Inhibition spécifique des récepteurs de la mélatonine par une approche d'ARN interférent

Introduction

Les récepteurs de la mélatonine sont largement décrits dans la littérature, ainsi que leurs voies de signalisation. Néanmoins, aucun anticorps spécifique ni même de ligand sélectif ne sont disponibles. Afin d'étudier les voies de signalisation et le(s) rôle(s) respectif(s) des récepteurs mélatoninergiques, nous avons mis au point une approche originale basée sur le principe d'ARN interférence. L'objectif est d'inhiber spécifiquement les récepteurs de la mélatonine exprimés de façon stable dans la lignée cellulaire CHO-K1 et dans des cultures primaires de *pars tuberalis*, structure cérébrale très riche en récepteurs MT. Cette approche technique permet même d'envisager son utilisation ultérieure *in vivo*.

Notre approche pour cette étude a été de valider l'efficacité des siRNA aussi bien au niveau transcriptionnel qu'au niveau fonctionnel. La quantité relative d'ARNm codant pour les récepteurs ovins MT_1 et MT_2 a été mesurée dans la lignée CHO exprimant de façon stable ces récepteurs ainsi que dans des cultures primaires de pars tuberalis ovine ayant subi ou non un traitement avec les siRNA par RT-PCR en temps réel. La fonctionnalité des récepteurs suite à un traitement avec des siRNA a été évaluée à l'aide d'un test de saturation avec la 2-[^{125}I]-iodo-mélatonine, en mesurant la capacité de liaison de ces récepteurs. L'effet des siRNA dirigés contre les récepteurs mélatoninergiques sur la signalisation AMPc a également été évalué, par un dosage ELISA et par Western-Blot.

Targeting knock-down of ovine melatonin receptors using RNA interference in CHO cells

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Abstract

The ovine species is an animal model for study of seasonality of reproduction. It is well established that ovine melatonin receptors, oMT_1 and oMT_2, are expressed in several brain areas, particularly in *pars tuberalis* and in premamillary hypothalamus, both target of melatonin for the control of reproduction. The question of respective involvement of MT_1 and MT_2 in the control of reproductive seasonality has yet to be resolved. In the absence of specific and selective ligands for MT receptors, we choosed to develop an approach using RNA interference to discriminate the respective roles of MT receptors. First, siRNAs designed to target MT_1 or MT_2 were assessed in CHO-K1 cell lines stably expressing oMT_1 or oMT_2 receptors. After siRNA treatment, binding capacity (B_{max}), but not affinity (Kd), of oMT_1 as well as oMT_2 receptors for melatonin was significantly decreased. Similarly, relative quantity of mRNA encoding for ovine MT receptors decreased after treatment with siRNA. However, no effect of siRNA treatment was observed on cAMP signalling and on phosphorylation of ERK proteins. Possible reasons of this absence of effect are discussed in this article.

Introduction

Melatonin is a neurohormone synthesized and secreted by the pineal gland only during the night. The most definitive physiological role of melatonin is to convey information about day length (photoperiod) to body physiology for the organization of functions that vary with season, such as reproduction, pelage, appetite, body weight, and sleep (reviewed in Dubocovich et al., 2010). Other roles were admitted to melatonin at high concentrations (micromolar and beyond), like anti-oxidative, anti-mitotic or immunological properties (Drazen and Nelson, 2001; Mao et al., 2010; Sliwinski et al., 2007).

Melatonin acts through three main targets: two G protein-coupled, seven-transmembrane receptors (MT_1 and MT_2) and one atypical binding site called MT_3. This last binding site has been purified in our laboratory and is designated as quinone reductase 2 (QR2) (Nosjean et al., 2000). Collectively, the repertoire of G protein-dependent signalling pathways activated by MT_1 and MT_2 receptors is very similar. Signal-transduction pathways triggered by MT_1 and MT_2 receptors were characterized and involve the activation of both pertussis toxin-sensitive and -insensitive G proteins. Indeed, both receptors preferentially couple to Gi proteins. MT_1, and most likely MT_2, couple also to Gq/11 proteins, although to a lesser extent (Brydon et al., 1999b; Jarzynka et al., 2006). G protein activation of the MT_1 receptor modulates several signal-transduction pathways. Melatonin typically inhibits forskolin-stimulated cAMP formation (Brydon et al., 1999a; Brydon et al., 1999b), protein kinase A activity (Morgan et al., 1994; Witt-Enderby et al., 1998) and phosphorylation of the cAMP-responsive element binding (CREB) (McNulty et al., 1994; McNulty et al., 1996; Witt-Enderby et al., 1998). Activation of the MT_1 receptor also increases the phosphorylation of the mitogen activated protein kinase kinases 1 and 2 (MEK1 and MEK2), the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) (Witt-Enderby et al., 2000) and c-Jun N-terminal kinase (JNK) via pertussis toxin-sensitive and -insensitive G proteins (Chan et al.,

2002). Similarly, activation of the MT_2 receptor inhibits forskolin-stimulated cAMP production (MacKenzie et al., 2002; Reppert and Weaver, 1995) and stimulates JNK (Chan et al., 2002) and phosphoinositide turnover (MacKenzie et al., 2002).

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). Sheep is a relevant and widely used species for *in vivo* studies on melatonin as it is less distantly related to human than rodents in terms of diurnal/nocturnal behaviour. Recent cloning of oMT_2 receptor (Coge et al., 2009) from ovine retina allows to study the pharmacology of two sub-types of ovine melatonin receptors. Unfortunately, specific and selective ligands were not available to study respective role of MT_1 and MT_2 receptors. An alternative approach consists in using siRNA methodology to inhibit specifically one subtype of melatonin receptor. Known since 1998 as small interfering RNA (siRNA), this physiological tool allows a transient but potent inhibition of specific gene expression. This phenomenon is based on the delivery of double-stranded RNA (dsRNA) into cells that causes degradation of the complementary mRNA (Hammond et al., 2000). In cells, the siRNA further assembles with protein components into a RNA-induced silencing complex (RISC), that will ultimately cause the degradation of the corresponding target mRNA. RNAi approach enables to perform a cellular knock-down of MT_1 and/or MT_2 receptors. RNAi is therefore a valuable tool for gene function discovery both *in vitro* and *in vivo* or for future potential therapy. The aim of the study was to determine whether siRNA molecules could specifically target MT_1 or MT_2 mRNA for knock-down in Chinese human ovary cells expressing MT_1 or MT_2. This would be of benefit to decipher functions of MT_1 and/or MT_2 receptors and more particularly implication of each MT receptors in signalling pathway.

Materiels and methods

Reagents

Ham F12 Glutamax, foetal bovine serum, geneticin. All culture media were from Invitrogen (Carlsbad, CA).

CHO-K1-oMT culture

CHO-K1 cells stably expressing oMT_1 and oMT_2 receptors were grown in Ham F12 Glutamax medium supplemented with 10% foetal bovine serum and 400 µg/mL geneticin (Audinot et al., 2008). CHO-K1-oMT cells were transfected with siRNAs directed against oMT receptors or control siRNA as described below.

siRNA design

All siRNAs were chemically synthetized by Invitrogen. Three siRNA sequences designed to target oMT_1 receptor mRNA were used: 5'AAUUCGUGCCGCUAUACAGCUUGCC3' (named siRNA 502), 5'CCGAUGCUCGUAGUCGUCUUCUGUU3' (named siRNA 691) and 5'UGGCUGUUUGUGGCUAGUUACUAUA3' (named siRNA 919). Each siRNA possessed its own control siRNA, described as follows: 5'GGCUCGAUGGAUGGCCACAGAAAUU3' (named control siRNA 502), 5' CCGGCUCGUAGUCGUCUUCUAUGUU3' (named control siRNA 691) and 5'UGGUUGUGUCGGGAUAUUUCCUAUA3' (named control siRNA 919). Three siRNA sequences designed to target oMT_2 receptor mRNA were used: 5'CAGGUAACCUGUUCUUGGUGAGUCU3' (named siRNA 735), 5'UGAGCAUGUUCGUGGUCUUUGUGAU3' (named siRNA 1266) and 5'ACGCCAUCAUCUAUGGGCUCCUGAA3' (named siRNA 1425). Each siRNA possessed its own control siRNA, described as follows: 5'CAGCAAGUCCUUGUUGUGGAGUUCU3' (named control siRNA 735),

5’AUCCGAACAAAGCCCAAGCAUAUCA3’ (named control siRNA 1266) and 5’ACGACUAAUCUGGGUCCUCUCCGAA3’ (named control siRNA 1425).

siRNA knockdown of oMT receptors level

For transfection, CHO-K1-oMT$_1$ and CHO-K1-oMT$_2$ cells grown at 30% confluence in Ham F12 Glutamax medium supplemented with 10% fetal bovine serum and antibiotics (400 µg/mL geneticin) in 24-well plates (for qRT-PCR) and in Petri dish 100mm (for binding assay) were transfected with 20 nM siRNA using GeneSilencer (Gene Therapy Systems, Inc) according to the manufacturer's instructions. Fluorescent nonspecific siRNA (BLOCK-iT^{TM} Alexa Fluor$^{®}$ Red Fluorescent Oligo, Invitrogen, Carlsbad, CA) was used to evaluate the transfection efficiency.

Forty-eight hours after transfection, cells were used either for 2-[^{125}I]-iodo-melatonin binding assay or for total RNA extraction procedure, as described as follows.

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

Cells were removed with trypsin and centrifuged at 900 rpm for 10 min. The resulting cell pellet was suspended in binding buffer (Tris/HCl 50mM, pH 7.4, 5 mM MgCl2, 1 mM EDTA) in order to obtain a solution with 13 000 cells/ml. Cells were incubated for 2 h at 37°C in binding buffer in a final volume of 250 µl containing 2-[^{125}I]-melatonin (specific activity: 2500 Ci/mmol, PerkinElmer, Massachusetts, USA). Non-specific binding was defined with 1 µM melatonin (Sigma-Aldrich, Saint-Quentin-Falalvier, France). Reaction was stopped by rapid filtration through GF/B unifilters, (PerkinElmer, Massachusetts, USA), followed by three successive washes with ice-cold buffer. Data were analysed by using the program PRISM (GraphPad Software Inc., San Diego, CA). The density of binding sites

(B_{max}) and the dissociation constant of the radioligand (K_D) values were calculated using non-linear regression model.

Quantitative RT-PCR from CHO-K1-oMT

Levels of mRNA for oMT_1 and oMT_2 receptors were measured using SYBR Green RT-PCR (iQ SYBR Green Supermix, Bio-Rad). Total RNA from 10 cm^2 well (plated at $3x10^6$ cells/well) was extracted using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from 2 µg RNA in a volume of 20 µl containing 250 ng oligodT (Promega), 1 mM dNTPs, 40 U/µl of RNasin, 1x RT PCR buffer, and 200 U/µl M-MLV reverse transcriptase (Promega). For the negative control, the reverse transcriptase was omitted. The RNA denaturation was performed at 70 °C for 10 min, and the RT was performed at 37 °C for 1 h.

For PCR, each reaction consists of a final reaction volume of 20 µl containing iQ SYBR Green Supermix (Bio-Rad) (1x), 3 µM of each gene-specific primer, and cDNA. Primers were designed using Beacon Designer Software (Premier Biosoft International, Palo Alto, CA, USA), and sequences are described in Table 1. The equivalent of 5–50 ng of starting RNA was used in each reaction. Each sample was assayed in duplicate. Quantitative real-time PCR was run on an iCycler from Bio-Rad. The amplification program consisted of 95 °C for 3 min followed by up to 34 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 20 s. Annealing temperatures are given in Table 1.

Prior to analysis, amplification efficiency was determined for each gene by generating a standard curve using serial dilutions of the cDNA, obtained after reverse transcriptase of the RNA, in abscissa and the corresponding cycle threshold (C_t) in ordinate. The slope of the log-linear phase reflects the amplification efficiency derived from the formula $E=(10^{-slope}-1)x100$.

Amplification efficiency was around of 80%, and was equivalent for test gene and the reference gene. Amplification was followed by melting curve analysis for each primer pair to verify the presence of one gene-specific peak and the absence of primer dimers. PCR products obtained with each set of primers were run on an agarose gel, and visualized using ethidium bromide and u.v. illumination to confirm amplification of a single product of the correct size. This was followed by sequencing each amplicon to confirm the identity of amplified product, and the results were matched with the National Center for Bioinformatics Computing (NCBI-BLAST) database. Quantification of specific mRNA levels was performed by the $\Delta\Delta C_t$ method with the internal reference gene *18S* (Bulletin #2; PE Biosystems, Foster City, CA, USA).

cAMP assay ELISA

CHO-K1 cells stably expressing either the oMT_1 or oMT_2 receptors grown to 50% confluence in 24-well plates were transfected with siRNA as previously described. Cells were incubated for 1 h with increasing concentrations of melatonin in the presence of 100 μM IBMX with or without 10 nM forskoline at 37 °C in a humidified 5% CO_2 incubator. After removing supernatant, cells were washed with phosphate buffer saline and lysed with 0,1M HCl. Intracellular cAMP was measured using a commercially available cAMP ELISA kit (Biomedical Technologies, Inc., Stoughton, MA, USA) according to the manufacturer's instructions. This assay is based on the competition between the cAMP from our sample and a cAMP tracer for a cAMP-specific rabbit antibody. The tracer is conjugated to acetylcholinesterase (AchE). The antibody –cAMP complex binds to a mouse anti-rabbit antibody and the resulting complex is revealed by a colorimetric reaction using a substrate to AChE. The intensity of the colour, determined spectrophotometrically is inversely proportional to the amount of free cAMP present in the sample.

Western-Blot analysis

CHO-K1 cells stably expressing either the oMT_1 or oMT_2 receptors grown to 50% confluence in 24-well plates were transfected with siRNA as previously described. Cells were serum-starved during 4 hours and were stimulated during 15 min with concentrations of melatonin (10^{-8} or 10^{-10}M) in absence or presence of 10^{-8}M forskolin. Cells were then lysed in lysis buffer and equivalent amounts of protein were electrophoresed on 10% SDS polyacrylamide gel, transferred to immobilon P membranes. The membranes were incubated overnight at 4 °C with anti-phospho-ERK 1/2 (1:3000) and then with secondary anti-mouse IgG conjugated to horseradish peroxidase (Biosource International, Armadillo, CA, USA). Signal was developed using the Pierce ECL Western Blotting detection kit (Rockford, IL, USA). To confirm that equal protein amount was loaded, the membranes were stripped and reprobed using a primary polyclonal antibody against total ERK2 (1:10,000).

Statistical analysis

Statistical analysis of the data was performed with one-way ANOVA. When significant differences were found, means were compared by Bonferroni's multiple-comparison tests).

Results

Efficaciency of siRNA transfection in CHO-K1-oMT cells

The efficiency of siRNA transfection in CHO-K1-oMT_1 and CHO-K1-oMT_2 cells was evaluated using Alexa Fluor red-labeled nonspecific siRNA. As observed in figure 1, fluorescence was found in cytoplasm of CHO-K1-oMT cells and around 50% of cells appeared fluorescent.

Inhibition of binding capacity of melatonin receptors expressed in CHO-K1

While B_{max} of CHO-oMT_1 untreated or treated with control siRNA 502 or control siRNA 691 were 56.18 ± 8.93, 61.70 ± 21.27 and 51.16 ± 21.48 $fmol.mg^{-1}$ of membrane protein, respectively, B_{max} of CHO-oMT_1 treated with siRNA 502 or 691 fall to 31.07 ± 6.04 and 24.90 ± 4.01 $fmol.mg^{-1}$ of membrane protein ($p<0.001$, figure 2A). These values represented an inhibition of 50% and 51%, respectively, in the binding capacity of oMT_1 after cell treatment with siRNA compared to control siRNA. Treatment with siRNA 919 did not modify the B_{max} of CHO-oMT_1 cells compared to control. Treatment of CHO-oMT_2 with siRNA 1425 decreased the binding capacity of oMT_2 ($p=0.01$) (figure 2B). The B_{max} values of CHO-oMT_2 untreated or treated with control siRNA 1425 were 69.65 ± 11.36 and 98.67 ± 0.62 $fmol.mg^{-1}$ of membrane protein and fell to 29.16 ± 1.13 $fmol.mg^{-1}$ of membrane protein after treatment of CHO-oMT_2 siRNA 1425. These values corresponded to a reduction of around 70% in the binding capacity of oMT_2 after a treatment with siRNA compared to corresponding control siRNA. Concerning the siRNA 736, it decreased the binding capacity of oMT_2 receptor of around 60%. Treatment with siRNA 1266 appeared to decrease the B_{max} of CHO-oMT_2 of around 70% compared to non transfected cells. However, the B_{max} obtained with the control siRNA 1266 was very variable from one experiment to another.

The affinity of melatonin receptors for 2-[^{125}I]-iodo-melatonin was not modified by siRNA treatment. Values of Kd were comparable between control and siRNA treated cells (figure 2C and 2D). Kd values for oMT_1 were comprised between 30.92 and 91.36 pM (p=0.13) (figure 2C) For oMT_2, Kd values were comprised between 4.83 and 14.34 pM (figure 2D). No significative difference was observed.

Considering these results, siRNA 502 and siRNA 1425 were selected to perform the following experiments.

Decrease of relative quantity of mRNA coding for ovine melatonin receptors

Total RNA of cells transfected with siRNA was analyzed by real-time RT-PCR and standardized by the level of 18S mRNA in each sample. In CHO-K1-oMT_1 cells, the relative concentration of oMT_1 mRNA decreased in a significative manner after treatment with siRNA 502 (p=0.007) (Figure 3A). A 60% inhibition was observed. Control siRNA did not modify the levels of oMT_1 mRNA compared to non transfected conditions. In CHO-K1-oMT_2 cells, the levels of oMT_2 mRNA tended to decrease of 70% after treatment with siRNA 1425 (p=0.059) (Figure 3B). Control siRNA did not modify the levels of oMT_2 mRNA compared to control conditions.

Inhibition of functionality of melatonin receptors

Effect on cAMP signalling

Melatonin receptors are coupled to Gi protein. Once activated by binding of melatonin, Gi protein inhibits activation of adenylate cyclise (AC), and consequently inhibits the production of cAMP from ATP. Visualization of activity of Gi protein is possible only after a treatment with forskolin (FSK), an activator of adenylate cyclase. CHO-K1 cells expressing oMT_1 and oMT_2 receptors were treated with corresponding siRNA. Intracellular cAMP production was

measured in 4 conditions: basal, MLT-stimulated, FSK-stimulated and FSK-MLT-stimulated. Figure 4 represents the ratio of the binding value of a sample to that of the maximum binding obtained in the absence of sample. This ratio is inversely proportional to the amount of cAMP present in the sample. As expected, MLT only had not effect on cAMP production whereas FSK reduced the ratio B/B_0 indicating an increase in the production of cAMP. Values of percentage of B/B_0 decreased to 25.34 ± 6.68 % and 36.17 ± 23.96 %, oMT_1 and oMT_2, respectively. In FSK-MLT-stimulated condition, MLT suppressed the effect of FSK on the level of cAMP (117.28 ± 52.53 % and 100.69 ± 20.28, for oMT_1 and oMT_2, respectively). Surprisingly, the treatment with oMT_1 or oMT_2 siRNAs did not affect the level of cAMP production (Figure 4).

Effect on phosphorylation of ERK proteins

G-protein activation of the MT receptors modulates several signal-transduction pathways. It was reported in CHO cells expressing human melatonin receptors that whether MLT typically inhibits FSK-stimulated cAMP production, PKA activity and phosphorylation of the cAMP-response element binding, it also increases the phosphorylation of the extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2). (Reviewed in: Jockers et al., 2008; Witt-Enderby et al., 2000). We chose this output to study the effect of siRNA designed to target oMT_1 and oMT_2 mRNA (Figure 5). The activation of the phosphorylation of ERK1/2 was followed by western blot analysis using an antibody which recognized the phosphorylated ERK1/2 with an apparent molecular weight of 42/44 kDa. Figure 5A and 5C shows that MLT induced the apparition of 42/44 kDa band in control cells or in cells treated with control siRNA. When cells were exposed to oMT_1 or oMT_2 siRNA and treated with MLT, the intensity of the band did not change suggesting that the level of phosphorylation of ERK

Discussion

In this present report, several siRNA molecules were assessed that were designed to target ovine MT_1 and MT_2 receptor mRNAs. Molecular mechanism of siRNA lies on degradation of the corresponding target mRNA, leading to inhibition of target protein production (Hammond et al., 2000). Firstly, the efficiency of siRNA transfection in CHO-K1-oMT_1 and CHO-K1-oMT_2 cells was evaluated using fluorescent nonspecific siRNA. Around 50% of cells appeared fluorescent suggesting that the transfection protocol allows the siRNAs to penetrate into cells. When siRNAs designed to target oMT receptors were used, the relative quantities of oMT_1 and oMT_2 mRNAs were studied. After cell treatment with siRNA 502 and siRNA 1425, inhibitions of around 60% and 70% respectively were observed. To study the impact of siRNA on binding capacity of MT receptors, a binding assay with 2-[^{125}I]-iodomelatonin was performed. Assay of binding ensured that ovine melatonin receptors had partially lost the capacity of binding the 2-[^{125}I]-iodomelatonin around of 50% and 70%, respectively for oMT_1 and oMT_2, after a siRNA treatment without modification of receptor affinity.

Despite the decrease in the levels of mRNA and in the binding capacity of oMT receptors, no effect of siRNA treatment was observed on signalling pathways of melatonin receptors. More specifically, cAMP production and phosphorylation of ERK proteins did not appear to be modified after treatment with siRNA against oMT_1 and oMT_2 receptors. Two explanations can be provided. First, siRNA against ovine MT receptors induced a cellular knock-down and not a knock-out. Population of persistent receptors can transmit the melatonin signal. In CHO-K1 cell line, the level of ovine melatonin receptors is supra-physiological with B_{max} around 600 fmol/mg of proteins (Coge et al., 2009). In our experiment, the B_{max} fell from around 80 to 20-50 fmol/mg proteins after siRNA induced knock-down. Other cell lines like Neuro2A or primary culture of *pars tuberalis* (PT) express a low level of melatonin receptors, with

respectively B_{max} of around 100 foml/mg of proteins for human melatonin receptors (personal communication) and 50 fmol/mg of proteins for ovine melatonin receptors (Piketty and Pelletier, 1993). Behaviour of ovine melatonin receptors, in terms of affinity for melatonin, is similar in these cells and in CHO-K1 over-expressing melatonin receptors, with Kd found in the range of 10-25 pM in both cells (Coge et al., 2009; Piketty and Pelletier, 1993). These results indicate that the same "MLT" message can be transmitted both by a low or a high expression level of melatonin receptors. Secondly, one can argue that the signalling targets we studied were not specific of the melatonin even if they are involved in signalling pathway of melatonin receptors. Functionality of melatonin receptors was measured at level of cAMP production since the best-established effect of melatonin in mammalian cells is the inhibition of forskolin-induced cAMP synthesis (Morgan et al., 1995; Vanecek and Watanabe, 1998). However, cAMP is involved in several cellular mechanisms, for example cellular energetic pathway and plays a role in the signalling of other receptors constitutively expressed in CHO-K1 cell line, as PGE2 and ATP receptors coupled Gs and Gq proteins respectively (Verdonk et al., 2006). Another output of melatonin receptors signalling, phosphorylation of ERK1/2 was studied. Similarly to cAMP, these members of the family of mitogen-activated protein (MAP) kinases are activated by a number of different extracellular stimuli (Reviewed in: Cargnello and Roux, 2011). Another output of signalling pathway could be studied such as cAMP response element binding protein (CREB). Melatonin is known to downregulate the phosphorylation of CREB in ovine PT cells and in CHO-K1-hMT_1 cells (McNulty et al., 1994; Witt-Enderby et al., 1998). The phosphorylation of CREB in ovine PT cells is regulated through both cAMP-dependent and cAMP-independent mechanisms (McNulty et al., 1996). Further studies are necessary to improve the ability of siRNA to inhibit the MT_1 and MT_2 mRNAs and the binding capacity of the receptors in order to decipher the functional impact of these inhibitions.

Final aim of siRNA against oMT$_1$ and oMT$_2$ melatonin receptors is their use *in vitro* and *in vivo*. In our laboratory, we tried to apply the siRNA approach in primary culture of ovine *pars tuberalis* (PT). Because of its embryonic origin and its tissue composition, it is established that primary culture of PT is very difficult to transfect. However, according to the immunofluorescent cells detected after transfection of a fluorescent nonspecific siRNA, transfection protocol was successful. Nevertheless, results did not show any decrease in the relative quantity of MT$_1$ mRNA. Despite the fact that PT corresponds to the cerebral region the most abundant in melatonin receptors, the *in vitro* expression level of melatonin receptors was relatively low when observed by quantitative RT-PCR. Consequently, it was difficult to observe a decrease of mRNA encoding oMT receptors induced by siRNA treatment.

The use of siRNA in vivo should allow to distinguish the respective roles of MT receptors in reading of photoperiodic message. A technique of gene knockdown involving siRNA in the ovine brain was developped. With this method, one gene can be targeted in a well-defined area thanks to the size of the brain in this species. This corresponds to a gene therapy technique allowing a local brain effect lasting for several months. To achieve this, adeno-associated viruses (AAV) were chosen, which are non-pathogenic and tested in several species including rodents (Hommel et al., 2003), monkeys (Forsayeth et al., 2006) and humans (Manno et al., 2003). One successful attempt was performed in sheep (Dufourny et al., 2008). AAV have several advantages including a small genome, generation of highly stable proteins, and most importantly, different cell tropisms depending on their serotypes: AAV serotypes 1 and 2 infect only neurons while AAV serotype 5 infects both neurons and glial cells (Buning et al., 2003; Davidson et al., 2000). Such viruses represent adapted tools to transfer siRNA in the brain.

Figures

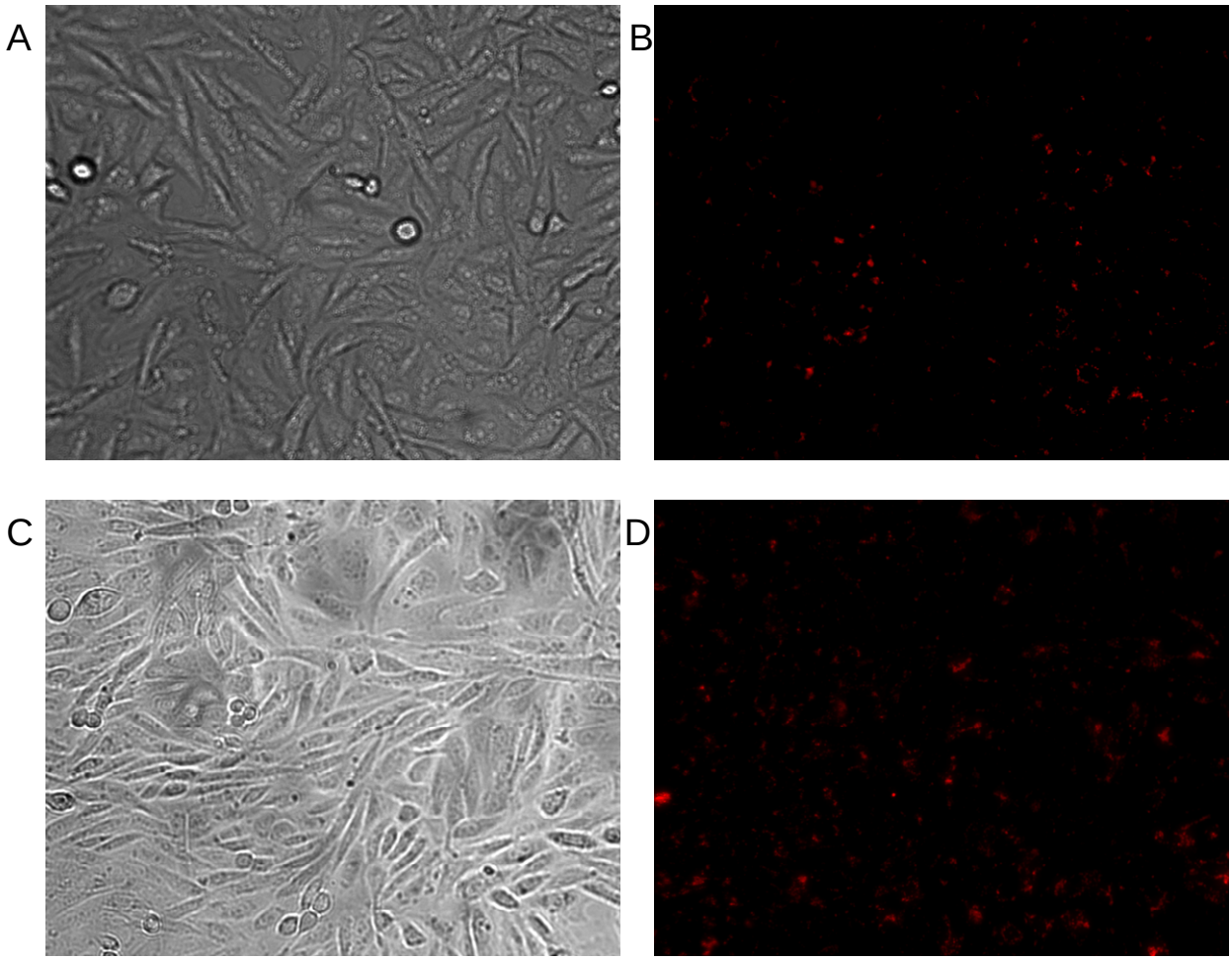
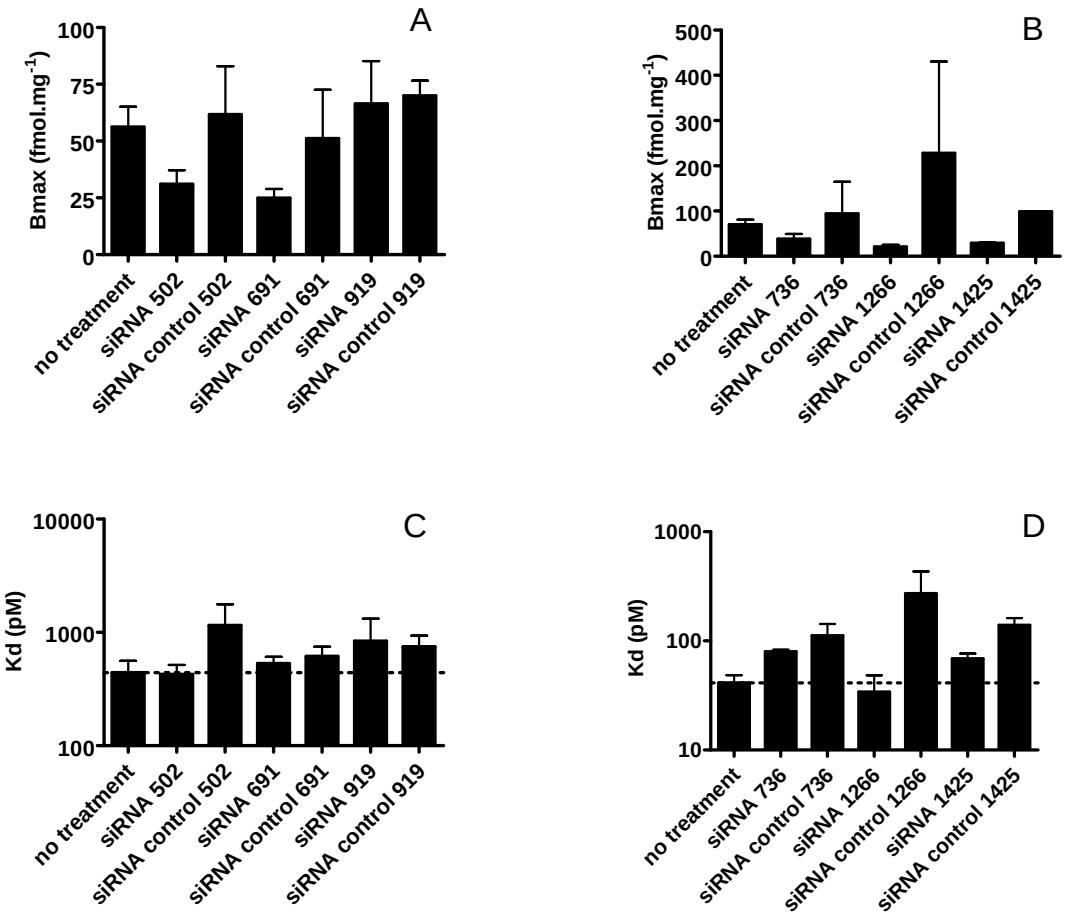


Figure 1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siRNA control 919
B
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300
200
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siRNA control 736
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siRNA 1425
siRNA control 1425
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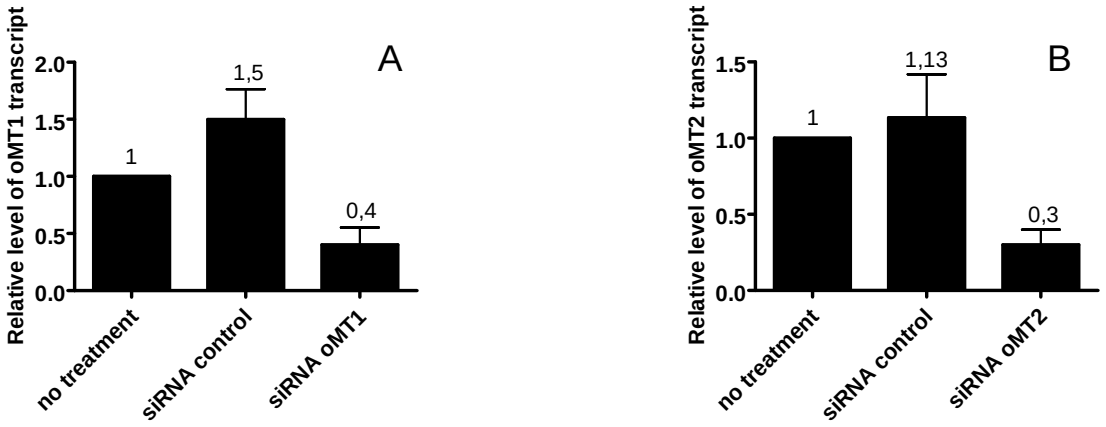
Figure 2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siRNA oMT1
B
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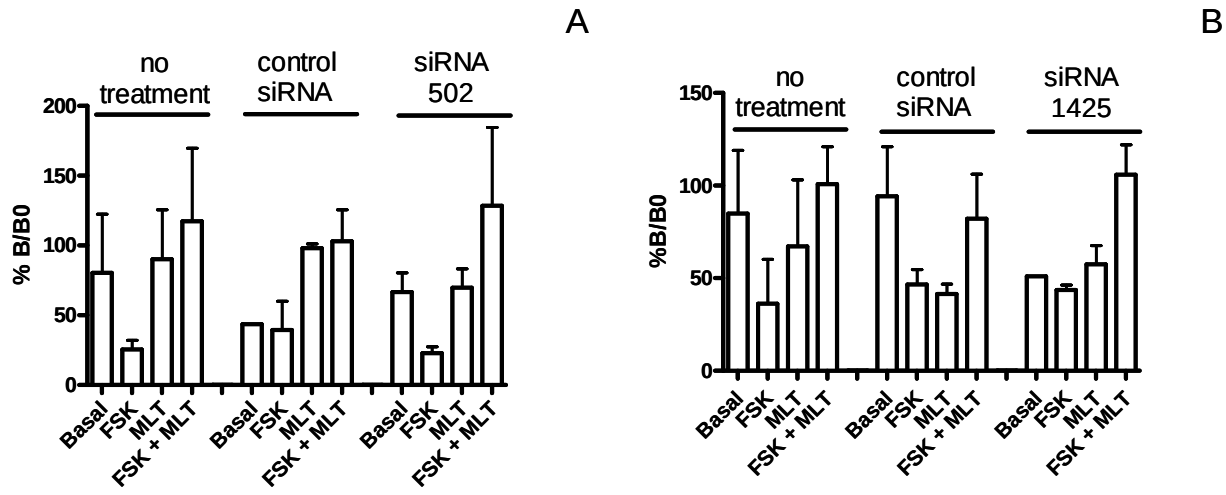
Figure 3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B
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control siRNA
siRNA 502
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%B/B0
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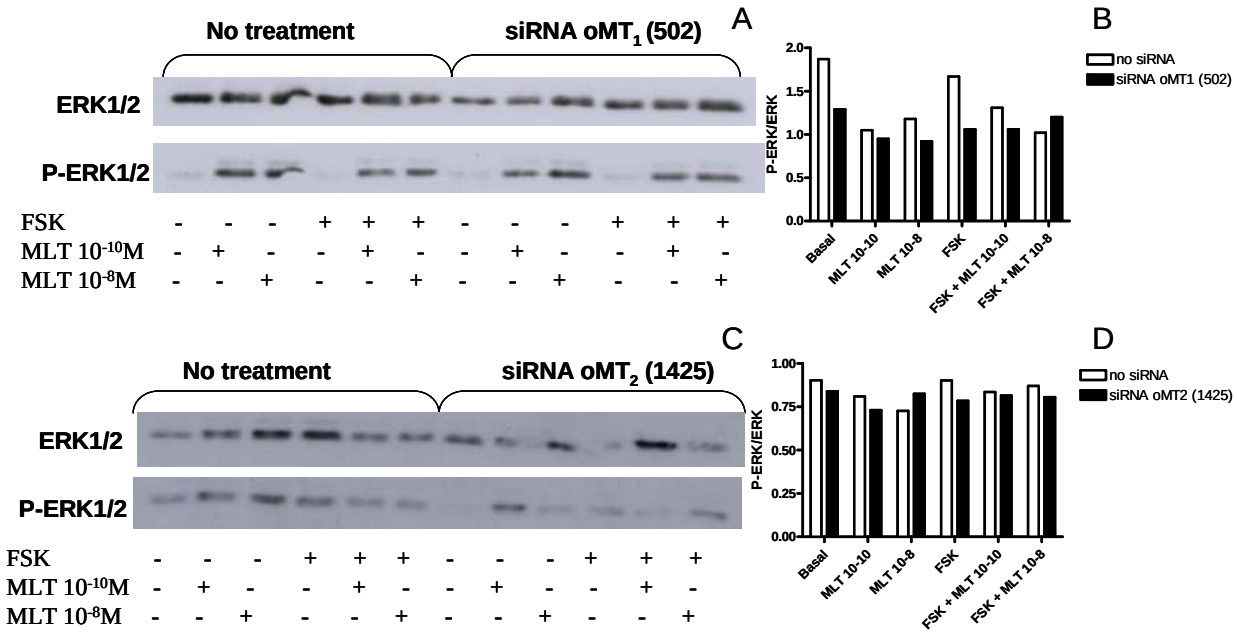
100
50
0
Basal
FSK
MLT
FSK + MLT

Figure 4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No treatment
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ERK1/2
P-ERK1/2
FSK
MLT 10-10M
MLT 10-8M
D
no siRNA
siRNA oMT2 (1425)
P-ERK/ERK
Basal
MLT 10-10
MLT 10-8
FSK
FSK + MLT 10-10
FSK + MLT 10-8

Figure 5

Primers	Sequence	Annealing temperatures
oMT_1 Rev	5’-GGCTCACCACAAACACATTCG-3’	62,6°C
oMT_1 For	5’-CCTCCATCCTCATCTTCACCATC-3’	64,6°C
oMT_2 Rev	5’-GCTTGCTCTCCGCCTTGAC-3’	64,5°C
oMT_2 For	5’-CGCTCGTGTGCTTCTGCTACC-3’	64,5°C
m18S Rev	5’-GTACTGAGCAGGATTACCATGGC-3’	51,9°C
m18S For	5’-GTGAAAGCGGGGCCTCACGATCC-3’	57,3°C

Table 1

Legends of figures

Figure 1: Incorporation of fluo-siRNA in CHO-K1-oMT1 (A and B) and CHO-K1-oMT2 (C and D) cells (20x) CHO-K1-oMT1 cells transfected with 20nM fluorescent siRNA were observed with transmitted light (A) and with red light (B). CHO-K1-oMT2 cells transfected with 20nM fluorescent siRNA were observed with transmitted light (C) and red light (D).

Figure 2: Binding characteristics of CHO-K1-oMT1 (A and C) and CHO-K1-oMT2 (B and D) treated or not with siRNA against oMT receptors. Bmax was measured in absence and in presence of siRNA at CHO-K1-oMT1 (A) and CHO-K1-oMT2 (B). Kd values were calculated in absence or in presence of siRNA at CHO-K1-oMT1 (C) and CHO-K1-oMT2 (D). Histograms represent each of three independent experiments. The data points and error bars represent mean of triplicate and standard error, respectively.

Figure 3: Relative levels of oMT1 (A) and oMT2 (B) transcript after treatment with or without siRNA. A) and B) histograms represent one of three independent experiments. The data points and error bars represent mean of triplicate and standard error, respectively.

Figure 4: Effect of siRNA treatment on cAMP signalling on CHO-oMT1 (A) and CHO-oMT2 (B). cAMP was measured using a competitive ELISA. cAMP concentration was expressed in % of B/B0. B0 is the maximum amount of tracer that antibody can bind in the absence of free cAMP. % B/B0 represents the ratio of the absorbance of a sample to that of the maximum binding (B0) well. Four experimental conditions were tested: basal, forskolin, melatonin and forskolin with melatonin. FSK: forskolin; MLT: melatonin. Histograms represent each of three independent experiments. The data points and error bars represent mean of duplicate and standard error, respectively.

Figure 5: Effect of siRNA treatment on phosphorylation of ERK proteins. CHO-K1 cells stably expressing oMT1 receptor (A and B) or oMT2 receptor (C and D) were stimulated with increasing concentrations of melatonin, in presence or absence of forskolin. ERK1/2 phosphorylation level was assessed by Western blotting using specific antibodies. All the blots were probed with an ERK1/2 antibody to normalize for total protein content. A) Representative Western blot for ERK phosphorylation levels are show for oMT1 (A) and oMT2 (C). ERK signals were quantified by densitometry and expressed as a ratio of phosphorylated ERK over ERK1/2, for oMT1 (B) and oMT2 (D).FSK: forskolin; MLT: melatonin.

Table 1: List of alls primers used.

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Conclusion

Dans la lignée cellulaire CHO-K1, l'interférence par ARN des récepteurs ovins MT_1 et MT_2 permet d'inhiber d'une part, la capacité de liaison pour la 2-[^{125}I]-iodo-mélatonine de l'ordre de 50% et 60%, respectivement et d'autre part, le niveau des ARNm codant pour ces récepteurs de l'ordre de 60% et 70%, respectivement. Toutefois, ces niveaux d'inhibition ne sont pas suffisants pour observer des effets du traitement par les siRNA sur la voie de signalisation majeure des récepteurs MT. L'optimisation des conditions expérimentales de ces études préliminaires est nécessaire avant d'élargir l'utilisation des siRNA à la culture primaire de pars tuberalis ou encore *in vivo*.