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Functional characterization of polymorphic variants for ovine MT1 melatonin receptors: Possible implication for seasonal reproduction in sheep

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ABSTRACT

In seasonal breeding species, the gene encoding for the melatonin MT_1 receptor (oMT_1) is highly polymorphic and numerous data have reported the existence of an association between an allele of the receptor and a marked expression of the seasonality of reproduction in ewes. This allele called "m" (previously named "−" allele) carries a mutation leading to the absence of a MnlI restriction site as opposed to the "M" allele (previously named "+" allele) carrying the MnlI restriction site (previously "+" allele). This allows the determination of the three genotypes "M/M" (+/+), "M/m" (+/−) and "m/m" (−/−). This mutation is conservative and could therefore not be causal. However, it is associated with another mutation introducing the change of a valine to an isoleucine in the fifth transmembrane domain of the receptor. Homozygous "M/M" and "m/m" animals consequently express structurally different receptors respectively named oMT_1 Val^{220} and oMT_1 Ile^{220}. The objective of this study was to test whether these polymorphic variants are functionally different. To achieve this goal, we characterized the binding properties and the transduction pathways associated with both variants of the receptors. Using a pharmacological approach, no variation in binding parameters between the two receptors when transiently expressed in COS-7. In stably transfected HEK293 cells, significant differences were detected in the inhibition of cAMP production whereas receptors internalization processes were not different. In conclusion, the possibility that subtle alterations induced by the non conservative mutation in "m/m" animals might modify the perception of the melatoninergic signal is discussed in the context of melatonin action.

1. Introduction

Seasonal reproductive activity for species living in temperate latitudes is characterized by alternating ovulatory and anovulatory periods triggered by annual variations in daylength. Photoperiodic cues are transduced into neuroendocrine signals by melatonin. In sheep, this hormone that controls seasonal breeding is secreted during the night

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by the pineal gland (Bitman et al., 1983). Melatonin acts through the link with specific high affinity receptors, MT1 and MT2 (Reppert et al., 1994; Cogé et al., 2009). However, only MT1 is thought to be the main receptor mediating melatonin action on the gonadotrope system to modulate GnRH pulsatile activity (Weaver and Reppert, 1996; Malpaux et al., 1998, 2001). Melatonin receptor 1A gene, named MTNR1A, consists of two exons divided by a large intron (Reppert et al., 1994). The exon II of MTNR1A gene coding for the ovine MT_1 receptors (oMT_1) is known to be highly polymorphic (Barrett et al., 1997; Messer et al., 1997; Pelletier et al., 2000) and a mutation at position 612 that induces the absence of a MnlI restriction site at position 605 (m or minus) characterizes an "m" allele. The presence or the absence of this mutation discriminates three genotypes "M/M", "M/m" and "m/m" previously described as +/+, +/− and −/− (Pelletier et al., 2000). Interestingly, an association between this allele and the expression of seasonality has been evidenced where the "m/m" genotype was associated to seasonal anovulation in spring in two different sheep breeds (Pelletier et al., 2000; Notter et al., 2003). This relationship was also observed in other breeds of sheep where "M/M" animals show lower level of seasonality (Chu et al., 2006; Mateescu et al., 2009; Carcangiu et al., 2009). This allele is characterized by three other mutations one of which at position 706 is non conservative and changes a Valine in an Isoleucine (V220I) in the 5th domain of the melatonin receptor (Pelletier et al., 2000). Thus animals from extreme "M/M" and "m/m" genotypes express structurally different melatonin receptor variants, respectively called oMT_1 Val^{220} and oMT_1 Ile^{220}. It has been postulated that this change alone (V220I) should not modify the receptor's functionality and consequently could not be responsible for the phenotypic differences reported in previous studies (Carcangiu et al., 2009; Mateescu et al., 2009). However, to our knowledge, data supporting this hypothesis are missing. The objective of this study was therefore to explore whether functional differences exist between oMT_1 Val^{220} and oMT_1 Ile^{220} in their binding properties and signal transduction pathways. To that purpose, transient and stable expression in COS-7 and HEK293 cells respectively were developed to analyze and compare ligand binding parameters, cAMP inhibition and internalization properties of the two natural variants of the receptor oMT_1 Val^{220} and oMT_1 Ile^{220}.

2. Materials and methods

2.1. Cloning of the oMT_1 Val^{220} and oMT_1 Ile^{220} ovine melatonin receptor

The protocol used to obtain the complete coding region of the oMT_1 receptor has already been described elsewhere (Mailliet et al., 2004). Briefly, exon I of the gene which shows a low degree of polymorphism (Migaud et al., unpublished observations) was obtained by amplification of genomic DNA using forward and reverse primers 5′-ATGGCGGGGCGGCTGTGGGGCT-3′ and 5′-TTCCCTGCGTTCCTCAGCTTC-3′, respectively. Polymerase Chain Reaction (PCR) was performed according to a high GC content method (Dutton et al., 1993) using a heat stable thermal polymerase (Deep Vent, Biolabs). For the cloning of exon II of the gene, 1 mg of total RNA extracted either from pars tuberalis (PT) of "M/M" or of "m/m" animals from the Merinos d'Arles breed (Pelletier et al., 2000) was reverse transcribed (SuperscriptII reverse transcriptase) with Oligo dT primers. One-tenth of each reaction provided templates for the polymerase (Platinium Taq) to amplify the two partial coding sequences with forward and reverse primers 5′-CTCATCTTCACCATCGTGG-3′ and 5′-AGCTTTAAACGGAGTCCACC-3′, respectively. The resulting PCR products were ligated into pGEM (Promega) in frame with the corresponding upstream coding region of the cDNA. Complete oMT_1 Val^{220} and oMT_1 Ile^{220} sequences obtained from "M/M" or of "m/m" animals respectively, were confirmed by DNA sequencing. Each cDNA (oMT_1 Val^{220} and oMT_1 Ile^{220}) was excised and subcloned in the pcDNA 3.1vA expression vector (Invitrogen) linearised by KpnI (Biolabs). The sequences of both constructs were confirmed by sequencing.

2.2. Transient and stable expression of the ovine MT_1 receptor in COS-7 and HEK293 cells

2.2.1. Transient receptor transfection

oMT_1 Val^{220} and oMT_1 Ile^{220} plasmid DNA (2 μg) were transfected simultaneously in COS-7 cells using the calcium phosphate method (Chen and Okayama, 1987). Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) (supplemented with 10% FCS (Invitrogen), glutamine 2 mM, streptomycin 100 μg/ml and penicillin 100 u/ml at 37 °C and 5% CO_2) and harvested for affinity assays 48 h after transfection.

2.2.2. Stable receptor transfection

HEK293 cells, maintained in Minimum Essential Medium Eagle (MEM, Invitrogen) supplemented (10% fetal calf serum (FCS), glutamine 2 mM, streptomycin 100 μg/ml and penicillin 100 u/ml) in an atmosphere of 95% air/5% CO_2 at 37 °C, were transfected with either pcDNA, oMT_1 Val^{220} or with oMT_1 Ile^{220} cDNA (2 μg) by a liposome-mediated transfection method using the transfection reagent Fugene (Roche). Stable populations were selected with 400 μg/ml of geneticin (G418, Gibco Invitrogen) and screened by binding with 2-[^{125}I]iodomelatonin.

2.3. Membrane preparation and binding assays

2.3.1. Membrane preparation

Transfected cells were harvested in phosphate buffer (phosphate 0.1 M, EDTA 2 mM, pH 7.4). After centrifugation (200 × *g*, 10 min at 4 °C), the cells were homogenised using an ultraturax (IKA Labortechnik) at 4 °C. The homogenate was centrifuged (15,000 × *g*, 45 min at 4 °C) and the resulting membrane pellet was resuspended in stock buffer (75 mM Tris/HCl, 2 mM EDTA, 12.5 mM $MgCl_2$, pH 7.4). The protein determination was performed with BC Assay Protein Quantification Kit (Uptima, Interchim). Aliquots of membrane preparation were stocked at −80 °C until use.