample as an exogenous standard. Reverse transcription was performed at 37°C for 50 min using oligo$(dT)_{15}$ primers (Promega, France) by ThermoScript reverse transcriptase (Invitrogen, France) onto DNAse-treated RNA.

Resulting target cDNAs were quantified by real-time PCR with iQ SYBR green supermix (Bio-Rad, France) using a iCycler system (Bio-Rad) with specific primers for ovine MT_1 receptor (5′-CCTCCATCCTCATCTTCACCATC; reverse 5′-GGCTCACC

ACAAACACATTCC), for ovine MT_2 receptor (5′-CGTCGTGT GCTTCTGCTACC and reverse 5′-GCTTGCTCTCCGCCT TGAC) and for *luciferase* (forward 5′-TCATTCTTCGCCAAA AGCACTCTG; reverse 5′-AGCCCATATCCTTGTCGTATCCC). A standard three-step protocol (95°C for 30 s, 60°C for 30 s, 72°C for 20 s) was repeated for 40–50 cycles, followed by acquisition of the melting curve. Amplicons were sequenced to check the identity of the amplified cDNA. The standard curve was deduced from serial dilutions (100–0.01 fg) of a plasmid including the target sequence incorporated in each run. A cDNA amount was used in triplicate PCR reactions, and the median value was considered (0 when not detected). For each sample, the data were normalized to the median value for exogenous luciferase.

Data analysis

All the results of real-time quantitative PCR assay were expressed as mean values (±SEM). One-way ANOVA was performed to assess time-related changes in oMT1 and oMT2 mRNA expression in the PT, PMH and MB of sheep kept under LD conditions. A probability of $P < 0.05$ is considered statistically significant.

Materials

The two radioligands 2-[^{125}I]-iodomelatonin (specific activity: 2000 Ci·mmol^{-1}) and [^{35}S]-GTPγS (guanosine-5′[γ-^{35}S]-triphosphate; specific activity: 1000 Ci·mmol^{-1}) were purchased from Perkin Elmer (Courtaboeuf, France). Melatonin was obtained from Sigma (St Louis, MO) and 4P-PDOT and luzindole (2-benzyl-*N*-acetyltryptamine) from Tocris (Bristol, UK). Compounds were dissolved in dimethylsulphoxide at a stock concentration of 10 mM and stored at –20°C.

Results

Cloning and sequence analysis of the ovine MT_2 receptor

Because the partial ovine MT_2 sequence released by Xiao *et al*. (2007) was rich in GC, we wondered if the complete cloning was impaired by a technical problem. We therefore tested all commercially available thermo-resistant reverse transcriptases (with stable activity at temperatures over 37°C) after denaturation of mRNA at 70°C for 5 min followed by 5 min on ice. Using Thermoscript reverse transcriptase, we successfully amplified by RT-PCR from sheep retina, a cDNA fragment corresponding to the complete coding region (1131 bp) of the mRNA for the ovine MT_2 receptor (Figure 1). 5′ and 3′ RACE analyses revealed 5′UTR of 514 bases and a short 3′UTR of 194 bases of ovine MT_2 mRNA (Figure 1). The nucleic acid sequence isolated by RT-PCR from sheep retina (Figure 1) revealed 67% of GC, which is higher than all other known MT_2 receptor sequences cloned so far. These results confirmed our starting hypothesis. The analysis of the initiation and stop codons identified only one open reading frame compatible with a receptor of 376 amino acids (Figure 1). The ovine MT_2 receptor cDNA isolated in the study possesses the residues T97, A99, A111, S154, L159, G243, A244, I276, I285, I307, K313, V323, S335 and D357, which corresponded to the major genotype (AA CC EE GG PP) described by Xiao *et al*. (2007) from five ovine breeds (Figure 1). With regards to sequence alignments between the human, rat and sheep MT_2 receptor sequences, there are global sequence identity and similarity [64% and 78% respectively (Figure 2)]. Local alignments are more significant with a 90% sequence similarity percentage in helices and 95% in extracellular loops, with the noticeable exception of the N-terminus region. Furthermore, there is no gap in this whole alignment. This result highlights the highly conserved structure of MT_2 receptors between species. The carboxy-terminal domain of ovine and bovine MT_2 receptors contains 10 additional amino acids compared with other species (Figure 2). The computational analysis of this carboxy-terminal region did not allow the identification of any potential specific property of ovine MT_2 and bovine MT_2 compared with MT_2 receptors from other species. The amplification of the ovine *MNTR1B* gene by PCR using cloning primers described in the *Methods* section showed that the coding region is composed of two exons spaced by a large intron (>10 kbp; data not shown) as described in human, rodent *MTNR1B* genes (Ensembl data bank: human ENSG00000134640, rat ENSRNO00000008972 and mouse ENSMU00000050901) and mouse *MTNR1A* gene (Roca *et al*., 1996).

Expression and subcellular localization of recombinant ovine MT_2 receptors in CHO-K1 cells

After the addition of a flag with three influenza HA epitopes at the amino-terminal end of the coding region, the modified ovine MT_2 receptor was stably transfected in CHO-K1 cells. Immunostaining of non-permeabilized CHO-K1/3HA-oMT_2 cells with a fluorescent anti-HA antibody demonstrated a major expression of ovine MT_2 receptors at the plasma membrane (Figure 3B). No fluorescent signal was detected from CHO-K1 cells transfected with the vector alone (Figure 3A).

Figure 1 Complete sequence of melatonin receptor 2 (MT_2) mRNA isolated from sheep retina. The sequence of ovine retinal MT_2 receptor (1838 bases) was obtained by RT-PCR using primer set derived from the bovine MT_2 receptor cDNA sequence (XM_607095 and contig Ensembl ENSBTAG 00000001270). 5′UTR and 3′UTR regions of ovine MT_2, obtained to 5′ and 3′ RACE experiments, correspond to 514 and 194 bases respectively. The coding region contains 1131 bases and encodes 376 amino acids. The polyadenylation signal (AATAAA) is underlined. The deduced amino acid sequence is shown using single-letter amino acid code. Nucleic and amino acid sequences are numbered on the right. The nucleic and amino acid sequences of first exon are in italic characters. The positions of the two nonsense mutations described in Siberian hamster MT_2 receptor cDNA are double underlined. Amino acids described to be essential for ovine breeds polymorphisms discrimination (Xiao *et al*., 2007) are highlighted in black. This sequence has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession no. EU679365.

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tgaggcatcgccaagacttctcaagtaaacagcaaataaacagcgcagggtcttccttct   60

cctgctgctgcctgcctgatctcttcgcgatgcctggaggtatgtattcgctttagaggg  120

gcagtgcccttctgacagccaatcagcaggtcctgacgagcagcaccttttccagtccga  180

gaatctgggtgtccctggacaccccagcttcttgtgcgggcgcgctcaggcgctgaggccgg  240

aaccgggccaggcacgtgctgggctccgggcgggcgcgccgtcgcaacctgctcctgggcga  300

gccaagcggagacgcgcttctttcagcagcgcctgcaccccaaatgctcctctccgtttt  360

tctccgccccctgcccacgcttaattaatcatctagtccaggtgcctcggcactggcctc  420

ctcgctggtgcgcgcgggctgggcgggggaaagggggcgccccgcctgcggagagactgggtg  480

cgccctggctggcgcggcgccggagcgtccgggg ATGCCTGAGAACGGCTCCTTCGCCAAC  540
                                    M  P  E  N  G  S  F  A  N    9
TGCTGCGAGGCGGGCGGCCGGGCCGAGAGCCCCCGCTGGACTGGCGCGGGCGGCGCGCGG  600
 C  C  E  A  G  G  R  A  E  S  P  R  W  T  G  A  G  G  A  R   29
CCCTCCGCGGCGCCCCGGCCTCCCTGGGTGGCTCCCGCGCTGTCCGCGGTGCTCGTCGTC  660
 P  S  A  A  P  R  P  P  W  V  A  P  A  L  S  A  V  L  V  V   49
ACCACCGCCGTGGACATCGTGGGCAACCTCCTGGTCATCCTCTCGGTGCTGGGCAACCGC  720
 T  T  A  V  D  I  V  G  N  L  L  V  I  L  S  V  L  G  N  R   69
AAGCTCCGGAACGCAGGTAACCTGTTCTTGGTGAGTCTGGCGTTCGCTGACCTGGCCGTA  780
 K  L  R  N  A  G  N  L  F  L  V  S  L  A  F  A  D  L  A  V   89
GCCCTGTACCCCTACCCGCTGACCCTTGCGGCCATCTTCCACGACGGCTGGGCCCTGGGG  840
 A  L  Y  P  Y  P  L  T  L  A  A  I  F  H  D  G  W  A  L  G  109
GAGGCGCACTGCAAGGCCAGCGCCTTCGTGATGGGCCTGAGCGTGGTCGGCTCCGTCTTC  900
 E  A  H  C  K  A  S  A  F  V  M  G  L  S  V  V  G  S  V  F  129
AACATCACCGCCATCGCCGTCGACCGCTACTGCTACGTCTGCCGCAGCGTGACCTACCAC  960
 N  I  T  A  I  A  V  D  R  Y  C  Y  V  C  R  S  V  T  Y  H  149
CGCCTCTGCCGCAGCCGGCACGCCGCCCTCTACGTCGGCCTCGTCTGGCTGCTCACCCTG 1020
 R  L  C  R  S  R  H  A  A  L  Y  V  G  L  V  W  L  L  T  L  169
CTGGTCCTGCTGCCCAACTTCTTCGTGGGGTCCCTGGAGTACGACCCGCGCGTCTACTCG 1080
 L  V  L  L  P  N  F  F  V  G  S  L  E  Y  D  P  R  V  Y  S  189
TGCACCTTCGCGCAGACGGCCAGCGCCGGGTACACGGCGGCCGTGGTGCTCGTGCACTTC 1140
 C  T  F  A  Q  T  A  S  A  G  Y  T  A  A  V  V  L  V  H  F  209
CTGCTGCCCGTGGCCGTCGTGTGCTTCTGCTACCTGCACATCTGGGTGCTGGTGCTGCGC 1200
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 F  L  S  M  F  V  V  F  V  I  F  A  I  C  W  A  P  L  N  C  269
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ttgctgtgaatacttaaaaaaaaaaaaaaaaaaaaaa                        1838
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Saturation assays and binding characteristics

To determine whether ovine MT_2 cDNA encodes a melatonin MT_2 receptor, binding and pharmacological properties were examined by stably expressing ovine MT_2 receptors in CHO-K1 cells. For comparison, binding and pharmacology of CHO-K1 cells expressing the human and rat MT_2 receptors and the ovine MT_1 receptor were assessed in parallel. Analysis of the saturation data using one site and two site binding hyperbola fits (*F*-test, GraphPad PRISM) revealed the presence of a single high-affinity binding site of 2-[^{125}I]-melatonin. Scatchard plot of the saturation data gave a K_D value of 0.041 ± 0.04 nM (pK_D = 10.39 ± 0.10) and a number of sites, B_{max} value of 599 ± 10 fmol·mg^{-1} protein in the CHO-K1/3HA-oMT_2 cells (Figure 4A). This pK_D value was similar to that of human MT_2 and rat MT_2 receptors when expressed in CHO-K1 cells (Table 1). The binding characteristics of recombinant ovine MT_2 receptors were determined by competition binding using 2-[^{125}I]-iodomelatonin as a radioligand and three reference ligands of melatonin MT_2 receptors (melatonin, 4P-PDOT and luzindole).

Figure 4B shows typical binding curves and Table 2 reports the calculated pK_i values for the ovine recombinant MT_2 receptor, as well as affinity values of these compounds for ovine MT_1, rat MT_2 and human MT_2 receptors. For the three compounds, competition curves were monophasic on membranes from cell lines expressing sheep, human and rat MT_2 receptors, showing that all curves were better fitted by a one-site analysis than by a two-site analysis (Figure 4B). The relative affinities of the three compounds studied here were similar for ovine, human and rat MT_2 receptors: melatonin > 4P-PDOT > luzindole (Table 2).

Table 1 Binding affinities (pK_D) and levels of receptor expression (B_{max}) in CHO-K1 cells expressing rat (rMT_2), human (hMT_2), ovine (oMT_2 and oMT_1) melatonin receptors

Receptors	pK_D ± *SEM*	B_{max} ± *SEM, fmol·mg^{-1} protein*
rMT_2	9.89 ± 0.61 (*n* = 4)	1485 ± 342 (*n* = 4)
hMT_2	9.97 ± 0.99 (*n* = 4)	2656 ± 282 (*n* = 4)
oMT_2	10.39 ± 0.10 (*n* = 4)	599 ± 10 (*n* = 4)
oMT_1	10.85 ± 1.15 (*n* = 4)	670 ± 25 (*n* = 4)

The affinity and density of binding sites were determined by saturation analysis of the binding of 2[^{125}I]-2-iodomelatonin to membranes prepared from stably transfected CHO-K1 cells. Non-specific binding was determined in the presence of 1 μM 2-iodomelatonin.

However, the ovine and rat MT_2 receptors presented 10-fold less affinity for the antagonists, 4P-PDOT and luzindole, compared with human MT_2 receptors. The specific MT_2 receptor antagonist 4P-PDOT was approximately 10-fold more selective for the ovine MT_2 receptor than for the ovine MT_1 receptor (Table 2).

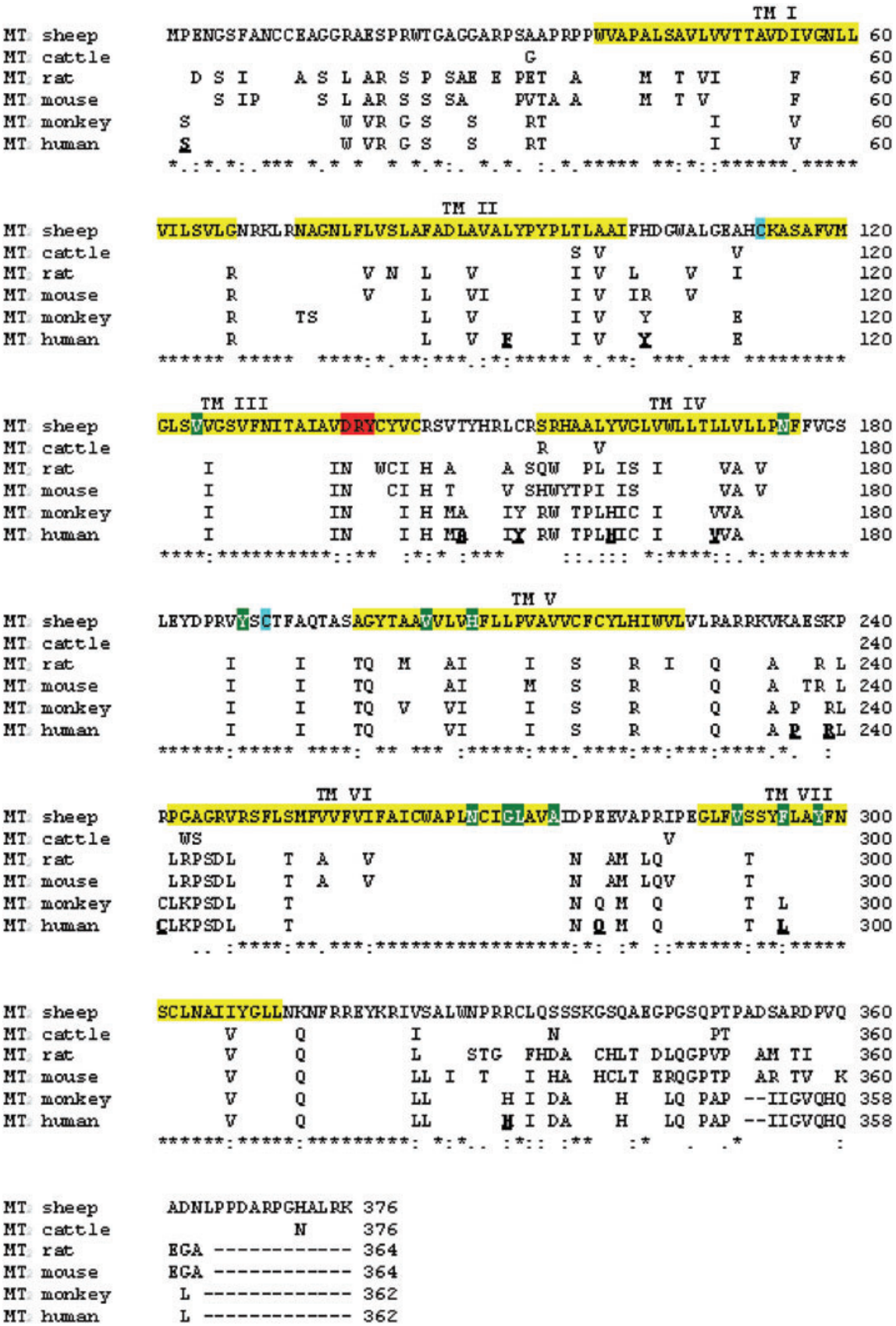
G protein coupling

Functional activity of ovine MT_2 receptors was studied using the [^{35}S]-GTPγS binding assay, cAMP accumulation assay and cellular dielectric spectroscopy. The agonist or antagonist activities of three compounds (melatonin, 4P-PDOT and luzindole) at the recombinant melatonin receptors were evaluated by using [^{35}S]-GTPγS binding assay (Table 3). Melatonin showed an agonist effect on ovine recombinant MT_2 receptors with an affinity close to that obtained upon activation of human and rat MT_2 receptors (Table 3). Furthermore, 4P-PDOT and luzindole revealed no agonist activity at ovine MT_2 receptors. Hence, the behaviour of 4P-PDOT as partial agonist on human MT_2 receptors seems to be a distinguishing feature of this receptor (Table 3). The antagonist affinities of 4P-PDOT and luzindole were consistent with the observations made with the competition binding data, with a well conserved overall pharmacology (Table 1 and Figure 4B). These results demonstrated that the ovine MT_2 receptor expressed in CHO-K1 cells could couple to a G protein in presence of melatonin.

Incubation of CHO-K1/3HA-oMT2 cells with 5 μM forskolin increased intracellular cAMP concentrations approximately 10-fold. This increase was inhibited by melatonin in a dose-dependent manner, with an IC_{50} of 0.81 ± 0.62 nM and maximal inhibition levels of approximately 80% (Figure 5). Melatonin alone had no effect on basal cAMP levels. Similar to all other melatonin receptors, the ovine MT_2 receptor is coupled to G_i protein in CHO-K1 cells.

To investigate if ovine MT_2 receptors can be coupled with other G proteins in CHO-K1 cells, we used a novel technology: cellular dielectric spectroscopy. This technology is based on the change of intercellular impedance (mainly resistance to an external electric current) of a monolayer of cells (Verdonk *et al.*, 2006; Peters *et al.*, 2007). Upon stimulation of a GPCR with an agonist, the signalling cascade will eventually lead to minute changes in cell adherence and/or cell shape, resulting in a change in the intercellular impedance. Interestingly, the kinetics and values of the impedance signal are

Figure 2 Alignment comparison of primary sequence of various mammalian melatonin MT_2 receptors. The deduced amino acid sequence of the ovine MT_2 receptor was compared with cattle (XP_001254950), rat (XP_345900), mouse (NP_663758), human (NP_005959) and chimpanzee (XP_522146) MT_2 receptor sequences. The bovine MT_2 sequence (XP_001254950) used here are deduced from bovine *MTNR1B* gene (Loc787599 entrezgene), which presents a deletion of one adenine residue at position 1126 compared with XM_607095 sequence and which reveals a carboxy-terminal region that better matches with other MT_2 of different species. The ClustalW algorithm was used to align these mammalian MT_2 sequences. Amino acid residues identical in the six MT_2 receptors are indicated by asterisks. Similar amino acids are indicated by dots or double dots. Amino acids not homologous to the ovine MT_2 sequence are indicated under this sequence. Deleted amino acids are indicated by a dash. The seven putative transmembrane domains (TMI to VII) designed by comparison with human rhodopsin receptor crystal are highlighted in yellow on ovine MT_2 sequence and numbered in roman numbers. The DRY sequence is highlighted in red. Thirteen amino acids described to be essential for 2-iodomelatonin binding to human MT_2 and MT_1 receptors (Conway *et al.*, 1997; 2000; Mseeh *et al.*, 2002; Gerdin *et al.*, 2003; Mazna *et al.*, 2005) are highlighted in green on the ovine MT_2 sequence. Twelve residues out of the 13 are conserved in all species; only the Phe^{295} of human MT_2 receptor is replaced by a Leu in the ovine sequence. The amino acids specific to human MT_2 versus ovine/rat MT_2 receptors are presented in bold and underlined. The two cysteine residues Cys^{113} and Cys^{190} engaged in a disulphide bridge between TMIII and E2 loop, necessary for structural conservation of MT_2 receptors, are highlighted in blue.


TM I
TM II
TM III
TM IV
TM V
TM VI
TM VII
MT sheep
MT cattle
MT rat
MT mouse
MT monkey
MT human

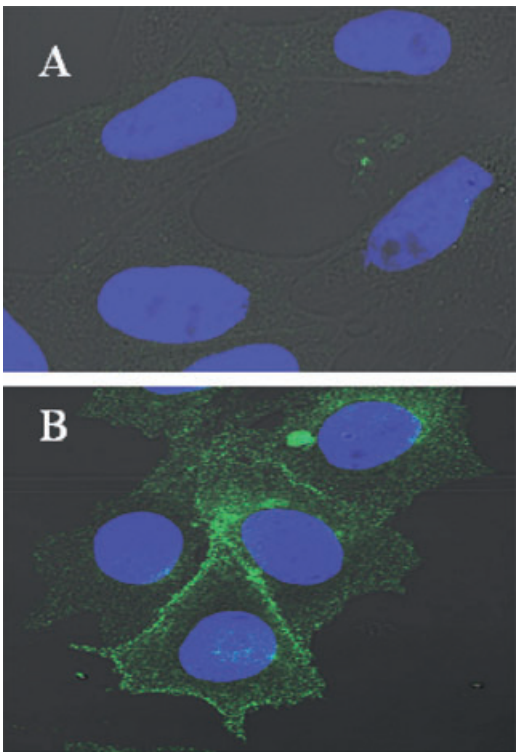


Figure 3 Subcellular localization of epitope-tagged ovine MT_2 receptor. Immunofluorescence studies were performed with transfected CHO-K1 cells grown on Labtech as described in the *Methods* section. CHO-K1 cells (A) and CHO-K1 expressing 3HA-oMT_2 receptor (B) were probed with mouse monoclonal Antibody HA directed against the N-terminal epitope tag present on recombinant receptor. Experiments were carried out with paraformaldehyde-fixed and non-permeabilized cells. The fluorescence images were obtained by using Alexa 488-conjugated goat anti-mouse IgG secondary antibody. CHO-K1 cell nuclei were stained with 4′, 6′-diamidino-2-phenylindole. CHO-K1 cells transfected with the vector alone were used as a negative control (A). Magnification is 800×. Each picture is representative of five independent experiments.

dependent on the G protein subtype involved in the cascade (see Verdonk *et al.*, 2006 for examples and theory). Typical G_i coupling kinetic upon stimulation of CHO-K1/3HA-oMT2 cells by melatonin were obtained, corroborating that the ovine MT_2 receptor is G_i-coupled (not shown). The EC_{50} value of melatonin in this system is 0.13 nM, in agreement with the high, sub-nanomolar affinity of melatonin for its receptors. In addition, after a 24 h treatment of cells with *Pertussis* toxin, a G_i protein inhibitor, the melatonin response was abolished (Figure 6A). Similar results were obtained with the human (Figure 6B, melatonin EC_{50} = 0.43 nM) and rat (Figure 6C, melatonin EC_{50} = 0.11 nM) MT_2 receptors, adding to the evidence that the ovine MT2 receptor is mainly coupled to G_i proteins when expressed in CHO-K1 cells, because no G_s nor G_q signals were detected in these functional studies.

Distribution of mRNA for ovine MT_2 receptors

The tissue expressions of MT_2 and MT_1 mRNAs in different regions of sheep brain were studied using highly stringent RT-PCR conditions as those used for the cloning of ovine MT_2 receptor cDNA. The low abundance of MT_2 receptors in brain tissue necessitated performing RT-PCR reaction with at least 30 cycles. The lengths of amplified DNA fragments were consistent with those expected from the structure of the MT_2 mRNA species. The specificity of PCR products was assessed by cloning and sequencing each amplified DNA. No signal was observed when either the mRNA or the reverse transcriptase was omitted from the first-strand cDNA conversion, which suggests that the signals observed were not due to any contaminating genomic DNA. A quantitative control (GAPDH) confirmed that each sample contained similar amounts of total cDNA (Figure 7). The ovine MT_2 receptor mRNA was expressed in pars tuberalis, choroid plexus and retina, moderately in mammillary bodies and poorly in hippocampus, premammillary hypothalamus, caudate nucleus and pineal gland (Figure 7). The ovine MT_1 receptor mRNA was strongly expressed in pars tuberalis and pineal gland and moderately in choroid plexus, premammillary hypothalamus and mammillary bodies. It is important to observe that the mRNAs for MT_1 and MT_2 receptor were co-expressed in all analysed brain tissues. However, this non-quantitative study suggests that the expression level ratio of the two mRNA may be not identical in all brain regions. We extended our investigations to three regions of the brain, namely *pars tuberalis*, premammillary hypothalamus and mammillary bodies, sampled at six time points during the night and day periods, by using a real-time PCR approach. The results clearly showed that the total amount of ovine MT_2 mRNA is extremely low, especially when compared with that for the ovine MT_1 receptor (Figure 8). Overall, the ratio between ovine MT_1 and MT_2 mRNAs was at least 600-fold, depending on the brain region and the time of the day. Both populations of mRNA varied with the period of the day and night, in a similar fashion in mammillary bodies, and differently in pars tuberalis and premammillary hypothalamus (Figure 8).

Two expression peaks of ovine MT_1 and MT_2 receptor mRNA were observed at ZT 6 and ZT 18 in mammillary bodies. Similarly, two expression peaks of ovine MT_1 and MT_2 mRNA were observed in premammillary hypothalamus, but the second peaks were not coincident for the two mRNAs (at ZT 14 for MT_2 mRNA and ZT 16 for MT_1 mRNA). In pars tuberalis, the levels of ovine MT_2 mRNA were stable except for a marked fall at ZT 18, whereas for the ovine MT_1 mRNA, levels were maximal at ZT 2 and minimal at ZT 10.

Discussion

Because it is scarcely possible to differentiate MT_1 and MT_2 receptors pharmacologically and no laboratory had so far isolated a full-length mRNA coding for functional ovine MT_2 receptors (Drew *et al.*, 1998; Migaud *et al.*, 2005; Xiao *et al.*, 2007), it was suggested that ovine *MTNR1B* gene had evolved into a pseudogene that produced no mRNA (Barrett *et al.*, 2003). This hypothesis was also partially based on the previous identification of two stop codons in the coding region of the MT_2 mRNA of the Siberian hamster, impairing any expression of MT_2 receptors in this species (Weaver and Reppert, 1996). Recently, the presence in the sheep genome of an orthologous gene of the human *MTNR1B* gene was confirmed by Xiao *et al.* (2007). However, these authors did not amplify ovine MT_2

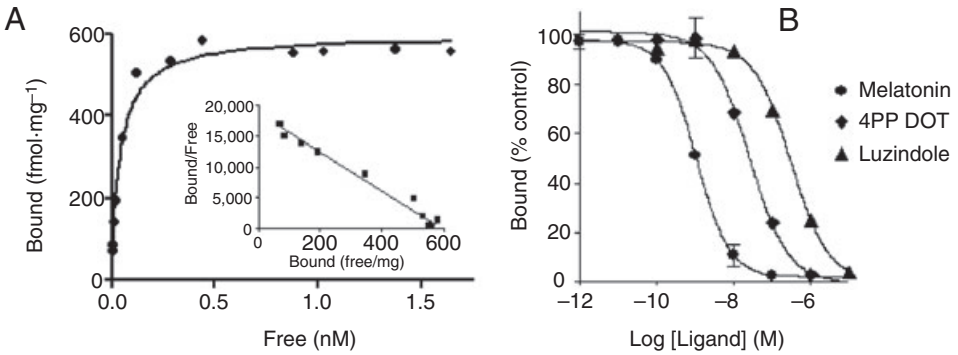


Figure 4 Binding characteristics of ovine MT_2 receptor expressed in CHO-K1 cells. Saturation binding experiments with [^{125}I]-2-iodomelatonin (A). Specific binding is represented as a direct plot (main graph) and as a Scatchard plot of the specific binding (inset). Competition binding experiments against [^{125}I]-2-iodomelatonin (B). Ligands evaluated are melatonin, 4-phenyl-2-propionamidotetraline (4P-PDOT) and luzindole. Points shown are from representative experiments performed in triplicates and repeated four times.

Table 2 Binding affinities (pK_i) of reference ligands to hMT_2, rMT_2, oMT_2 and oMT_1 receptors

	hMT_2	*rMT_2*	*oMT_2*	*oMT_1*
Melatonin	9.46 ± 0.05	9.11 ± 0.36	9.16 ± 0.15	9.74 ± 1.44
4-phenyl-2-propionamidotetralin	8.96 ± 0.19	7.44 ± 0.11	7.77 ± 0.20	6.85 ± 0.66
Luzindole	7.57 ± 0.02	6.47 ± 0.66	6.67 ± 0.28	6.80 ± 0.35

Binding competition studies were performed using [^{125}I]-2-iodomelatonin and a concentration range of each compound. Values of pK_i were calculated from the IC_{50} values using the method of Cheng and Prussoff (1973). Concentration–response curves were analysed by non-linear regression. Binding affinities (nM) are expressed as mean pK_i ± SEM of at least three independent experiments.

Table 3 Compound potency and efficacy as agonist or antagonist, on [^{35}S]-GTPγS binding at human, rat and ovine (hMT_2, rMT_2 and oMT_2) melatonin receptors

	MT_2 receptor agonist EC_{50} (E_{max}) nM	*MT_2 receptor antagonist K_b (I_{max}) nM*
Melatonin	(h) 0.36 ± 0.06 (100)	nd
	(r) 0.9 ± 0.01 (100)	nd
	(o) 1.37 ± 0.17 (100)	nd
4-phenyl-2-propionamidotetralin	(h) 1.28 ± 0.08 (28)	(h) 1.47 ± 0.14 (53)
	(r) Inactive (<10)	(r) 19.9 ± 5 (90)
	(o) Inactive (<10)	(o) 36.2 ± 1.7 (80)
Luzindole	(h) Inactive (<10)	(h) 16.4 ± 5.4 (85)
	(r) Inactive (<10)	(r) 284 ± 102 (99)
	(o) Inactive (<10)	(o) 393 ± 123 (104)

Agonist and antagonist activities of the compounds were evaluated using a [^{35}S]GTPγS binding assay. Concentration–response curves were analysed by non-linear regression. Agonist potency was expressed as EC_{50} ± SEM (nM) while the maximal efficacy, E_{max} ± SEM, was expressed as a percentage of that observed with melatonin 1 μM (=100%). Antagonist potency to inhibit the effect of melatonin (3 nM) was expressed as K_B ± SEM while the maximal inhibition I_{max} ± SEM was expressed as a percentage of that observed with melatonin 3 nM (=100%). Data are mean of at least four independent experiments. Inactive, no dose–response effect. nd, not determined.

mRNA and did not demonstrate the existence of any MT_2 receptor mRNA in this species. Altogether, these observations led us to test the hypothesis that a technical difficulty had impeded the amplification of ovine MT_2 receptor mRNA, that is, a high percentage of GC in ovine MT_2 mRNA. Indeed, the published mRNA sequence for bovine MT_2 receptors (XM_607095) contains 66% of GC, which is higher than MT_2 mRNA sequences from rat/mouse (55% GC) or human (60% GC). The search for MT_2 mRNA was performed in sheep retina as Reppert *et al*. (1995) had demonstrated in humans, using RT-PCR and binding assay, that the MT_2 receptor is highly expressed in this tissue. This observation has been confirmed in numerous species (Alarma-Estrany and Pintor, 2007). The amplification from sheep retina of an mRNA corresponding to an MT_2 receptor that exhibited high percentage of GC (67%), confirmed our starting hypothesis. The cDNA isolated from sheep retina does not contain the two nonsense mutations described in the Siberian hamster MT_2 receptor at positions 1062 [TAC (Tyr) vs. TAA (Stop)] and 1177–1179 [CAC (His) vs. TGA (Stop)] (Weaver *et al*., 1996). This cDNA led to the expression of a 376-amino-acid protein that has 95%, 73%, 72%, 68%, 67% and 58% identity with cattle, human, chimpanzee, mouse and rat MT_2 receptor mRNAs respectively. As for all the other mammalian MT_2 receptors (Audinot *et al*., 2003; 2008; Mailliet *et al*., 2004), the ovine MT_2 receptor presents a strong affinity for 2-[^{125}I]-iodomelatonin (pK_D = 10.39 ± 0.10).

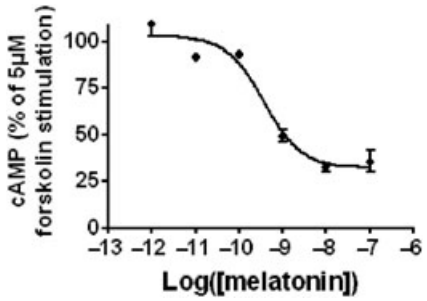


Figure 5 Modulation of forskolin-stimulated cAMP accumulation by the 3HA-tagged ovine MT_2 receptor in CHO-K1 cells. CHO-K1 cells stably transfected with ovine MT_2 receptor cDNA were stimulated with forskolin (5 μM) in the presence of the indicated concentrations of melatonin. Intra-cellular cAMP levels were determined as described in the *Methods* section. Data represent the means ± SEM of three independents experiments performed in triplicate and repeated three times. Data are expressed as per cent of mean forskolin-stimulated value (100%). The potency of melatonin in this assay was 0.81 ± 0.62 nM. Control native CHO-K1 cells did not respond to melatonin in this assay (not represented).

Furthermore, the pharmacological profile of the ovine MT_2 receptor is similar to MT_2 receptors of other species: melatonin > 4P-PDOT > luzindole. The affinity of this receptor for its natural agonist, melatonin, was 24- and 300-fold stronger than for the reference antagonists 4P-PDOT and luzindole respectively. Although the primary sequence of the ovine MT_2 receptor exhibits a higher identity with the human receptor (73%) than for the rat receptor (58%), the pharmacology of ovine MT_2 receptors is closer to the rat than to the human receptors.

The two antagonists 4P-PDOT and luzindole were less selective for ovine and rat MT_2 receptors (pK_i values were 7.77 ± 0.20 and 6.67 ± 0.28 for sheep, 7.44 ± 0.11 and 6.47 ± 0.66 for rat) than for the human MT_2 receptor (pK_i values 8.96 ± 0.19 and 7.57 ± 0.02). To explain this paradox, we searched for all the common amino acids between rat and ovine MT_2 and different from human MT_2 receptors: P2S (amino-terminal end), L91F [transmembrane (TM) domain II], H103Y (external loop 1) T147A, Y152C (internal loop 2), Y160H, L169V (TMIV), A236P, K239R, R241C (internal loop 3), E278Q (external loop 3), F295L (TMVII) and R331H (carboxy-terminal end). Only the residue 295 is close to the putative antagonist binding domain of the MT_2 receptor (Grol and Jansen, 1996) and can interfere with 4P-PDOT and luzindole binding. This residue is also essential for the binding of 2-iodo-melatonin to human MT_2 receptors (Mazna *et al.*, 2005). The Leu^{295} residue described as essential for the binding of 2-iodomelatonin in the human MT_2 receptor is replaced by a phenylalanine residue in all other MT_2 receptor sequences. It is important to note that the MT_1 receptor, which also binds 2-iodomelatonin, presents a tyrosine residue at the same position, 7.40 (Tyr^{282} for human MT_1), using the nomenclature of Baldwin *et al.* (1997). In this nomenclature, the residues of the TM domain, which are conserved in all GPCRs and are essential for structural conservation of GPCRs, have a specific position corresponding to the number of TM, following the number 50. The positions of other residues are determined following the position of the conserved residues (Reppert *et al.*, 1994). These results suggest that the phenyl substituent

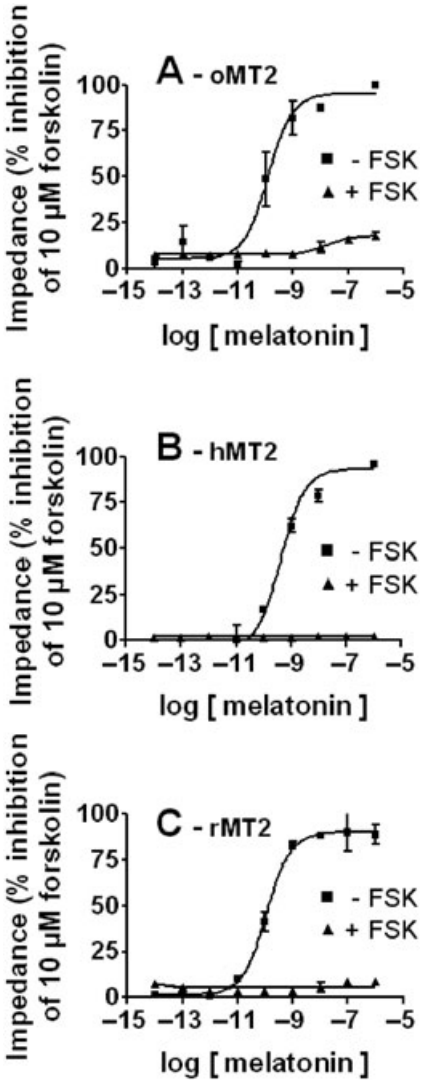


Figure 6 Subtype of G protein coupling of ovine MT_2 receptor. Cellular dielectric spectroscopy of CHO-K1/3HA-oMT$_2$ cells stimulated by melatonin showed a direct increase in impedance, typical of a G_i coupling [see Verdonk *et al.* (2006) for theory and examples]. Melatonin stimulation of CHO-K1 cells expressing ovine MT_2 receptors (A) was compared with CHO-K1 cells expressing either human MT_2 (B) or rat MT_2 (C) receptors. A dose–response of melatonin was obtained alone or after pre-treatment of cells with *Pertussis* toxin.

in the luzindole and 4P-PDOT antagonist molecules could interact with residues towards the TMVII, where the amino acid 295 is located, yielding a variation in binding between rat/ovine and human receptors.

4P-PDOT and luzindole revealed no agonist activity at ovine MT_2 receptors, in line with the results obtained with rat MT_2 receptors (Audinot *et al.*, 2008). Hence, the behaviour of 4P-PDOT as a partial agonist on human MT_2 receptors seems to be a particular feature of this receptor (Table 3). This result is in accordance to previous studies (Browning *et al.*, 2000).

In contrast to all other melatonin receptors, the ovine MT_2 receptor possesses a DRY motif, not a NRY motif as reported in other species (MT_1 and MT_2 from human, monkey, rat, mouse) (Figure 2), just downstream from the third TM domain and an arginine residue (Arg^{246}) at position 6.30

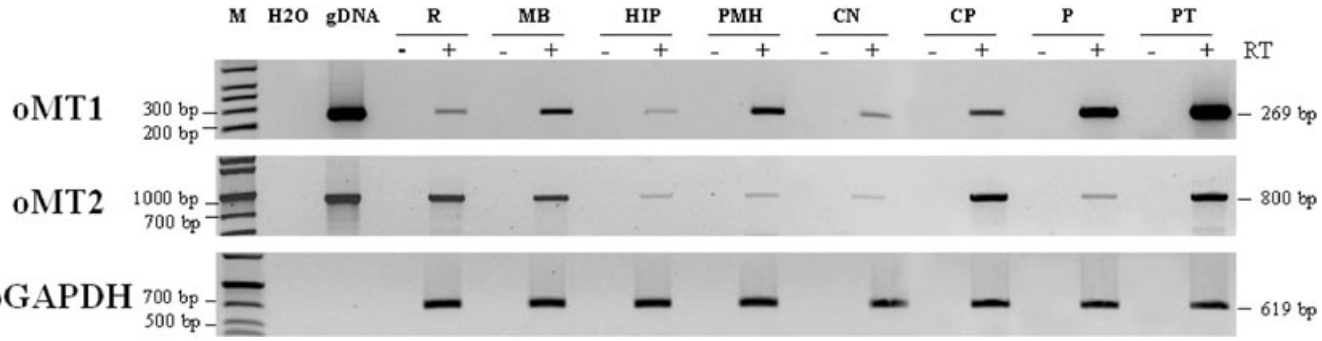


Figure 7 Distribution of mRNA melatonin receptors MT_1 and MT_2 in sheep brain tissues. Animals were killed between 06:00 and 12:00 h (late night and morning). Total RNA (4 μg) of sheep retina (R), mammillary bodies (MB), hippocampus (HIP), premammillary hypothalamus (PMH), caudate nucleus (CN), choroid plexus (CP), pineal gland (P) and pars tuberalis (PT) was amplified by RT-PCR as described in the experimental section. After 35 PCR cycles for ovine MT_1 and MT_2 receptors (oMT1 and oMT2) and 25 cycles for ovine GAPDH (oGAPDH), PCR products were analysed using a 2%w/v agarose gel stained with ethidium bromide. Control experiments without reverse transcriptase (–) revealed no product. The lengths of amplicons were estimated by molecular mass markers (Gel Pilot 1 kb Plus Ladder; M) and indicated in base pairs (bp) on the right. Each PCR product was purified and identified by sequencing on both strands. GAPDH amplification was used as an internal standard. Each picture is representative of three independent experiments.

(Baldwin nomenclature). The inactive state of a GPCR receptor depends on the ionic lock between the residues R3.50 (Arg^{138}), DRY motif of TMIII and D/E6.30 (near to the cytoplasmic end of TMVI) (Ballesteros *et al.*, 2001; Smit *et al.*, 2007). The charge-neutralizing mutation of residue 3.50 or 6.30 leads to a significant increase of constitutive activity. In ovine MT_2 receptors, the residues 3.50 and 6.30 are identical (Arg^{138} and Arg^{246} respectively) and thus present the same positive charge (Figure 2). This conflict induces a repulsion of the cytoplasmic ends of TMVI and TMIII and the destruction of the ionic lock. The repulsion of the cytoplasmic ends of TMVI and TMIII have been described in other receptors to be sufficient to cause constitutive receptor activation, consistent with an increased accessibility of the resulting open cytoplasmic face of the receptor structure to docking G proteins (Roka *et al.*, 1999; Parnot *et al.*, 2002; Smit *et al.*, 2007). In human and rat MT_2 receptors, the residues 3.50 and 6.30 correspond to residues Arg^{138} and Asp^{246} respectively. Thus the ionic lock is preserved. Nelson *et al.* (2001) have demonstrated that the mutation of Asn^{124} to an aspartic acid or glutamic acid residue significantly decreased the efficacy of melatonin for the inhibition of cAMP. N124D and E mutations in the MT_1 receptor also strongly compromised the efficacy and potency of melatonin for inhibition of K^+-induced intracellular Ca^{++} fluxes.

Our functional studies showed that ovine MT_2 receptors expressed in CHO-K1 cells were preferentially coupled to G_i protein. In these cells, we have not observed the other signal transduction pathways, inhibition of guanylyl cyclase and phosphoinositide production, which have been described for MT_2 receptors (Boutin *et al.*, 2005). It would be important to check that the ovine MT_2 receptor is not coupled with other G proteins in brain tissues.

The tissue distribution of MT_2 receptors is poorly described, mainly due to the lack of specific radioligands and antibodies and to the low expression of the MT_2 mRNA (Dubocovich and Markowska, 2005; Pandi-Perumal *et al.*, 2008). So far, the various studies reported lead to the consensus observations that MT_1 receptors are detected in many brain areas and that MT_2 receptors are more restrictively expressed, confined to a few brain areas (Dubocovich and Markowska, 2005; Pandi-Perumal *et al.*, 2008). As described in other species, our RT-PCR study in sheep brain showed that MT_1 receptor mRNA was expressed in all analysed brain tissues. However, strong expression of MT_1 mRNA was detected in pars tuberalis, pineal gland and premammillary hypothalamus. These results were in accordance with previous results obtained with various methods: *in situ* hybridization or binding on tissue slides in other species including sheep (Stankov *et al.*, 1991; Malpaux *et al.*, 1998; Musshoff *et al.*, 2002; Poirel *et al.*, 2003; Migaud *et al.*, 2005; Savaskan *et al.*, 2005; Brunner *et al.*, 2006; Wu *et al.*, 2006). These results validate our RT-PCR protocol. Contrary to previous observation in other species, we have observed that MT_2 mRNA was, similarly to MT_1 mRNA, expressed in all analysed sheep brain tissues. These results can be explained by the use of strong stringent and specific RT-PCR protocol (high number of cycles, >35 and high temperatures, >42°C), which allowed the amplification of GC-rich mRNA. The mRNA for MT_2 receptors was strongly expressed in retina as observed in other species [human and rat (Reppert *et al.*, 1995; Savaskan *et al.*, 2002; Sallinen *et al.*, 2005)] and which confirmed our choice of the tissue to clone the MT_2 receptor cDNA. MT_2 mRNA was also expressed in choroid plexus, mammillary bodies and pars tuberalis. As described in human, rat and mouse hippocampus (Reppert *et al.*, 1995; Wan *et al.*, 1999; Wang *et al.*, 2005), the MT_2 mRNA was expressed at low level in this area of the sheep brain. This low expression level of MT_2 receptor mRNA cannot be associated with a low biological action of this receptor. In mouse hippocampus, where a very low expression of MT_2 mRNA is observed, the action of melatonin involved MT_2, but not MT_1 receptors (Wang *et al.*, 2005).

The real-time quantitative RT-PCR demonstrated that ovine MT_2 mRNA expression level was much lower (15–500-fold) than ovine MT_1 mRNA expression in mammillary bodies, premammillary hypothalamus and pars tuberalis. Using real-time quantitative RT-PCR, Sallinen *et al.* (2005) have also observed a co-expression of rat MT_1 and MT_2 mRNA in all analysed rat brain and peripheral tissues and a higher rat MT_1 mRNA expression level compared with that for the receptor MT_2.

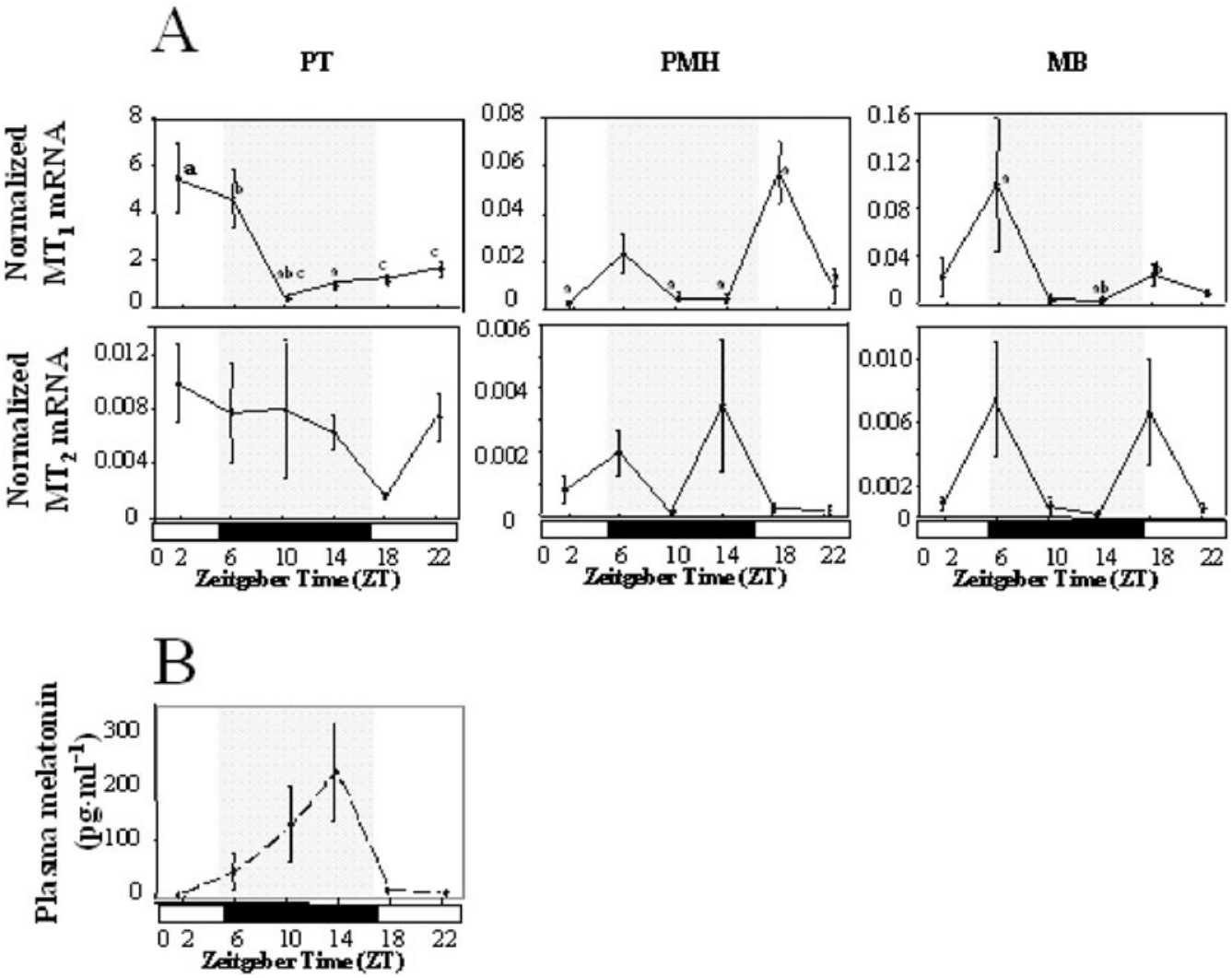


Figure 8 Circadian variation in expression of mRNA for MT_1 and MT_2 receptors in sheep brain tissues. Light–dark variations in MT_1 and MT_2 mRNA expression in sheep brain tissues. Tissues and blood were collected at six time points of the day–night cycle (ZT 1.5, 6, 10.5, 13.5, 18 and 22.5 (n = 5 structures per condition) and light–dark variations in MT_1 and MT_2 mRNA expression in the sheep pars tuberalis (PT), premammillary hypothalamus (PMH) and mammillary bodies (MB) were analysed. For each sample, the data were normalized to the median value for exogenous luciferase. The gene/luciferase values were then compared through ZT and genes within tissues. Normalized expression level of ovine MT_1, ovine MT_2 mRNA (A) and plasma melatonin concentrations (pg·mL^{-1}, B) were measured to estimate the daily changes in these parameters. Data are presented as the mean ± SEM and mean values with different letters are significantly different (ANOVA, $P < 0.05$). Open bars and shaded areas as well as solid bars represent the light and the dark phases respectively.

The real-time PCR on sheep brain area confirmed the high level of expression of ovine MT_1 receptor transcripts in the pars tuberalis and we showed substantial levels of ovine MT_2 mRNA expression in this structure. We showed daily variations of ovine MT_1 mRNA expression under LD conditions, with significantly higher levels of expression during the daytime in the pars tuberalis and premammillary hypothalamus and during the early phase of the night-time in the mammillary bodies. Daytime increase in MT_1 mRNA levels was also reported in rodent ventral tegmental area, nucleus accumbens (Uz *et al.*, 2005) and hypothalamus (Sallinen *et al.*, 2005) using qPCR techniques. These data suggest a previously suspected down-regulation by melatonin of its receptor densities in these structures (Sallinen *et al.*, 2005). Our results showed that the MT_2 receptor mRNA is expressed in the hypothalamus in sheep, as in rodents (Sallinen *et al.*, 2005), although at low levels. Such a low level of expression prevented any significant diurnal variations to be observed, although these levels were likely to be more elevated during the night-time. Ovine MT_2 mRNA was also expressed at low levels in the mammillary bodies, where no significant diurnal variations were obtained.

However, although being much less expressed than MT_1 receptors as demonstrated by this study, MT_2 receptors might also contribute to the melatonin responses in tissues in which they are expressed, as shown in mice carrying a targeted disruption of MT_1 receptors (Jin *et al.*, 2003).

The complexity of the mechanism regulating the melatonin receptor expression makes it almost impossible to compare different studies on tissue melatonin receptor expressions. Indeed, melatonin receptor mRNA level varies on a circadian basis with expression levels affected by light and melatonin

concentration in plasma. Moreover, melatonin down-regulates some of its receptor population (MT_1 and MT_2) (Sallinen *et al.*, 2005). The co-localization of MT_1 and MT_2 receptors in sheep brain tissues can perhaps favour the cross-regulation of these receptors. A cross-regulation between melatonin receptors has already been observed by Imbesi *et al.* (2008) in mouse cerebellar granule cells. In these cells, physiological concentrations (low nanomolar) of melatonin decreased the activity of extracellular signal-regulated kinase (ERK). Deficiencies of both MT_1 and MT_2 receptors transformed the melatonin inhibition of ERK into a melatonin-induced ERK activation. Ayoub *et al.* (2004) have demonstrated that the MT_2 receptor was preferentially engaged in heterodimers with MT_1 receptors, rather than forming MT_2 receptor homodimers, in cells co-expressing both receptors. The same authors have observed that the pharmacological profiles of MT_1/MT_2 receptor heterodimers were different from those of melatonin receptor homodimers.

In conclusion, our data demonstrated unambiguously the existence of a functional MT_2 receptor in sheep. The co-expression of MT_1 and MT_2 receptors in all analysed sheep brain tissues suggest a possible cross-regulation of both receptors. The melatonin regulation of seasonal breeding in sheep may be more complex than previously proposed, based solely on the MT_1 receptor. It remains to determine whether these two receptors are expressed in the same cells and whether they interact to mediate the effects of melatonin on reproduction. The biological role of the ovine MT_2 receptor remains also to be elucidated.

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Conflicts of interest

The authors declare no conflicts of interest.

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