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MTNR1A Melatonin Receptors in the Ovine Premammillary Hypothalamus: Day-Night Variation in the Expression of the Transcripts[1]

Martine Migaud,[2] Agnès Daveau, and Benoît Malpaux

Physiologie de la Reproduction et des Comportements, UMR 6175 INRA-CNRS-Université de Tours-Haras Nationaux, 37380 Nouzilly, France

ABSTRACT

Melatonin regulation of reproductive functions in sheep is mediated by action in the premammillary hypothalamus (PMH). The aim of this study was to identify the high-affinity melatonin-receptor subtypes expressed in this structure. To achieve this, we used reverse transcription-polymerase chain reaction (RT-PCR) and developed in situ hybridization techniques (ISH). By using RT-PCR, we detected a band corresponding to the MTNR1A melatonin-receptor cDNA in the PMH as well as in the pars tuberalis (PT). On the opposite, MTNR1B melatonin-receptor transcripts were not detected using degenerate primers in any of the structures considered, confirming the lack of expression of this receptor subtype in sheep. The expression of *MTNR1A* mRNA was further confirmed in the PMH by ISH with a ^{35}S-labeled ovine *MTNR1A* riboprobe. We next investigated the variation in the expression of *MTNR1A* mRNA between the end of the day and the end of the night (absence and presence of melatonin, respectively). *MTNR1A* transcript expression was greater at the end of the night than at the end of the day in the PMH. In contrast, *MTNR1A* mRNA expression was lower at the end of the night than at the end of the day in the PT. No significant variation in the *MTNR1A* mRNA expression was observed in a more dorsal hypothalamic area. Overall, these results show that *MTNR1A* transcripts are expressed in the ovine PMH and that their expression follows a diurnal rhythm, which is different from the pattern of expression observed in the PT.

hypothalamus, melatonin, neuroendocrinology, receptors, seasonal reproduction

INTRODUCTION

Most species that live in temperate climates use the annual variations in day length as temporal cues to initiate changes in their reproductive condition. Photoperiodic information is conveyed from the retina through a multisynaptic pathway to the pineal gland. From there, the variations in the annual lighting conditions are converted into rhythmic synthesis of the pineal hormone, melatonin. Synthesis and secretion of this indolamine follow a circadian rhythm with high levels at night and low levels during the day in both blood and cerebrospinal fluid [1]. The variations in the duration of nocturnal melatonin secretion serve as an endocrine messenger of the season to adjust the neuroendocrine status of seasonal animals and regulate the pulsatile secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus [2]. In sheep, long periods of melatonin secretion (i.e., short days) stimulate the GnRH system.

The use of the specific radioligand 2-[^{125}]I melatonin in in vitro studies using autoradiography has allowed the detection of high-affinity melatonin binding sites in several areas of the brain and pituitary [3] and has revealed putative central nervous system (CNS) targets for the control of reproduction by melatonin. Although the pars tuberalis (PT) expresses a higher density of high-affinity melatonin receptors than any other CNS or pituitary areas in most species, this structure does not seem to mediate the effects of melatonin on neurons involved in neuroendocrine control of reproduction but appears rather involved in the photoperiodic control of prolactin secretion [4]. Functional studies have demonstrated the hypothalamic localization of the melatonin target in sheep and hamsters. A hypothalamic target has been strongly suggested by lesion studies in Syrian hamsters [5, 6]. In ewes, a strong correlation has been observed between the proximity of melatonin microimplants to an area of binding, the premammillary hypothalamic (PMH) [7], and the ability of the implants to stimulate luteinizing hormone secretion [8]. This area is also potentially involved in the generation of endogenous circannual rhythms [9].

Melatonin action is mediated through specific receptors. In mammals, two high-affinity melatonin-receptor subtypes have been cloned and characterized, MTNR1A melatonin receptor [10] and MTNR1B melatonin receptor [11], also known as MT_1 and MT_2, respectively. MTNR1A and MTNR1B share similar binding properties and pharmacological characteristics [12]. However, whether they are both involved in the central control of seasonal reproduction and expressed in the ovine PMH remains to be determined. The lack of pharmacological analogues (agonists or antagonists) highly specific for one or the other of each receptor subtype makes the identification of the subtype involved difficult. However, the implication of MTNR1B receptors does not seem likely because seasonality of reproduction is not altered in two species of hamster presenting a natural knockout of the gene encoding this receptor [13]. No expression of MTNR1B has ever been detected in sheep so far [14]; however, it has not been assessed in the hypothalamus.

The purpose of this study was, therefore, to undoubtedly identify melatonin-receptor subtype(s) expressed in the ovine PMH and in other brain areas. Because no specific antibodies for MTNR1A and MTNR1B receptors are currently available to allow immunohistochemical experiments to be carried out in this model as well as in other models of seasonal mammals, we performed reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization experiments to address this question.

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[2]Correspondence: Martine Migaud, PRC, Domaine de l'Orfrasière, INRA UMR 6073, 37380 Nouzilly, France. FAX: 33 247 427 743; e-mail: migaud@tours.inra.fr

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MATERIALS AND METHODS

Animals and Tissue Preparation

All experiments were performed according to the A37801 authorization granted by the French Ministry of Agriculture and Fisheries. For the RT-PCR experiments, tissue samples (PT, PMH, dorsal hypothalamus [dHP], and mammillary bodies [MB]) were collected from adult animals of various breeds in the morning from the local abattoir, immediately frozen in liquid nitrogen, and kept at −70°C until RNA extraction. Total RNA was obtained by the guanidium thiocyanate extraction method first described by Chomczynski and Sacchi [15] from 100–300 mg of each tissue. Total RNA was extracted from Chinese hamster ovary (CHO) cells expressing human recombinant MTNR1B receptor subtypes [16] (CHO-hMT_2; donated by Drs. Boutin and Delagrange, Servier Research Institute, France) using RNeasy total RNA kit (Qiagen, France) according to the manufacturer's instructions.

For the in situ hybridization (ISH) studies, 11 adult INRA 401 breed ewes kept under natural photoperiod were divided in two groups of five and six animals and killed in September, the early part of the breeding season, (sunrise, 0715 h; sunset, 2025 h) by decapitation by a licensed butcher in an official slaughterhouse. The first group of five ewes was killed at the end of the day (from 1700 to 1745 h) and the second group of six ewes at the end of the night (from 0500 to 0545 h) in the dark under red light. Brains from both groups were immediately removed, their hypothalamus dissected out and rapidly frozen by immersion in isopentane precooled to −50°C in nitrogen. They were then stored at −70°C until sectioning.

Plasma Melatonin Concentration

Blood samples from each animal were collected before decapitation. Plasma melatonin-concentration measurements were performed using a well-validated radioimmunoassay [17]. Results are expressed in picograms/milliliter ± SEM.

Oligonucleotide Primers and RT-PCR

Specific sense and antisense 19–20mer oligonucleotide primers (Sigma Genosis, UK) were directed toward selected regions of exons 1 and 2 of the gene coding *MTNR1A* receptor, respectively [10], to avoid the amplification of genomic DNA. The sequences 5′-CTGTCCGTGTATCGGAA-CAAG-3′ (bases 244–264) and 5′-GCCATATAGTAACTAGCCAC-3′ (bases 947–928) correspond to the upper-strand and lower-strand primers, respectively. The ovine *GAPD* (AF030943) served as a control for the quality of cDNA, and forward and reverse primers were 5′- GTGATGC TGGTGCTGAGTAC-3′ (bases 127–146) and 5′-GTAGAAGAGTGAGT GTCGC-3′ (bases 745–727), respectively. Degenerate primers for MTNR1B gene were designed from the previously reported rat (AF141843), mouse (U57554), hamster (U57555), and human (NM_ 005959) sequences encoding MTNR1B receptors. The sequences of the primers were as follows: sense primer, 5′-TACTGSTRCATCTGYCAY-AG-3′, and antisense primer, 5′-ACATGGTTAGRAARCTSSGC-3′, to produce a band at 346 base pairs (bp). Total RNA was primed with oligodT and converted into cDNA using a reverse-transcriptase (Superscript II; Invitrogen, France) for 50 min at 37°C. After enzyme inactivation (10 min at 70°C), cDNAs were subjected to PCR amplification. PCRs were run in 50 μl containing 100 mM dNTPs, 2 mM $MgCl_2$, 0.8 μM primers, 2 μl cDNA, and 1 U *Taq* polymerase (Platinum Taq; Invitrogen). PCRs were preceded by a 3-min predenaturation step at 94°C, then run for 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min followed by an 8-min extension period. A PCR control was performed by replacing the cDNA sample with water. Ten microliters of the PCR were loaded in parallel with the molecular weight marker (100-bp ladder; NEB, Ozyme, France) on a 1% agarose gel containing ethidium bromide. To confirm the identity of the sequence, the amplified cDNA fragment of 706 bp obtained from PMH cDNA was subcloned into pCRII vector (Invitrogen) and sequenced (Genome Express, France).

Radiolabeled MTNR1A cRNA and Sense Control Probes

The template for the transcription was a PCR product amplified from sheep genomic DNA and subcloned into pCRII (Invitrogen) [17]. The final 106-bp fragment of the probe (from the ATG to the NotI restriction site) was obtained by deleting a 125-bp NotI fragment. Integrity of the sequence was confirmed by automated DNA sequencing (Genome Express). One to 3 μg of linearized plasmid were transcribed with Sp6 (NEB) and T7 (RNA transcription kit; Stratagene, France) with ^{35}S-[UTP] (Amersham, France) to produce sense and antisense riboprobes, respectively, according to the manufacturer's instructions. The probes were purified using Sephadex G-50 spin columns (Amersham).

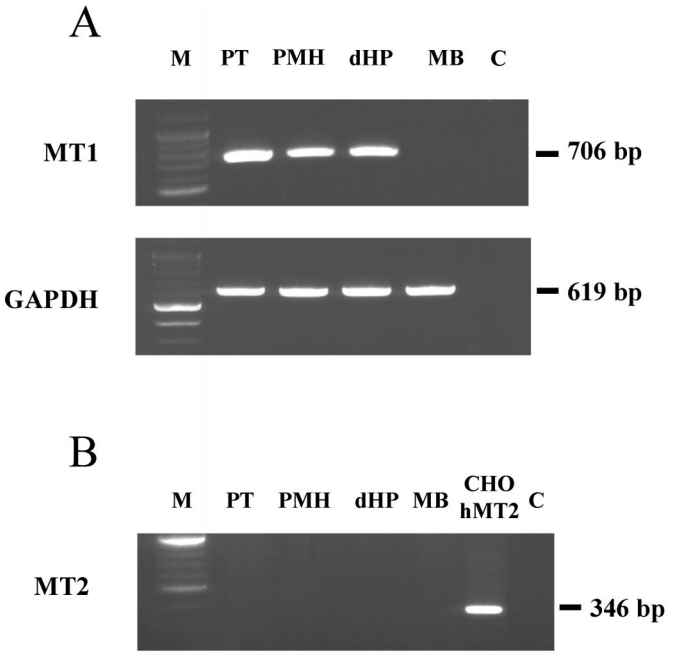

FIG. 1. **A**) RT-PCR demonstration of *MTNR1A* gene expression in ovine premammillary hypothalamus. Upper photograph: RT-PCR produced a 706-bp band in the pars tuberalis (PT), in the PMH, and in the dHP. Lower photograph: The amplification of gene transcripts for *GAPD* (605 bp) serves as a control for the quality of RNA. **B**) Absence of detection of *MTNR1B* transcripts except in CHO overexpressing human *MTNR1B* receptors. M, Molecular weight marker; MB, mammillary bodies; CHO-hMT_2, CHO cells expressing human recombinant MTNR1B melatonin-receptor subtypes; C, negative PCR control.

In Situ Hybridization

Coronal hypothalamic sections (20 μm) from the posterior hypothalamus were obtained using a cryostat, collected on 3 amino propyl tri ethoxy silane (Sigma, France) -coated slides and stored at −70°C before use. For both PMH and dHP, three sections at 200-μm intervals were analyzed per animal. Paired sections taken from each ewe from both groups were treated. ISH was performed according to Sibony and coworkers [18] and modified as previously described [19]. Incubation of each hybridization solution containing 200 000 dpm of the sense or antisense hybridization riboprobe was followed by two washes (saline-sodium citrate [SSC] 4×, dithiothreitol (DTT) 10 mM) at 50°C for 30 min and (SSC 2×, DTT 10 mM, 50% formamide) at 55°C for 20 min, respectively. Sections were next RNase treated (10 μg/ml in NaCl, 0.5 M Tris 10 mM, pH 7.5, EDTA 5 mM, pH 8.0) for 1 h at 37°C. After two washes (NaCl, 0.5 M, Tris 10 mM, pH 7.5, EDTA 5 mM, pH 8.0, and SSC 0.1×), sections were dehydrated, air dried, and coated with liquid NTB2 emulsion (Kodak, France), diluted twice with sterile demineralized water, and exposed for 5 wk. Following development and fixation using standard procedures, sections were counterstained with 0.1% neutral red.

Quantification was performed as previously described for serotonin receptors of class II (5HT2-R) [9, 19] using a computerized image-analysis system (Biocom Histo 500; Les Ulys, France). All observations and quantifications were conducted by an experimenter unaware of the animal group. The quantitative analysis was performed using dark background to detect all the grains. Due to the nature of the PT tissue, distinction of individual cell labeling was not possible. Determination of the labeling was then carried out using a 20-mm^2 area as an arbitrary surface unit. Between 5 and 21 measurements per section were made and between 2 and 4 sections were analyzed per animal. Results were expressed as the number of grains per surface unit. For PMH and dHP sections, a cell was considered positively labeled if it contained at least 20 silver grains. This cutoff is equal to the average plus 4 SDs of the number of grains obtained

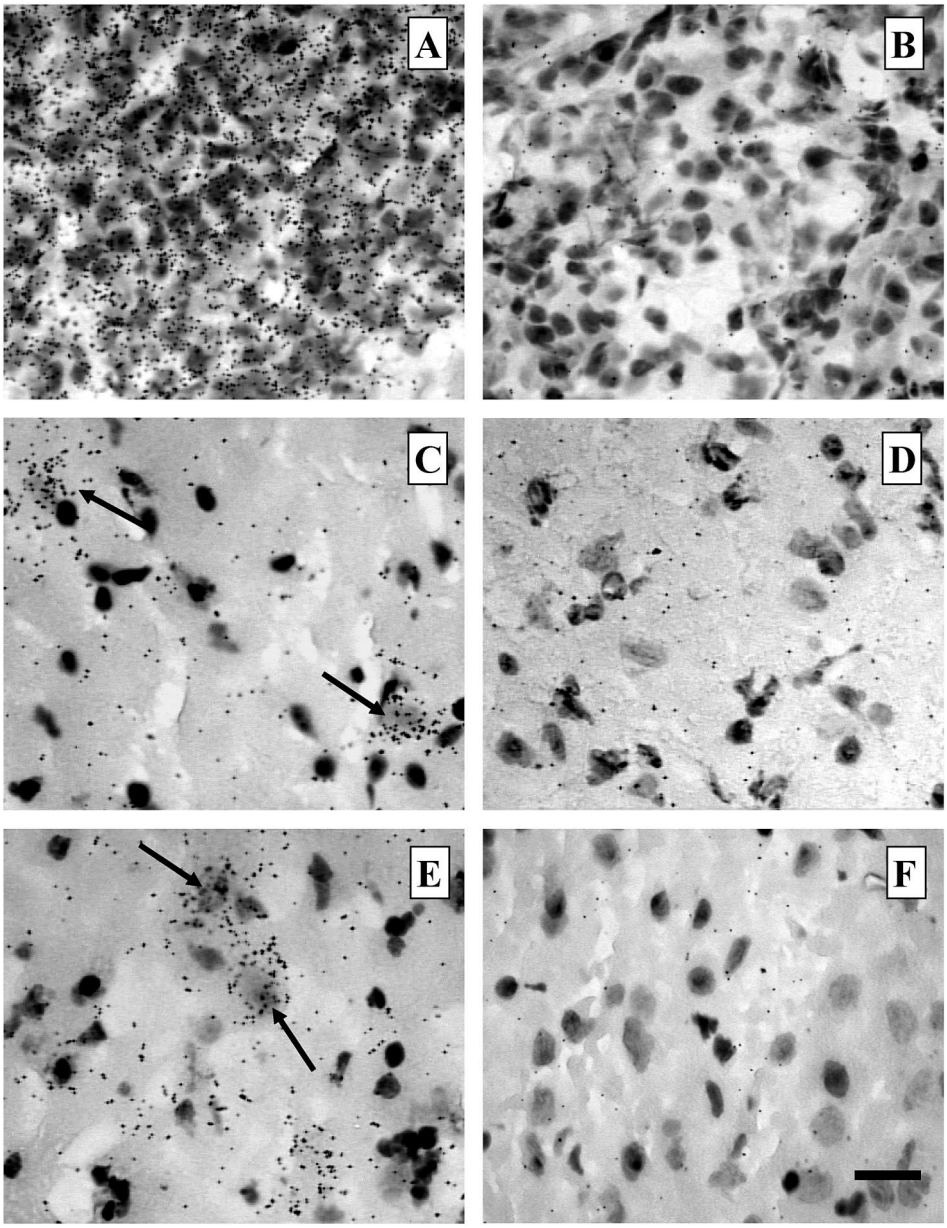

FIG. 2. Bright-field microscopy images of PT (**A**, **B**), PMH (**C**, **D**), and dHP (**E**, **F**) after in situ hybridization with antisense (**A**, **C**, **E**) or sense (**B**, **D**, **F**) riboprobes for MTNR1A receptors. Arrows indicate representative PMH and dHP cells hybridized to radiolabeled antisense riboprobe for *MTNR1A* receptor mRNA. Only a very low level of background is observed when the sense control probe was used. **A**–**F**) Sections taken from the same animals. Bar = 20 μm.

in sections hybridized using the sense control probe. The PMH corresponds to a 2- × 4-mm area positioned at the base of the hypothalamus, next to the third ventricle and limited dorsally by the fornix. The dHP area corresponds to a 1- × 4-mm area limited dorsally by the mammillothalamic tract (MT) and located exactly above the PMH area. All the labeled cells were counted in both areas. Results are expressed as the mean (±SEM) number of labeled cells and of the mean (±SEM) number of silver grains per labeled cell.

Statistical Tests

Density labeling in ISH was compared between groups using unpaired *t*-test analysis.

RESULTS

Mean melatonin concentration was 5.4 ± 0.6 pg/ml for animals slaughtered at the end of the day and 179.4 ± 23.7 pg/ml for those killed at the end of the night.

The specific MTNR1A receptor primers enabled the amplification of the predicted PCR products (706 bp) from the PT, the PMH, and the dHP cDNA (Fig. 1A, upper photograph). However, no band could be detected from MB cDNA, a structure where no melatonin-binding site could be found. Sense and antisense primers were designed from exon I and exon II, respectively, to exclude genomic DNA contaminant. RNA integrity was tested using specific sense and antisense primers for ovine GAPD (Fig. 1B), and a band of the expected size (619 bp) could be observed in all the samples (Fig. 1A, lower photograph). To confirm the base composition of the PCR product obtained from PMH cDNA, the band was excised, subcloned, and sequenced, and the data confirmed the cDNA was the one expected for *MTNR1A* (AY680863) [10].

To assess the presence of hypothetical MTNR1B receptors in the ovine structures, degenerate primers designed from the sequence of the human, murine, rat, and hamster MTNR1B receptors were used with each cDNA. No band could be detected with these primers, although a band of the expected size (346 bp) was observed using cDNA obtained with total RNA extracted from CHO cells expressing human recombinant MTNR1B, indicating the validity of the primers used (Fig. 1B).

ISH analysis using *MTNR1A* antisense riboprobe re-

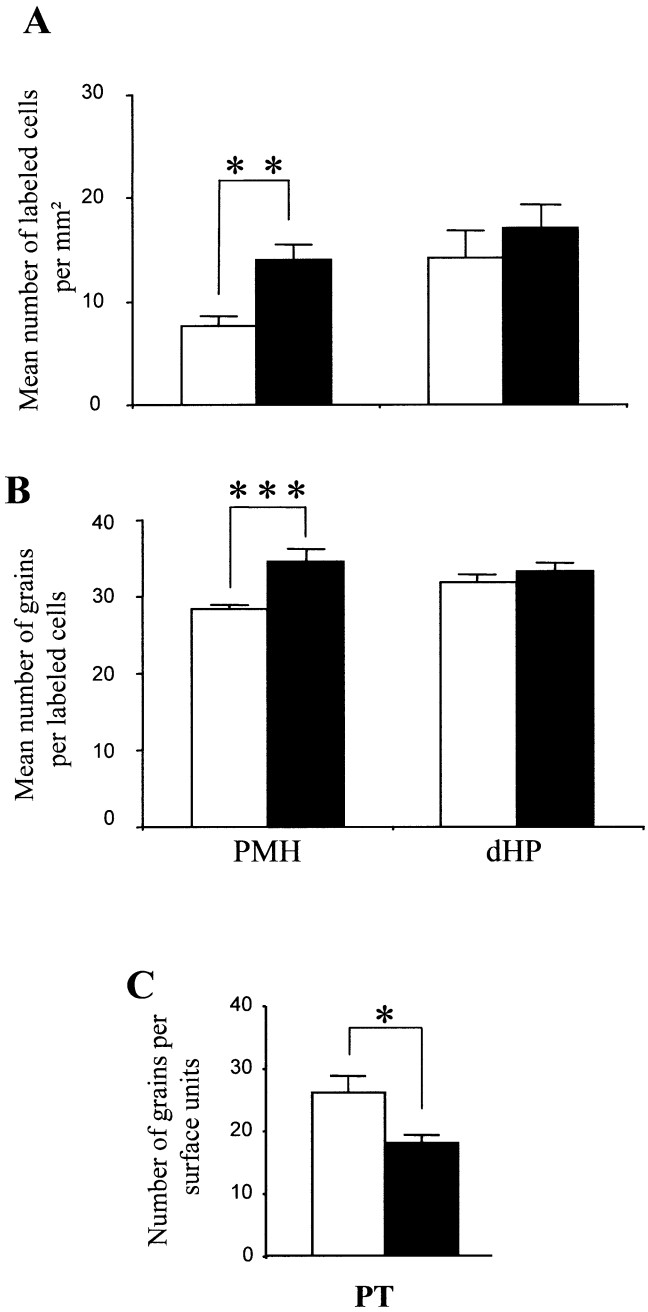

FIG. 3. Quantitative ISH in PMH, dHP, and PT. **A**) Mean number of labeled cells per mm² cell in the PMH and in the dHP at the end of the day (open bars) and at the end of the night (black bars). **B**) Density of silver grain per labeled cell in the PMH and in the dHP at the end of the day (open bars) and at the end of the night (black bars). **C**) Density of silver grain per surface unit in the PT at the end of the day (open bars) and at the end of the night (black bars). Means ± SEM are shown. *: $P \leq 0.05$ assessed by Student *t*-test.

vealed labeling in the PT, where most of the cells displayed a high number of silver grains (Fig. 2A), in the PMH (Fig. 2C), and in the dHP (Fig. 2E). Only background staining could be detected in either of these areas with the control sense riboprobe (Fig. 2, B, D, and F) showing the specificity of the labeling.

Quantitative ISH in the PMH showed that the density of labeled cells per mm² differs significantly between the end of the day and the end of the night (7.6 ± 1.0 versus 14.2 ± 1.4, $P < 0.005$; Fig. 3A), whereas no difference is observed in the dHP (14.1 ± 2.5 versus 17.1 ± 1.8; Fig. 3A). In addition, the density of silver grains per labeled cell in the PMH was significantly higher in the labeled cells at the end of the night than at the end of the day (28.4 ± 0.5 versus 34.3 ± 1.8, $P < 0.001$; Fig. 3B), whereas no significant difference between night and day was observed in the densities of silver grains per labeled cell in the dHP (31.9 ± 1.4 versus 33.2 ± 1.2; Fig. 3B). Analysis of the *MTNR1A* transcript expression in the PT revealed that *MTNR1A* mRNA expression level was significantly higher at the end of the day than at the end of the night (26.8 ± 2.7 and 18.0 ± 1.27 grains per surface unit, respectively, $P < 0.005$; Fig. 3C).

DISCUSSION

This study provides the first demonstration of *MTNR1A* transcript expression in the ovine PMH, the melatonin target for its reproductive effect, using two different techniques. The RT-PCR experiments show expression of *MTNR1A* in the PT, PMH, and dHP. PT was used as a positive control in this study because the ovine MTNR1A was initially cloned from a PT cDNA library [10]. The expression of *MTNR1A* mRNA in the PMH and dHP is in agreement with previous autoradiographic [7, 8] and binding [20] data showing the presence of high-affinity melatonin binding sites of moderate densities in the PMH and low levels of binding densities in other areas of the hypothalamus, especially in the dHP. No *MTNR1A* transcript expression could be detected in the MB, consistent with the absence of binding sites observed in this area [7].

The absence of any detectable band when degenerate primers for *MTNR1B* were used in RT-PCR experiments is in agreement with data published so far indicating that MTNR1B receptors are not expressed in sheep [14, 21]. The absence of detection of the *MTNR1B* mRNA reported here suggests that MTNR1A is the only high-affinity melatonin-receptor subtype accounting for the binding level observed in those ovine structures.

We confirmed the presence of *MTNR1A* mRNA in the ovine PMH using ISH. To our knowledge, this is the first demonstration in this species of MTNR1A-labeled cells in this area. A low density of labeled cells is found in the PMH, whereas dense labeling was observed in the PT. These results are in agreement with binding data, where the maximal number of binding sites was shown to be 20–100 times lower in the PMH than in the PT [7, 8]. Interestingly, a recent study has shown that three nuclei could be differentiated within the PMH, the caudal hypothalamic arcuate nucleus, the ventral premammillary nucleus, and the ventral tuberomammillary nucleus, each with distinct neurochemical characteristics [22]. The coupling of ISH and immunohistochemical techniques should indicate whether and how *MTNR1A* transcript expression is regionalized between the three nuclei of the PMH. The use of both techniques should also shed light on the neurochemical characterization of the *MTNR1A* mRNA-positive cells. In the Siberian hamster, suprachiasmatic nucleus *MTNR1A* transcripts were shown to colocalize with arginine vasopressin and retinoid Z receptor β [23].

Rhythmic regulation of *MTNR1A* transcript expression in the sheep PT, with lower levels found at the end of the night, is in line with the daily pattern of expression found in rat SCN using quantitative RT-PCR and ISH [24, 25] and in hamster PT and SCN by quantitative ISH [26, 27]. In these structures and species, melatonin may downregulate the expression of *MTNR1A* mRNA by cAMP-depen-

dent and -independent mechanisms, as shown in ovine PT cells in culture [28]. However, more time points are needed to examine the detailed temporal variations of the *MTNR1A* mRNA expression in intact as well as in pinealectomized ewes to check whether these variations are melatonin dependent.

Transcriptional mechanisms of *MTNR1A* gene expression seem to be differently regulated according to the structure considered because, in ovine PMH, a higher level of gene expression is observed at the end of the night (high level of melatonin) when compared with the end of the day (low level of melatonin). Such a region-specific regulation has also been observed for the rat PT and SCN [25, 27]. In both cases, melatonin-independent mechanisms involved in the regulation of *MTNR1A* gene expression cannot be excluded. In contrast, no day-night variation in the expression of *MTNR1A* transcripts could be observed in the dHP. Previous functional studies have shown that melatonin microimplants placed into the PMH were able to stimulate the GnRH system, whereas microimplants located 1 mm outside the PMH area were ineffective [8]. In addition, a day-night change in the expression of the neuronal activation marker Fos was observed in this region, with a higher number of Fos-expressing cells during the day than during the night [29]. This effect, which is pineal dependent in the PMH but not in other brain areas, may rely on the day-night variation in the *MTNR1A* transcript expression observed in the PMH in this study. The absence of day-night variation in *MTNR1A* mRNA expression in the dHP, an area located more than 1 mm above the PMH and therefore not involved in the melatonin-dependent stimulation of GnRH neurons, supports this hypothesis. It is then tempting to speculate that melatonin, through the inhibitory G-protein signaling pathway linked to the activation of *MTNR1A* receptors, induces a decrease in nocturnal Fos expression in the ovine PMH.

In conclusion, the evidence of the presence of *MTNR1A* transcripts, together with the lack of expression of the *MTNR1B* receptor subtypes, suggests that melatonin may act through the activation of MTNR1A receptors in the PMH to control seasonal reproduction. The next step will be to test this hypothesis by specifically inactivating their expression in this area.

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