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MELATONIN FROM SLOW-RELEASE IMPLANTS UPREGULATES CLAUDIN-2 IN THE OVINE CHOROID PLEXUS

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In ewes, the turnover rate of cerebrospinal fluid (CSF), mainly produced by choroid plexus (ChP), is photoperiodically modulated and is higher during short days (SDs) than long days (LDs). We demonstrated that, melatonin from continuous slow-release implants increases the expression of aquaporins, water channel-forming proteins engaged in transcellular water transport (across the plasma membrane of the cells), in the ovine ChP. This study evaluated the effect of slow-release melatonin implants on the expression of claudin-2 (CLDN2), a pore-forming protein that allows the paracellular passage (between the cells) of select inorganic cations and water, in the ovine ChP. The studies were conducted on ovariectomized, estradiol-implanted ewes during seasonal anestrus (May/June). The ewes were implanted with slow-release-melatonin implants (n = 6, Melovine 18 mg) or sham-implanted (n = 6). Blood samples were collected for melatonin and prolactin measurements. The ewes were sacrificed 40 days after the melatonin/sham implantation, and the ChPs from the brain ventricles were collected for real-time PCR and Western blot analyses. Plasma melatonin concentration reached the median value of 120.4 pg/ml (range: min/max = 29.6/447.0) or was below the detection limit 40 days after the melatonin/sham implantation, respectively. The area under the curve of the plasma prolactin concentration was significantly ($P < 0.05$) higher in sham-implanted ewes than in melatonin-implanted ewes. CLDN2 expression in the ChP was significantly ($P < 0.05$) higher in melatonin-implanted ewes than in sham-implanted ewes at both the mRNA and protein levels. This is the first evidence for the photoperiodic regulation of CLDN2 expression in the ovine ChP, since it has been shown that slow-release melatonin implants during LDs, mimicking SDs, increased the expression of CLDN2. This may partially explain the higher turnover rate of CSF observed in ewes during SDs.

Key words: *choroid plexus, claudin-2, slow-release melatonin implants, ewes, ovariectomy, photoperiodic regulation*

INTRODUCTION

Sheep exhibit a wide spectrum of seasonality, mainly in their reproductive cycles, voluntary food intake, fat metabolism and pelage and horn growth (1). Seasonal changes in day length (photoperiod) provide the primary environmental cue that synchronizes animal physiology to environmental living conditions. Rhythm of melatonin release by the pineal gland is a biochemical translation for daily rhythm and changes in photoperiod. Melatonin acts through its MT1 and MT2 receptors on several target sites in the brain, including the choroid plexus (ChP) (2). The ChP is a secretory organ responsible for cerebrospinal fluid (CSF) secretion and provides a barrier between the blood and the CSF (3). In contrast to the blood-brain barrier, which is located in the brain capillaries and sealed by tight junctions (TJs), the capillaries of the ChP are fenestrated, and the TJs in the choroidal epithelium form the blood-CSF barrier (BCSFB). In sheep, photoperiod was demonstrated to be key factor for modulating the passage of molecules through the brain barriers. For example, in rams, the amount of leptin that enters the CSF after an intravenous leptin injection is greater in long days (LDs) than in short days (SDs) (4). Similarly, in ewes, the passage of progesterone and estradiol from the peripheral blood into the CSF is greater in LDs than in SDs and seems to be regulated by mechanisms involving the pineal gland (5, 6). Additionally, access of *ortho*-substituted polychlorinated biphenyl (PCB153) to the brain and its effect on gonadotropin and thyroid hormones in adult ewes is modulated by photoperiod (7). Moreover, CSF protein content is also seasonally regulated in ewes. However its significance for expression of seasonal functions is not known (8). In an attempt to explain photoperiod-driven changes to the access of molecules in the brain of ewes, we found downregulation of specific TJ proteins (occludin, afadin-6, zonula occludens 1 and 2, and cadherin) in the ChP during LDs (9), which suggests photoperiodic plasticity of ChP. Based on the discovery by Thiery *et al.* (10) that in ewes, the turnover rate (TOR) of CSF is higher during SDs than LDs, we suggested that variations in the CSF secretion rate with photoperiods would result in the dilution or concentration of hormone molecules. The higher expression of vascular endothelial growth factor (VEGF) receptor 2 and its coreceptor neuropilin 1 in the ovine ChP during SDs compared to LDs (11) is consistent with this concept, mainly because the VEGF system is involved in maintaining the fenestrated phenotype of ChP capillaries, which is essential for CSF secretion (12). Recently we demonstrated that melatonin from continuous slow-release

implants that are used to master reproduction in small ruminants by advancing the onset of breeding seasons (13) increases the expression of aquaporins (AQPs), which are water channel-forming proteins that are engaged in transcellular water transport (across the plasma membrane of the cells) in the ovine ChP (14). In addition to the transcellular movement of water, some epithelial cell layers facilitate the paracellular (between the cells) transport of water and simple solutes *via* claudin proteins forming selective pores within the TJs. Numerous claudins (CLDNs) are expressed in the ChP, including CLDN1, -2, -3, -5, -9, -11, -12, -19, and -22 (15). Among these, only CLDN2, which was first discovered by Furuse *et al.* (16), is a pore-forming protein that is characterized as both monovalent cation- and water-selective channel, and as such, may be connected to the mechanism of CSF secretion (15, 17). Therefore, the aim of this study was to validate the hypothesis that slow-release melatonin implants modulate the expression of CLDN2 in the ovine ChP.

MATERIALS AND METHODS

Animals and treatment

The studies were performed on 12 female Polish Blackheaded Mutton sheep (3 – 5 years old, body condition score 2.5). Briefly, at the beginning of May, ewes were ovariectomized and subcutaneously implanted with an estradiol (E2) that maintained plasma E2 concentrations of 2 – 5 pg/ml (6). After two weeks of recovery, the ewes were subcutaneously melatonin-implanted with melatonin slow-release implants (n = 6, 18 mg of melatonin per implant, Melovine, Ceva Sante Animale, France) or sham-implanted (n = 6) for approximately 40 days (from 15 May to 25 June). The ewes were maintained indoors under natural lighting conditions and fed a constant diet of hay, straw, and commercial concentrates, with water and mineral licks available *ad libitum*. Blood samples were collected during the day through jugular vein venipuncture directly before melatonin/sham implantation, 25 days after implantation and approximately 40 days after melatonin/sham implantation for melatonin measurements and through a catheter inserted into the jugular vein (every 30 min for 3 hours) on the day of sacrifice for prolactin measurements. The animals were killed by a licensed butcher and then decapitated, and the ChP from the lateral ventricles was collected, immediately frozen in liquid nitrogen and stored at –80ºC until further analysis using real-time PCR and Western blot methods.

All animal experiments were conducted in accordance with the Polish Guide for the Care and Use of Animals and approved by the Local Ethics Committee (No. 25/2012).

Hormone measurement

To validate the slow-release melatonin implant action, the concentration of melatonin and prolactin in the blood plasma was assayed according to previously described methods (18). For melatonin measurements, the sensitivity of the assay was 16.8 ± 8.0 pg/ml, and the intra- and interassay coefficients of variation were 10.5% and 13.2%, respectively. For prolactin measurements, the sensitivity of the assay was 2 ng/ml, and the intra- and interassay coefficients of variation were 9% and 12%, respectively.

Gene expression

One portion of the ChP from each animal was cut into small pieces, and RNA isolation and RT (reverse transcription) were performed as described previously (14) with the use of the NucleoSpin RNA kit procedure (Marcherey-Nagel, Germany), including the genomic DNA digestion step, for RNA isolation and DyNAmo cDNA synthesis kit (Life Technologies, USA) for RT-PCR. The resulting cDNA was stored at –20ºC until further analysis. Real-time PCR analyses were performed with the ViiA 7 Real-Time PCR system (Applied Biosystems by Life Technologies, USA) using a DyNAmo SYBR Green qPCR kit (Thermo Scientific, USA). Specific primers, synthesized by Genomed (Poland), for determining the expression of the housekeeping genes (beta actin (*ACTB*), histone deacetylase 1 (*HDAC1*) and glyceralaldehyde-3-phosphate dehydrogenase (*GAPDH*)) and *CLDN2* were chosen based on literature (19-21) or designed (accession number DQ152939.1).

The following primers were used:
CLDN2 forward 5'-GCCTGGAATCTTCACGGGAT-3';
CLDN2 reverse 5'-TCCAGCTACCAGGGAGAACA-3' (product size: 129 bp);
ACTB forward 5'- GCCAACCGTGAGAAGATGAC-3';
ACTB reverse 5'-TCCATCACGATGCCAGTG-3';
HDAC1 forward 5'-CTGGGGACCTACGGGATATT-3';
HDAC1 reverse 5'-GACATGACCGGCTTGAAAAT-3';
GAPDH forward 5'-TGACCCCTTCATTGACCTTC-3'; and
GAPDH reverse 5'- GATCTCGCTCCTGGAAGATG-3'.

To confirm the primer specificity for *CLDN2*, the PCR product was sequenced (Genomed; Poland) and separated by electrophoresis on 2% agarose gels supplemented with GelRed Nucleic Acid Gel Stain (Biotium, USA) and examined under UV light (Gel Logic100, KODAK, USA). Each real-time PCR (20 µl) contained 3 µl of diluted RT product, 0.1 µM forward and reverse primers and 10 µl of DyNAmo SYBR Green PCR master mix (Thermo Scientific, USA). The following protocol was used: 95°C for 15 min for the hot start *Thermus brockianus* DNA polymerase, 35 cycles of 95ºC for 10 s (denaturation), and 60ºC for 1 min (annealing and extension). After the completion of the cycles, a final melting curve analysis under continuous fluorescence measurement was performed to evaluate the specificity of amplification. The results were analyzed with real-time PCR Miner (on-line available: http://www.miner.ewindup.info/version2), which is based on the algorithm that was developed by Zhao and Fernald (22).

Protein expression

The second portion of the ChP was cut into small pieces and homogenized while frozen as described previously (14) with 500 µl of ice-cold lysis buffer consisting of 100 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.2% SDS, 0.5% sodium deoxycholate and 1% protease and phosphatase inhibitor cocktail (Thermo Scientific, USA). For protein quantification, a Bradford kit (BioRad, USA) was used. SDS-polyacrylamide gels (12%) were loaded with 75 µg of the proteins and transferred to a 0.45 µm Immobilon-P membrane (Millipore, Germany) using a semidry technique (Trans-blot SD, BioRad, USA). Molecular weight standards were included for each immunoblot. The membranes were then blocked with 5% nonfat milk in TBST (Tris-buffered saline with 0.5% Tween-20) buffer for 1.5 h at room temperature, extensively washed in TBST and incubated overnight at 4ºC with the rabbit polyclonal anti-CLDN2 (ab133164, Abcam, UK; working dilution 1:500) antibody solution. In our studies, we detected CLDN2 proteins using polyclonal antibodies designed against human CLDN2 (*Fig. 1*), but these antibodies were predicted to work with 12 other species, including sheep. The protein sequences of human (NP_065117.1) and sheep (W5QC17) CLDN2 have 94% shared identity. The specific binding of anti-Cldn-2 antibodies is shown in *Fig. 1* and is identical to the manufacturer's data, which presented a specific band of ~27 kDa MW. Next, the immunoblots were incubated for 1.5 h at room temperature with goat anti-rabbit biotin-conjugated antibodies that were included in the WesternDot

625 Western Blot kit and were visualized with the Qdot 625 streptavidin conjugate (W10142, Invitrogen by Life Technologies, USA) according to the manufacturer's instructions. Then, the blots were examined under UV light (ChemiDoc Touch Imaging System, BioRad, USA). The blots were stripped and reprobed with a rabbit polyclonal anti-GAPDH antibody conjugated to horseradish peroxidase (sc-25778, Santa Cruz Biotechnology, USA) as the protein loading control, which was then detected with the enhanced chemiluminescence Super Signal West Dura Kit (Thermo Scientific, USA) and imaged with the ChemiDoc Touch Imaging System (BioRad, USA).

Data analysis

Hormone concentrations are presented as the mean ± SD of melatonin (pg/ml) and the mean ± SD area under the curve (AUC) of prolactin (ng/ml). The real-time PCR results are presented as the relative gene expression of *CLDN-2* versus that of the 3 reference genes (*ACTB*, *GAPDH*, *HDAC1*). The Western blot data are presented as the relative optical density of the CLDN2 protein versus the loading control (GAPDH). All expression values (mRNA and protein) are presented as the mean ± SEM, and the CLDN2 values obtained for each group were normalized to the control group values, which are presented as 1. However, statistical analysis were assessed on raw data. The significances of the differences regarding the expression data and prolactin concentration were analyzed by the Mann-Whitney U test. Melatonin concentration was analyzed using repeated measures two-way analysis of variance (two-way ANOVA) with multiple comparison Uncorrected Fisher's LSD post-hoc tests (PRISM 8, Graph Pad, USA). Statistical significance was defined as $P < 0.05$.

RESULTS

At the beginning of the experiment, the mean plasma concentration of melatonin did not differ between the sham-implanted and melatonin-implanted ewes (*Fig. 2A*). Twenty-five

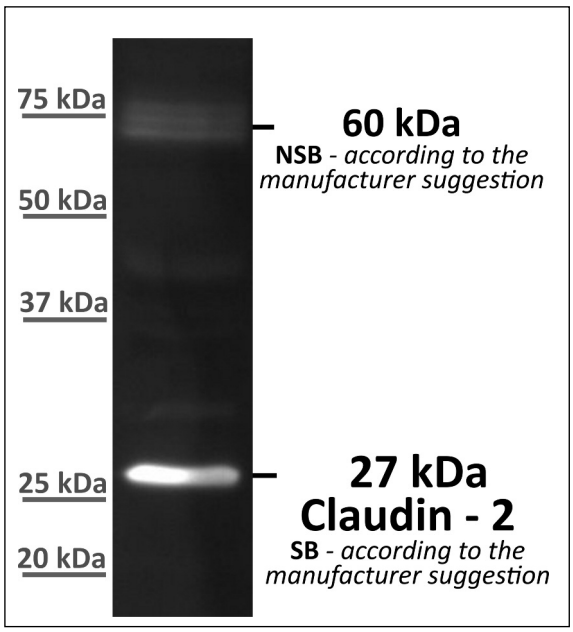

Fig. 1. Specificity of the claudin-2 antibodies in western blot analyses of the ovine choroid plexus homogenates. SB, specific binding; NSB, nonspecific binding.

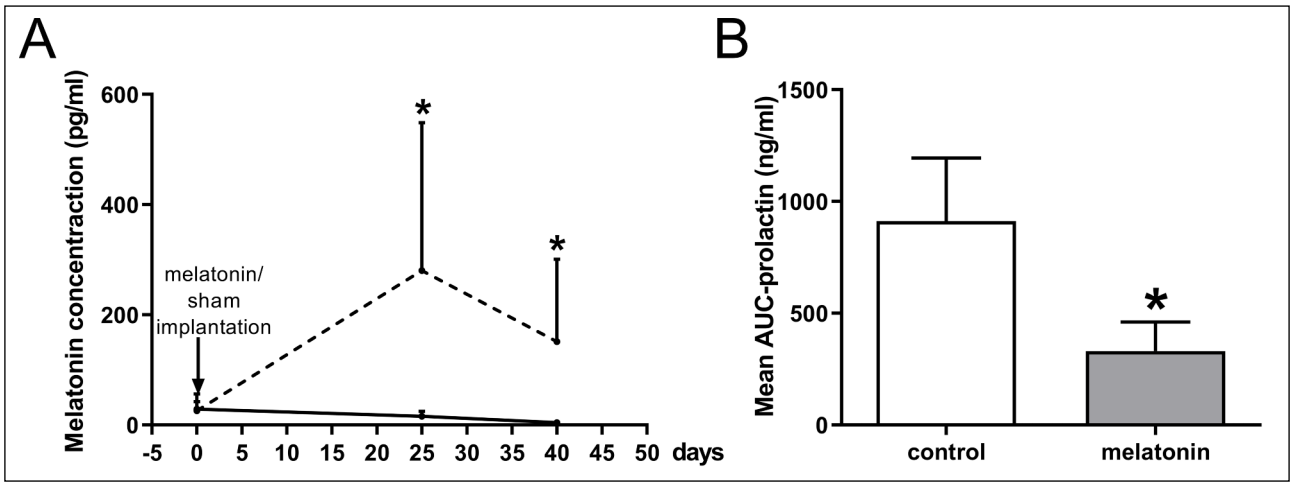

Fig. 2. Slow-release melatonin implantation in ovariectomized, estradiol-treated ewes during long days. (A) Mean (± SD) concentration of melatonin in melatonin-implanted (dotted line) and sham-implanted (solid line) ewes before implantation/sham implantation and at 25 and 40 days after implantation/sham implantation. (B) Mean (± SD) area under the curve (AUC) of the prolactin concentration 40 days after melatonin/sham implantation in melatonin- and sham-implanted ewes. *$P < 0.05$.

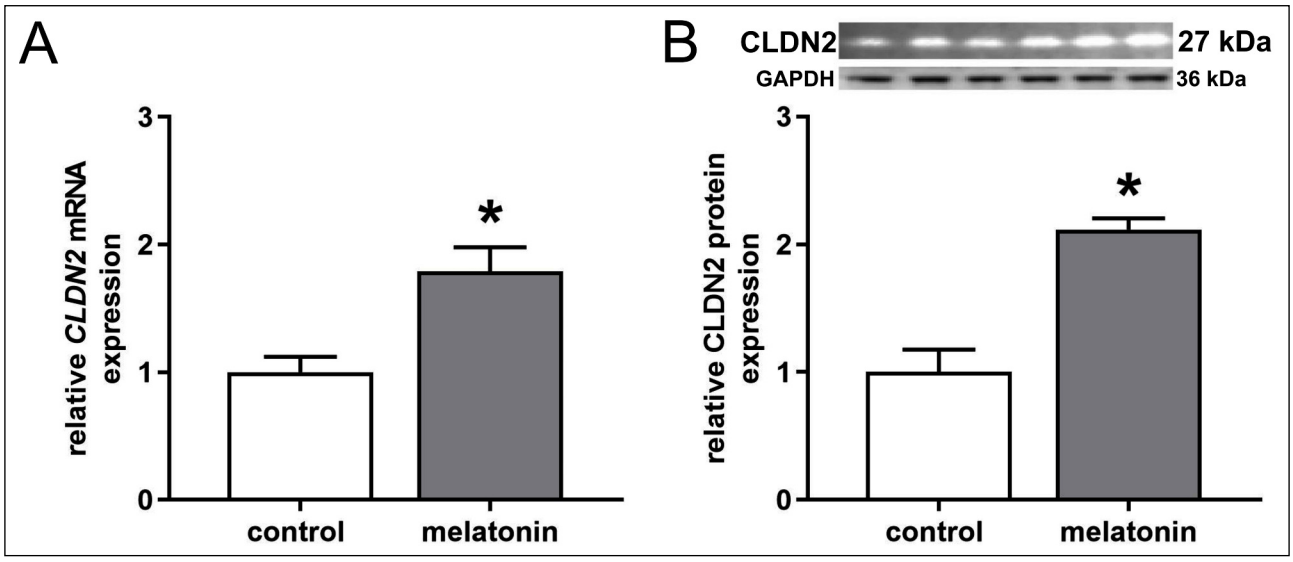

Fig. 3. Effect of melatonin implantation on claudin-2 (CLDN2) in the ovine choroid plexus. (A) Mean (± SEM) mRNA relative expression of CLDN2 versus 3 reference genes, in melatonin- (gray bars) and sham-implanted (white bars) ewes. (B) Mean (± SEM) protein expression level of CLDN2 in melatonin- (gray bars) and sham-implanted (white bars) ewes. Bars indicate the relative optical density that was normalized to GAPDH as an internal control. Western immunoblot for CLDN2 that contains 3 bands corresponding to each group is presented in the top of panel. *P < 0.05.

days after melatonin implantation, the melatonin concentration of the melatonin-implanted ewes increased significantly (P < 0.05) and was significantly higher (P < 0.05) compared with the sham-implanted ewes (median 151.02 pg/ml, min = 42.84, max = 760.52 versus median 18.63 pg/ml, min = 0, max = 27.54, respectively) and remained significantly higher (P < 0.05) up to Day 40 (median 120.41 pg/ml, min = 29.6 max = 447.04 versus below the detection limit) when the ewes were sacrificed (*Fig. 2A*). The effect of the melatonin implants was analyzed by examining the mean area under the curve (AUC) of the plasma prolactin concentration and a significantly (P < 0.05) lower AUC of the plasma prolactin concentration was observed in melatonin-implanted compare to sham-implanted ewes, 40 days after implantation (*Fig. 2B*).

The slow-release melatonin implant significantly (P < 0.05) upregulated CLDN2 expression at both the mRNA (*Fig. 3A*) and protein levels (*Fig. 3B*).

DISCUSSION

In our study, we demonstrated the presence of CLDN2 in the ovine ChP what is in agreement with previous observation on mice, rats and humans (23-25). CLDN2, together with CLDN1 and -3, are localized in the apicolateral border of ChP epithelial cells (25). Moreover, CLDN1 and -2 colocalize exactly with the transmembrane TJ protein occludin and the cytoplasmic protein ZO-1 in the ChP epithelial cells (24). As indicated by Steinemann *et al.* (26), CLDN2 is present in the epithelial cells of the ChP but not in the endothelial cells of cerebral blood microvessels. The role of CLDN2 in CSF production is not well known. The minor reduction (by ~25%) in CSF secretion observed in AQP1-null mice (27) suggests that water transport by ChP epithelial cells by AQP1-facilitated transcellular route accounts for only part of the total CSF production, with remaining water transport occurring through paracellular and non-AQP1-mediated transcellular routes (28). In a preliminary study we found a similar expression level for the *CLDN2* and *AQP1* in the ovine ChP (unpublished data).

The presented studies have shown the impact of melatonin from continuous slow-release implants on CLDN2 expression in the ovine ChP. To date, only studies from Sommansson *et al.* (29) have demonstrated the direct impact of melatonin administration on CLDN2 expression, but their studies were in the duodenum. The authors showed that in male Sprague-Dawley rats, 4 weeks of oral melatonin treatment significantly upregulated *CLDN2* mRNA expression in this tissue, but 2 weeks was not sufficient. In our study, ewes were under the influence of exogenous melatonin for almost 6 weeks. In the sheep, a low prolactin level is indicative of exposure to SDs (30) and/or exogenous melatonin treatment (31). A significantly lower AUC of the plasma prolactin concentration observed in melatonin-treated ewes reflects the proper action of the melatonin implants. Moreover, it allows to conclude that melatonin-implanted ewes transitioned their physiology to that of SDs, as observed previously (18).

The model of ovariectomized, estradiol implanted ewes used in this as well as in other studies (5-11, 14, 19) preserves estradiol receptors in the brain and prevents the variability resulting from the changing level of sex hormones. This allows to take into account photoperiodic but not reproductive status of the ewes. Therefore further study are necessary to evaluate the impact of sex hormones on melatonin action on CLDN2 expression.

So far, the mechanism of melatonin action on CLDN2 expression in the ChP is not known. It has been demonstrated that activation of the JAK/STAT pathway (mediated by IL-22) upregulates CLDN2 expression and subsequently leads to the increased permeability of the TJ barrier in intestinal epithelium (32). The JAK/STAT pathway may also be activated in the ChP through prolactin receptors (33), which are abundant in this structure (34, 35). However, a parallel increase in *CLDN2* mRNA expression and a decrease in plasma prolactin concentration were observed in melatonin-implanted ewes, which rather excludes prolactin participation. Therefore, further studies are necessary to explain this mechanism.

In summary, we obtained evidence for the photoperiodic regulation of CLDN2 expression in the ovine ChP, since it has been shown that slow-release melatonin implants during LDs, mimicking SDs, increased the expression of CLDN2 at both the

mRNA and protein levels, which may partially explain the higher TOR of CSF observed in ewes during SDs.

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