Melatonin from slow-release implants did not influence the gene expression of the lipopolysaccharide receptor complex in the choroid plexus of seasonally anoestrous adult ewes subjected or not to a systemic inflammatory stimulus

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Highlights

►Lipopolysaccharide receptor complex (TLR4, MD-2, CD14) expression in choroid plexus►Lipopolysaccharide (LPS) increases mRNA expression for TLR4 and CD14 but no for MD-2►Plasma LPS binding protein (LBP) level increases 4-6 h after LPS treatment►No effect of melatonin implantation on LBP and mRNA expression for TLR4, MD-2, CD14

Melatonin from slow-release implants did not influence the gene expression of the lipopolysaccharide receptor complex in the choroid plexus of seasonally anoestrous adult ewes subjected or not to a systemic inflammatory stimulus

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Key words: Toll-like receptors, CD14, lipopolysaccharide, choroid plexus, melatonin implantation, ewes

Abstract

This study was designed to determine the effect of melatonin from continuous slow-release implants on (i) the mRNA expression for the lipopolysaccharide (LPS) receptor complex, which includes toll-like receptor 4 (TLR4), myeloid differentiation-2 factor (MD-2) and cluster of differentiation-14 (CD14) in the choroid plexus (CP) and (ii) on the plasma concentration of LPS binding protein (LBP) in ewes subjected or not to a systemic inflammatory stimulus (intravenous injection of LPS). Experiments were conducted in ovariectomized, oestradiol-implanted ewes during seasonal anoestrus (May/June). In experiment 1, the ewes were divided into four groups: sham-implanted and placebo-treated (C/C), melatonin-implanted and placebo-treated (M/C, Melovine 18 mg), sham-implanted and LPS-treated for 3 h (C/LPS, 400 ng/kg of body mass) and melatonin-implanted and LPS-treated for 3 h (M/LPS). In experiment 2, the ovariectomized ewes were melatonin-implanted or sham-implanted and treated with LPS for 6 h (M/LPS and C/LPS, respectively). Plasma

melatonin concentrations reached 4.5 ± 1.5 pg/ml in the C/C group, 151.4 ± 56.4 pg/ml in the M/C group, 7.8 ± 4.3 pg/ml in the C/LPS group and 240.6 ± 93.0 pg/ml in the M/LPS group one month after melatonin or sham implantation. Plasma cortisol concentrations remained at basal level throughout the experiment in the control ewes (C/C and M/C), whereas in the LPS-treated ewes, mean cortisol concentrations increased from 13.7 ± 2.4 ng/ml to 92.0 ± 10.2 ng/ml and from 15.4 ± 5.9 ng/ml to 100.0 ± 10.7 ng/ml in the C/LPS and M/LPS groups, respectively. The LPS treatment significantly ($p \leq 0.05$) increased the mRNA expression for TLR4 and CD14 but had no effect on the MD-2 mRNA expression in the C/LPS and M/LPS groups. Plasma LBP concentration was not affected within 3 h of the LPS-treatment (experiment 1) but increased significantly at 4-6 h after LPS-treatment in the C/LPS and M/LPS groups (experiment 2). Melatonin from the slow-release implants did not change the mRNA expression of all the examined genes or the plasma LPB concentration. These results allow us to infer that melatonin implantation does not disturb the CP potential to detect the specific microbial components derived from gram-negative bacteria.

Introduction

The inflammatory stress caused by bacterial and viral infections induces a strong response from the immune system that has profound effects on an animal's productivity, including reproduction (Battaglia et al., 1997, 2000). A decreased concentration of luteinizing hormone following lipopolysaccharide (LPS, a principal cell-wall component of gram-negative bacteria) administration has been documented in cows (Suzuki et al., 2001), sheep (Daniel et al., 2003) and pigs (Jana et al., 2004). Our previous studies demonstrated that the peripheral inflammation induced by LPS decreases the expression of gonadotropin-releasing hormone (GnRH) and the mRNA of its receptors in the preoptic area in anoestrous ewes (Herman et al., 2010) and that IL-1β is one of the most important factors in this process (Herman et al., 2012).

In general, LPS action in the brain is limited due to its poor penetration of brain barriers (Borghetti et al., 2009; Singh and Jiang, 2004; Banks and Robinson, 2010). Two barriers protect the brain: the blood-brain barrier (BBB), located within the endothelial cells of the brain microvessels, and the blood-cerebrospinal fluid (CSF) barrier (BCSFB), located in the epithelial cells of the choroid plexus (CP) (Skipor and Thiery, 2008). These barriers form the interface between the interstitial fluid surrounding the neural tissue (BBB) and CSF (BCSFB)

and molecules, including pathogen components circulating in the blood. Therefore, most effects of peripherally administered LPS on the brain are likely mediated through the LPS receptors located outside the BBB (Banks and Robinson, 2010). The CP is ideally positioned to transmit signals from the periphery to the brain and therefore is an important pathway by which inflammation in the peripheral tissues may affect the central nervous system. Moreover, microorganisms belonging to the different groups of pathogens, including viruses, bacteria, fungi and parasites, use the BCSFB to reach the brain (Schwerk et al., 2015). Characteristic features of infectious microorganisms are recognized by specialized receptors of the innate immune system, termed pattern recognition receptors (PRRs). An important group of PRRs is the toll-like receptor (TLR) family (Akira et al., 2006). In a previous study, we demonstrated the presence of transcripts for numerous TLR receptors (TLR1-7 and TLR9-10) in the ovine CP, which indicates the potential of the CP to sense the presence of many components of microorganisms (Skipor et al., 2015).

TLR4 is known as a receptor for LPS and the endogenous ligands involved in the inflammatory response even in the absence of infection (Takeda and Akira, 2005). TLR4 alone is not sufficient for the total response to LPS, and additional components, such as CD14 (cluster of differentiation-14) and MD-2 (myeloid differentiation-2), are necessary for the maximum induction of LPS signalling (Wright et al., 1990; Nagai et al., 2002). After systemic administration, LPS is recognized by circulating acute-phase protein - LPS binding protein (LBP) - which forms a ternary complex with CD14, thus enabling LPS to be transferred to the TLR4-MD-2 complex located in the cell membrane (Palsson-McDermott and O'Neill, 2004). The stimulation of the TLRs by their respective ligands triggers numerous signalling factors, thereby leading to the production of chemokines and cytokines, adhesive and co-stimulatory molecules and enzymes (Takeda et al., 2003). Stimulation through the TLRs is therefore essential for the early identification and eradication of infectious microorganisms.

Melatonin, a neurohormone mainly produced and secreted at night by the pineal gland, has been demonstrated to modulate the expression of TLR4 in the brain (Sharman et al., 2008) and in macrophages (Xia et al., 2012). Extended melatonin treatment induces the physiological adaptations associated with winter and enhances certain aspects of immune function (Bartness et al., 1993; Demas and Nelson, 1998). In sheep and goats, seasonally breeding animals, melatonin from continuous slow-release implants is used to advance the onset of the breeding season by mimicking the stimulatory effect of short days (Chemineau et al., 1992). So far, the effect of exogenous melatonin on the LPS receptor complex in the choroid plexus is not known. Due to the fact that melatonin has been demonstrated to affect

TLR4 and MD-2 expression on mRNA level, the aim of our study was to evaluate the influence of melatonin from slow-release implants on (i) the gene expression of TLR4, MD-2, and CD14 in the CP and (ii) on the LPS binding protein (LBP) concentration in the blood plasma of seasonally anoestrous ewes subjected or not to a systemic inflammatory stimulus.

Materials and 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. 26/2012).

Experiment 1.

Adult Blackface ewes (n = 28, 3-5 years old, body condition score 2.5) 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. At the beginning of May (seasonal anoestrus) all the ewes were ovariectomized and subcutaneously implanted with an oestradiol (E2) implant that maintained plasma E2 concentrations of 2-4 pg/ml (Thiéry et al., 2006). In the middle of May, the ewes were implanted with melatonin (n=14, 18 mg of melatonin per implant, Melovine, Ceva Sante Animale, France) or sham-implanted (n=14). After approximately 40 days, animals in each group were randomly divided into 2 groups; one group was intravenously treated with LPS (*Escherichia coli* O55:B5, Sigma Aldrich, USA, at a dose of 400 ng/kg of body weight) to induce an immune stress, and another, with saline (placebo) according to the schedule presented in Fig. 1A. The dosage of LPS and the time response course were based on our previous study (Herman et al. 2013) and literature (Singh and Jiang, 2004), accordingly. Four groups of ewes participated in the experiment: sham-implanted and placebo-treated (C/C, n=7), melatonin-implanted and placebo-treated (M/C, n=7), sham-implanted and LPS-treated (C/LPS, n=7) and melatonin-implanted and LPS-treated (M/LPS, n=7). The body temperature was measured 1 h before and after saline/LPS administration. Blood samples were collected during the day through jugular vein venipuncture (one month after melatonin implantation) for melatonin measurement and through a catheter inserted into the jugular vein (at 1 h before and every 30 min for 3 h after LPS administration) for cortisol and LBP measurement. After centrifugation in heparinized tubes, the plasma was stored at -20 °C until being assayed. Ewes were euthanized 3 h after LPS administration; the brains were removed and dissected; and the choroid plexuses were collected from the lateral ventricles, immediately frozen in liquid nitrogen, and stored at -80 °C until use.

Experiment 2.

Due to the lack of LBP response to LPS treatment (3 h) in experiment 1 additional experiment was performed with prolonged LPS treatment. Ovariectomized and E2-implanted Blackhead ewes (n=14, 3-5 years old, body condition score 3) were implanted with melatonin (n=7) or sham-implanted (n=7) in the middle of May, and after 40 days, all animals were intravenously treated with LPS (C/LPS and M/LPS group). Blood plasma was collected from the jugular vein 1 h before and 4 and 6 h after LPS administration for LBP measurement (Fig. 1B).

The concentration of melatonin in the blood plasma was assayed by radioimmunoassay (RIA) according to the method described by Misztal et al. (1996). The sensitivity of the assay was 16.8 ± 8.0 pg/ml, and the intra- and inter-assay coefficients of variations were 10.5 and 13.2%, respectively. The concentration of cortisol in the blood plasma was assayed by RIA using a method described by Kokot and Stupnicki (1985).The sensitivity of the assay for cortisol was 0.95 ng/ml, and the intra- and inter-assay coefficients of variation were 10 and 12%, respectively. The level of LPS BP in the blood plasma was determined using a commercial LBP ELISA Kit (Hycult Biotech, The Netherlands) designed for sheep. The plasma samples collected from experiments 1 and 2 were analysed separately.

The total RNA from the CP was isolated using NucleoSpin RNAII (Marcherey-Nagel, Germany) according to the manufacturer's protocol. The initial homogenization of the frozen CP was carried out in a Lysing Matrix D (MP Biomedicals, France) that was filled with lysing buffer from NucleoSpinRNA (RA1) using a FastPrep24 instrument (MP Biomedicals, France) at an oscillation speed of 6.5 m/s for 30 s. The step for genomic DNA digestion was included in the isolation procedure. The purity and concentration of the isolated RNA were quantified spectrophotometrically using a NanoDrop 1000 instrument (Thermo Fisher Scientific, USA). One microgram of total RNA was retained for further use in a RT (reverse transcription) reaction. The RT reaction was performed with DyNAmo cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the protocol supplied by the manufacturer. The resulting cDNA was stored at -20 °C until further analysis.

Expression pattern of the TLR4 complex (TLR4, MD-2 and CD14) and TLR2 (positive control for response to LPS) in the ovine CP was determined by real-time PCR. Specific primer pairs for the different genes were used according to the literature or were designed using Primer Express Software v. 3.0 (Applied Biosystems by Life Technologies, USA) or

Primer-BLAST (National Center for Biotechnology Information) and were synthesized by Genomed (Poland) or by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences (Poland) and are presented in Table 1.

Real-time PCR was performed on a ViiA 7 instrument (Applied Biosystems by Life Technologies, USA). Each real-time PCR reaction (10 µl) contained 3 µl of diluted (1:10) cDNA, 0.2 µM of the forward and reverse primers and 5 µl of mastermix from a DyNAmo SYBR Green qPCR Kit with ROX (Thermo Fisher Scientific, USA). The following protocol was used: 95 °C for 10 min for Hot Start modified Tbr DNA polymerase, followed by 35 cycles of 15 s of denaturation at 95 °C, 30 s of annealing at X °C (Table 1) and 30 s of extension at 72 °C. The last cycle was performed to evaluate the specific amplification by a final melting curve analysis under continuous fluorescence measurement. The products of the real-time PCR for the TLR4, MD-2, CD14 and TLR2 were sequenced by Genomed (Poland). The results were analysed using Real-Time PCR Miner (available online: http://www.miner.ewindup.info/version2) based on the algorithm developed by Zhao and Fernald (2005).

All data are presented as the mean ± standard error of the mean [SEM]. Real-time PCR results are presented as the relative gene expression of all examined genes vs. the mean of 3 reference genes (GAPDH, ACTB, and HDAC1). In experiment 1, the significance of the differences among the groups for the melatonin (measured one month after the time of subcutaneous implantation) and cortisol (total area under the curve, AUC, from 1 h before to 3 h after the time of LPS administration) plasma concentration, and expression of all genes were analysed using a two-way analysis of variance (two-way ANOVA) with multiple comparisons of Tukey's *post-hoc* tests using GraphPad PRISM 6 (San Diego, USA). The body temperature and LBP plasma concentration were analysed by a two-way ANOVA in experiment 1 or a one-way ANOVA in experiment 2 (GraphPad PRISM 6) for repeated measures. Statistical significance was set at $p \leq 0.05$.

Results

One month after the ewes were implanted with melatonin (before the LPS treatment), the plasma melatonin concentration (experiment 1) in the sham-implanted ewes was, during the day, at low levels (C/C group - 4.5 ± 1.5 pg/ml and C/LPS group - 7.8 ± 4.3 pg/ml),

whereas in the melatonin-implanted ewes, the plasma melatonin concentration was significantly ($p \leq 0.05$) higher at 151.4 ± 56.4 pg/ml and 240.6 ± 93.0 pg/ml in the M/C and M/LPS groups, respectively (Fig. 2A). The plasma melatonin concentration was similar ($p > 0.05$) between the C/C and C/LPS groups and between the M/C and M/LPS groups.

The mean body temperature (experiment 1) in all ewes 1 h before the LPS/saline administration was 38.9 ± 0.1°C and increased significantly ($p \leq 0.05$) 1 h after LPS administration to 40.0 ± 0.1°C and 39.7 ± 0.1°C in the C/LPS and M/LPS groups, respectively. Melatonin treatment did not influence ($p > 0.05$) body temperature in all the melatonin-implanted ewes.

The plasma concentration of cortisol (experiment 1) increased from 13.7 ± 2.5 ng/ml to 92.0 ± 10.2 ng/ml and from 15.4 ± 5.9 ng/ml to 100.0 ± 10.7 ng/ml after LPS injection in both the C/LPS and M/LPS groups, respectively. In contrast, the plasma cortisol concentration remained at basal levels throughout the entire experiment in the LPS-untreated ewes (Fig. 2B). The AUC of the plasma cortisol profiles was significantly ($p \leq 0.05$) higher in LPS-treated ewes than the untreated ones (Fig. 2C). No effect of melatonin ($p > 0.05$) was observed on the cortisol according to the AUC.

The mean level of LBP in the blood plasma of the ewes before LPS and 3 h after LPS administration (experiment 1) was similar ($p > 0.05$) in all groups (Fig. 3A). In contrast, an increased level of circulating LBP relative to the period before LPS administration was observed in both the C/LPS and M/LPS groups 4 and 6 h after the LPS challenge (experiment 2) (Fig. 3B). No effect of melatonin on the plasma LBP concentration was observed in experiments 1 or 2.

The mean (± SEM) mRNA expression of the TLR4 complex (experiment 1), determined by real-time PCR, in the ovine CP is presented in Fig. 4. The mean mRNA expression of the TLR4 (Fig. 4A), TLR2 (Fig. 4B) and CD14 (Fig. 4C) was significantly higher in the LPS-treated than placebo ewes, regardless of the melatonin treatment, which did not influence ($p > 0.05$) these parameters. In the placebo-treated ewes, the relative level of the mRNA expression for the TLR4 and TLR2 was similar, but in LPS-treated ewes, the level of the mRNA expression was higher for the TLR2 than TLR4. The mRNA expression of MD-2 was similar in all groups (Fig. 4D).

Discussion

In the present study we investigated the effect of melatonin from continuous slow-release implants on the expression of the LPS receptor complex transcripts (TLR4, MD-2 and CD14) in the CP and on the plasma concentration of LBP in ewes subjected or not to a systemic inflammatory stimulus. Endogenous melatonin can act directly on the CP through two types of melatonin receptors, MT1 and MT2, which are expressed in the ovine CP (Coge et al., 2009). Melatonin is transported to its targets via the CSF and blood (Legros et al., 2014). In sheep, the mean CSF melatonin concentrations in the third ventricle reach 70 pg/ml in the daytime and approximately 1500 pg/ml during the night-time, whereas in plasma, these concentrations reach 8 pg/ml and approximately 120 pg/ml, respectively (Skinner and Malpaux, 1999). In our study, daytime plasma melatonin concentrations in melatonin-implanted ewes were similar to those reported by Skinner and Malpaux (1999) for plasma collected at night. However, the concentration of melatonin in the CSF in melatonin-implanted ewes was not measured in our study.

Our study shows that transcripts of all components of the LPS receptor complex (TLR4, MD-2 and CD14) are constitutively expressed in ovine CP and therefore support the role of the CP in the innate immune response. The similar level of mRNA expression for the TLR4 and TLR2 found in the CP of non-stimulated ewes is in agreement with that reported before for ovine CP (Skipor et al., 2015). In the present study, we observed that LPS challenge upregulates the expression of the TLR4, TLR2 and CD14 mRNA but has no effect on the MD-2 mRNA in the ovine CP. This result is in agreement with previous observations that the LPS upregulates the TLR4 gene expression in the hypothalamus in Blackface anoestrous ewes (Herman et al., 2013). Interestingly, Haziak et al. (2014) did not observe any increase in the TLR4 mRNA expression in the hypothalamus in anoestrous ewes when LPS was administered 2 times within 2 weeks. The effect of the LPS on the mRNA expression for TLR4 has been the object of seemingly conflicting results. Laflamme and Rivest (2001), using an in situ hybridization method, observed a slight decrease in an mRNA signal in the CP in LPS-challenged rats. Stridh et al. (2013) demonstrated that ultra-pure LPS in a dose of 0.3 mg/kg had no effect on the TLR4 mRNA expression in mice CP. In contrast, Singh and Jiang (2004) demonstrated a time-dependent increase in the mRNA expression for TLR4 in the brain endothelial cells, following systemic LPS injection. These discrepancies in the effect of LPS on the TLR4 gene expression seem to be related to the different times of sample collection, doses used, and tissue and species differences. The increase in the mRNA expression for the CD14 and TLR2 in the ovine CP during the LPS challenge, as found in our

study, is in agreement with the increase described for the CP in rats and mice (Laflamme and Rivest, 2001, Stridh et al., 2013). Augmented TLR2 mRNA expression may indicate lipopeptide impurities that are commonly present in the LPS, which account for the TLR2 being responsive to LPS (Fitzgerald et al., 2004). According to Takeda and Akira (2005), the TLR2 binds to a variety of microbial components, which include lipoproteins/lipopeptides, peptidoglycans and lipoteichoic acid, lipoarabinomannan, glycoinositolphospholipids, phenol-soluble modulin, zymosan, glycolipids and atypical LPS found in *Leptospira interrogans* and *Porphyromonas gingivalis*. From the other side, it has been demonstrated by Sakai et al. (2004) that IL-1β up-regulates the TLR2 but not TLR4 mRNA expression in human endothelial cells. Moreover, the presence of glucocorticoids enhanced IL-1β-induced expression of TLR2 mRNA. Considering the fact that in our study, the concentration of cortisol increased after LPS administration as well as mRNA expression for IL-1β in the CP (data not presented) we can suggest that IL1-β and cortisol are responsible for higher expression of TLR2 comparing to TLR4, found in this study.

We have found that melatonin from slow-release implants did not alter the mRNA expression for all components of the LPS receptor complex and the TLR2. The only data concerned with the effect of melatonin on the LPS receptor complex in healthy animals were published by Perreau et al. (2007) and Sharman et al. (2008). Authors demonstrated that melatonin did not alter the expression level of the mRNA for CD14 in the brain of old male mice (Perreau et al., 2007) but modulated the expression of TLR4 and MD-2 in an age-related manner (Sharman et al., 2008). In healthy old mice, the mRNA for TLR4 was higher in the old than the young mice and was much more attenuated by melatonin (Sharman et al., 2008). The MD-2 mRNA expression was also higher in old mice, and melatonin treatment also augmented that expression in old mice but not in young mice (Sharman et al., 2008). Ewes in our study were 3-5 years old; therefore, we can conclude that our results are similar to those described for young healthy mice, in which there was no effect of melatonin on the LPS receptor complex. Regarding LPS in the stimulated ewes, we also did not observe any effect of melatonin on the mRNA expression for the TLR4, MD-2 or CD14. The lack of melatonin effect on the CD14 mRNA expression in ovine CP is in agreement with data obtained by Perreau et al. (2007). However, our results contrast with the results obtained by Sharman et al. (2008) for LPS-treated mice. They observed melatonin-induced downregulation of brain mRNA expression for TLR4 in young mice and upregulation in old mice, as well as upregulation of the mRNA expression for MD-2 in both young and old mice. Altogether, this

may imply the existence of different mechanisms for melatonin regulation of immune processes in different tissues or species.

Despite the significant increase in core body temperature in ewes after LPS administration, which is a marker of the acute-phase response, we did not observe any increase in the plasma LBP concentration in ewes within the 3 h after LPS treatment (experiment 1). However, the plasma concentration of LBP after LPS treatment was on the mean level of 12.5 ± 2.5 μg/ml what allow LBP to bind LPS and initiate LPS response. Indeed, in LPS treated ewes we observed increased mRNA expression of pro-inflammatory cytokines in the CP (data not presented). Apparently, in ewes, more than 3 h (4-6 h in the second experiment) are necessary to observe an increase in the plasma LBP level following LPS administration. In cows, a rise in plasma LBP concentration was observed within 8 h of the LPS challenge (Bannerman et al., 2003). Regardless of this, melatonin from slow-release implants did not influence plasma LPB concentration.

The present study demonstrates that melatonin from subcutaneous slow-release implants (the treatment used on farms) did not alter the mRNA expression for all components of the LPS receptor complex in the ovine CP or in the plasma LPB concentration in ewes subjected or not to a stimulation of the immune system by LPS. These results allow us to infer that these implants do not disturb the potential ability of the CP to detect specific microbial components indicative of gram-negative bacteria. Further studies will be needed to evaluate exogenous melatonin action on the activation of the TLR4 signalling pathway in CP. The effect of melatonin on the mRNA expression of pro-inflammatory cytokines (IL-1β, TNFα and IL-6) and their receptors in the CP of ewes subjected or not to a systemic inflammatory stimulus is the subject of our present study.

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Figure and table captions:

Fig. 1. Schematic diagrams of the experimental design. A - Experiment 1. Blood samples were collected during the day one month after melatonin implantation for melatonin measurement and 1 h before and then every 30 min for 3 h after lipopolysaccharide (LPS)/saline administration for cortisol and LPS binding protein (LBP) measurement. B - Experiment 2. Blood samples were collected 1 h before and then 4 and 6 h after LPS administration for LBP measurement.

Fig. 2. Experiment 1: Mean (±SEM) concentration of (A) plasma melatonin one month after the ewes were implanted and before LPS administration and (B) plasma cortisol before and after LPS treatment in sham-implanted and placebo-treated (C/C), melatonin-implanted and placebo-treated (M/C), sham-implanted and LPS-treated (C/LPS) and melatonin-implanted and LPS-treated (M/LPS) adult ewes. C - Mean (±SEM) area under the curve (AUC) for cortisol concentration in the blood plasma. Different lowercase letters indicate significant differences at $p \leq 0.05$.

Fig. 3. Mean (± SEM) concentration of plasma lipopolysaccharide binding protein – LBP. A - Experiment 1. Blood samples were collected 1 h before and 3 h after treatment in sham-implanted and placebo-treated (C/C), melatonin-implanted and placebo-treated (M/C), sham-implanted and LPS-treated (C/LPS) and melatonin-implanted and LPS-treated (M/LPS) adult ewes. B - Experiment 2. Blood samples were collected 1 h before, 4 h after and 6 h after treatment in sham-implanted and LPS-treated (C/LPS) and melatonin-implanted and LPS-treated (M/LPS) adult ewes. Different lowercase letters indicate significant differences at $p \leq 0.05$ for all comparisons.

Fig. 4. Experiment 1: The effect of sham implantation (white bars) and melatonin (grey bars) on the mean (±SEM) relative TLR4 (A), TLR2 (B), CD14 (C) and MD-2 (D) mRNA expression in the choroid plexus in placebo- and lipopolysaccharide (LPS)-treated adult ewes. Different lowercase letters indicate significant differences at $p \leq 0.05$.

Table 1

Sequences of the oligonucleotide primers used for RT-PCR.

Table 1

Gene	Primers (5'→3')	Product size	Reference	Temp. of primers annealing
TLR2	Forward: GGCTGTAATCAGCGTGTTCA Reverse: GATCTCGTTGTCGGACAGGT	159 bp	Chang et al. (2009)	60 °C
TLR4	Forward: TGGATTTATCCAGATGCGAAA Reverse: GGCCACCAGCTTCTGTAAAC	152 bp	Chang et al. (2009)	60 °C
CD14	Forward: TGACACAATCAAGGCTCTGC Reverse: CGACACGTTACGGAGACTGA	211 bp	NM_ 001077209.1	60 °C
MD-2	Forward: CCTGTTTTCTTCCATATTTACTG Reverse: AATAACTTCTTTGCGCTTTGG	256 bp	Wolfs et al. (2009)	55 °C
GAPDH	Forward: TGACCCCTTCATTGACCTTC Reverse: GATCTCGCTCCTGGAAGATG	143 bp	Herman et al. (2010)	60 °C
ACTB	Forward: GCCAACCGTGAGAAGATGAC Reverse: TCCATCACGATGCCAGTG	122 bp	Herman et al. (2013)	60°C
HDAC1	Forward: TCCACATCCCAGACTTTCTACGA Reverse: GGTCCCAATCCCTTTCCTCTA	115 bp	BC_108088.1	60 °C

TLR-toll-like receptor; CD14-cluster of differentiation; MD-2-myeloid differentiation factor 2; GAPDH-glyceraldehyde-3-phosphate dehydrogenase; ACTB-β-actin; HDAC1-histone deacetylase 1