The Role of Endogenous Carbon Monoxide (CO) in the Functioning of Biological Clocks in Pig and Wild Boar Hybrids During Long- and Short-day Seasons

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Research Article

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Abstract

Mature males of a wild boar-pig crossbreed during long- and short-day seasons were used for the study, which demonstrated that the chemical light carrier CO regulates the expression of biological clock genes in the hypothalamus (preoptic area - POA and dorsal part of hypothalamus - DH) via humoral pathways. Autologous blood with experimentally elevated concentrations of endogenous CO (using lamps with white light-emitting diodes) was infused into the ophthalmic venous sinus via the right dorsal nasal vein.

The results showed that elevated endogenous CO levels through blood irradiation induced changes in gene expression involved in the functioning of the main biological clock. Changes in the expression of the transcription factors *Bmal1*, *Clock* and *Npas2* had a similar pattern in both structures, where a very large decrease in gene expression was shown after exposure to elevated endogenous CO levels. The changes in the gene expression of *PER 1-2*, *CRY 1-2*, *REV-ERB α-β* and *ROR β* are not the same for both POA and DH hypothalamic structures, indicating that both structures respond differently to the received humoral signal.

The obtained results indicate that CO is a chemical light molecule whose production in organisms depends on the amount of light. An adequate amount of light is an essential factor for the proper functioning of the main biological clock.

Introduction

Every living organism functions according to rhythms, both annual and circadian. The master biological clock makes organisms adjust to rhythms. The master biological clock is located in the suprachiasmatic nuclei (SCN) of the preoptic part of the hypothalamus (POA). Hormonal and synaptic information from the SCN orchestrates the rhythmic gating of various physiological, biochemical, and even behavioral processes. Clock function is based on the 24-hour oscillator of the transcription factor genes BMAL1/CLOCK or its paralogue NPAS2 in which an E-box binds. Heterodimeric transcription factors drive the expression of the *PER 1–2, CRY 1–2* and *REV-ERB α-β* (NR1D1 and NR1D2) genes. It is a cell autonomous, autoregulatory transcriptional/translational feedback loop (TTFL). These genes negatively feedback on their own expression via the suppression of E-box-mediated transcription. Although this is a self-regulating transcriptional loop, ancillary transcriptional pathways contribute to the robustness and phasing of the core clock oscillation, i.e., *REV-ERB α* or *β* and *ROR β* genes interloop [1, 2]. The expression of the core clock components, i.e., The genes *BMAL1, CLOCK*, and *NPAS2* constitute [3] according to the same pattern in summer and winter. With a constant gene and protein expression pattern of the analysed transcription factors, the change in the expression pattern of genes under their control during summer and winter proves the important role of lags in the functioning of regulatory loops, thus determining the circadian cycle [4].

The main factor that influences the functioning of this clock rhythm is the light signal reaching the SCN from the retina via the neural pathway, the so-called nonforming image signal. This signal is further

transmitted to the paraventricular nucleus (PVN) in the dorsal part of the hypothalamus (DH). Furthermore, this signal leaves the hypothalamus and is directed to the pineal gland, where the day-night signal is decoded into a hormonal signal [5, 6].

The SCN neural network precisely establishes the length of the day due to its plasticity within its own network. Thus, short days are encoded through the synchronization (clustering) of single-cells oscillation which leads to a narrow peak in the rhythm of the SCN. In contrast, long days are encoded by desynchronization (dispersion) of activity patterns of SCN neurons and this looks like a broad peak. However, the activity patterns of individual SCN neurons appear to be less sensitive to changes in photoperiod; studies on clock genes expression showed different peak in response to duration of photoperiod, and others showed changes in clock genes expression even within the SCN itself between dorsal and ventral part. In conclusion, seasonal coding depends on the SCN integrity and plasticity, but rhythms are generated in single cells of the network. (cited in [7]).

The amount of available endogenous CO whose formation and availability depend on the amount of light seems to allow tuning to short and long photoperiods. This signal is a chemical signal delivered through the humoral pathway independent of the neural signal, which may explain how single SCN cells can synchronize. Even changes in CO level occur seasonally and depend on the photoperiod [8, 9].

BMAL1, CLOCK, NPAS2, and, PER2, REV-ERB α and β proteins, which are products of biological clock genes, belong to a group of proteins called basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) containing PAS A and PAS B domains. These domains have the ability to bind Fe(II) heme groups. These proteins with a bound Fe(II) heme group become susceptible to gaseous agents such as CO, which by binding to them inhibits the ability to dimerize with other proteins inhibiting their biological activity. Thus, heme groups bound to particular proteins can act as cofactors for these proteins. (cited in [10]).

In recent years, it has been demonstrated that light information can be transmitted to hypothalamic structures via the humoral pathway by means of a gas transmitter, carbon monoxide [9, 11]. The CO formed under light influence in the retina reaches the perivascular complex as a result of haemoglobin decomposition and the participation of haem oxygenase and outflowing blood. It then enters the arterial blood through countercurrent permeation and reaches the hypothalamic structures [12–14]. The amount of CO in the blood flowing out of the eye has also been shown to depend on the amount of daylight that the retina receives [8]. The humoral phototransduction system, originally described by Oren et al., is a simpler and more inert system than the signal transmitted via the neural pathway, but nevertheless, it does not require high energy expenditure and transmits information on a wider scale simultaneously (POA and DH, as well as other hypothalamic structures, receive the signal in the same way in equal intensity, exerting a biological effect [9].

Following these findings, the authors investigated the possibility of CO formation in blood itself under the influence of condensed light and the effect of such enriched blood on the regulation of the function of the main oscillator of the biological clock in the POA part of the hypothalamus and DH in domestic pig and wild boar hybrids. These animals are a good research model of organisms whose nature of existence is

described as diurnal. The fact that the animals are hybrids is also important for this experiment because the high genetic variability makes it possible to highlight traits that disappear in the course of breeding in domestic pigs, such as seasonally regulated reproduction manifested by a clear manifestation of estrous symptoms in the period from October to March and disappearance in the period from May to October [15]. This feature is important because it guarantees high sensitivity of these animals to such environmental stimuli as the length of the light day. The aim of this experiment was to demonstrate that light, as an environmental signal, regulates the diurnal biological clock via humoral pathways. This is important for all biological processes because they all occur in relation to the internal biological clock. Any disturbance caused by a change in the amount of light available can have consequences for the functioning of biological processes in farm animals.

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Material And Methods

Study design

All experimental protocols were approved by Local Ethics Commission for Animal Experiments in Lublin, Poland (University of Life Sciences in Lublin, Akademicka str. 13, 20–950, Lublin, Poland; Consent no. 8/2007), consistent with Polish legal regulations (act of 21 January 2005) which determined the terms and conditions for experiments on animals. Mature males of a wild boar-pig crossbreed (12 months of age, body mass ~ 100–120 kg, n = 24) were used for the study. The animals were housed in an experimental farm of the Physiology and Reproduction of Animals Department, Rzeszow University in Kolbuszowa, near Rzeszow, Poland. They were kept under natural illumination and had *ad libitum* access to water and standard food. Two groups, control (C) and experimental (light treated, LT), with twelve animals each, were used in the study. The experiments were performed in the second half of June during the days with the longest periods of light in the summer and in the second half of December during the days with the shortest periods of light in the winter. This paragraph was originally described in Gilun et al. 2013. [9]

In summer, the animals were kept in an open-sided shed and exposed to approximately 30,000 lx of natural illumination during the day. The mean ambient temperature was 24°C during the light phase and 12°C during the nocturnal phase. In winter, the animals were housed in a room with windows and exposed to between 40 and 50 lx of natural illumination at the eye level of the animals during the day. The mean temperature during the day and night was 12°C. A dim red spotlight was used to assist in the experimental treatment during the nocturnal phase. This paragraph was originally described in Gilun et al. 2013. [9]

Surgical procedures

All methods are reported in accordance with ARRIVE guidelines. The animals were premedicated with atropine (0.05 mg/kg I.M.; Biowet, Gorzow Wielkopolski, Poland) and azaperone (Stresnil 2 mg/kg I.M.;

Janssen Pharmaceutica, Beerse, Belgium). General anesthesia was induced with thiopental sodium (Thiopental, I. V, Sandoz GmbH, Austria). A silastic catheter (o.d., 2.4 mm; i.d., 1.8 mm) was inserted into the right dorsal nasal vein (DNV) toward the ophthalmic sinus. This catheter placement allowed for the infusion of autologous plasma with experimentally elevated concentrations of CO (Fig. 1). An additional catheter (o.d. 2.4 mm; i.d., 1.8 mm) was inserted into the jugular vein (JV) to collect systemic venous blood. This paragraph was originally described in Gilun et al. 2013. [9]

Preparation and infusion of autologous blood with elevated concentrations of carbon monoxide achieved by bright light exposure

Systemic venous blood was repeatedly collected under sterile conditions from each animal and heparinized. The CO concentration in blood was estimated using a standard addition method described in Koziorowski et al. 2012 [8]. The average concentration was 1.51 nmol/ml in June and 0.89 nmol/ml in December. This autologous blood was pumped at 8.3 ml/h through a syringe into a clear plastic spiral cannula wrapped around a standard 2.5 cm diameter illuminated white fluorescent bulb (Narva LT-T8Standard). The spiral cannula was placed approximately 20 cm between two lamps with white light-emitting diodes (LEDs) (Lumie Desklamp). Blood exiting the spiral cannula drained, via a catheter placed in an external nasal vein, into the animal's ophthalmic venous sinus. The measured illuminance at the surface of the cannula was approximately 10,700 lux. This illuminance is comparable to that used to treat winter depression and was intended to represent the natural summertime increase in light that we have observed to be associated with elevated CO in ophthalmic venous blood in these animals. After two hours of bright light exposure, the CO concentration was measured again. Autologous blood with an increased concentration of CO, up to 5.1 nmol/ml in June and 2.1 nmol/ml in December, was infused at a rate of 8.3 ml/h with the use of a pump (SEP21S, Ascor, Poland) for 48 h into the ophthalmic sinus [11].

Preparation of preoptic area and dorsal hypothalamus tissue for total RNA isolation

After the autologous blank or light-treated blood was infused, the animals of the control (C) and experimental groups (LT) were transported to a properly adapted slaughterhouse. Three from each group were sacrificed during the summer day, during the summer night, during the winter day and during the winter night through electrical stunning and exsanguination. The head was then immediately cut, the skull opened, and the hypothalamus was isolated from the brain. The POA and DH tissues were collected, frozen in liquid nitrogen (–196°C) and then stored at − 70°C until required for analysis. This paragraph was originally described in Gilun et al. 2013. [9]

Total RNA isolation

Ice-cold brain tissues suspended in 1 ml of Tri-Reagent solution (Molecular Research Center, Cincinnati, OH, USA) in RNase-free tubes with ceramic beads were homogenized in a FastPrep-24 apparatus (MP Biomedicals LLC, Solon, OH, USA) for 45 s. Following homogenization, total RNA was isolated using a previously described method. Total RNA was isolated from the chloroform fraction using a Total RNA kit

(A&A Biotechnology, Gdansk, Poland). RNA integrity was checked by 2% agarose gel electrophoresis. The quality was expressed as the absorbance ratio at 260:280 nm, and the amount in the sample was measured with a NanoDrop 2000c (Thermo Fisher Scientific Inc., Waltham, MA, USA). This paragraph was originally described in Gilun et al. 2013. [9]

Total protein isolation

After chloroform removal (see step RNA isolation), total protein was isolated according to Chomczynski's protocol [16].

Real-time PCR analysis

Real-time PCR was performed with a Maxima First Strand cDNA Synthesis kit for qPCR (Thermo Fisher Scientific Inc., Waltham, MA, USA), and all steps were conducted according to the manufacturer's instructions. Briefly, aliquots of the RNA samples (1 µg) were treated with RNase-free DNase for 10 min at room temperature. Next, the RNA samples were denatured for 10 min at 70°C prior to the synthesis of complementary DNA. Both oligo dT and random hexamers (50 pmol each primer) were used for first-strand cDNA synthesis. In addition, the reaction mixture (per sample) contained 10 mM dNTP mix, 200 U Reverse Transcriptase M-MuLV RNase H Minus in 5×RT buffer (250 mM Tris-HCl, 250 mM KCl, 20 mM MgCl, 20 mM DTT, pH 8.3 at 25°C), and 20 U RiboLock RNase inhibitor. cDNA synthesis was carried out in a PCR Thermal Cycler (Bio-Rad, Hercules, CA, USA) according to the thermal cycle program of 25°C for 15 min, 50°C for 25 min and 85°C for 5 min and then held at 4°C. Then, the RT products (cDNA) were diluted 20-fold with nuclease-free water and used for real-time PCR analysis, as described below. This paragraph was originally described in Gilun et al. 2013. [9]

The cDNA was subjected to quantitative PCR analysis using a real-time PCR system (ABI 7900HT; Applied Biosystems, Foster City, CA, USA). The reaction mixture (per sample) contained 5 µl cDNA (equivalent to 12.5 ng RNA), 5 µl primers (forward and reverse), 2.5 µl TaqMan probe and 12.5 µl Maxima probe PCR mix (Thermo Fisher Scientific Inc., Waltham, MA, USA). Each cDNA sample was assayed in duplicate. Thermal cycling was initiated at 95°C for 10 min for DNA polymerase activation. Forty PCR steps were performed, each consisting of heating at 95°C for 15 s and 60°C for 60 s. All primers and probes (see *Table 1*) were designed using Primer Express Software v3.0 (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated through the comparison of the genes of interest with the reference gene (*Sdha*) and was expressed in arbitrary units. For result calculation, the real-time PCR Miner algorithm was used [17]. This paragraph was originally described in Gilun et al. 2013. [9]

Western Blot

The acetone-precipitated proteins were washed three times with 95% EtOH with 5% glycerol and dissolved in Tris buffer containing 0.01% sodium dodecyl sulfate (SDS). After total protein determination with a Pierce™ BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), samples containing 25 µg of total proteins and NovexTM Tris-Glycine SDS Sample Buffer (2x) were fractionated on 10% SDS-polyacrylamide gels (SDS-PAGE) and wet transferred to a nitrocellulose membrane (Bio-Rad, Germany).

To block nonspecific signals, 1% blocking solution (BM Chemiluminescence Western Blotting Kit; Mouse/Rabbit, Roche, Switzerland) was used. Then, the membranes were incubated overnight at 4°C with the following primary antibodies: anti-BMAL1 rabbit IgG, anti-CLOCK rabbit IgG and anti-NPAS2 rabbit IgG (Santa Cruz Biotechnology, USA) diluted 1:200 in 0.5% blocking solution. The next day, the membranes were washed 3 times for 10 min in Tris-buffered saline with Tween (TBS-T) and incubated for 60 min at room temperature with goat anti-rabbit IgG-HRP conjugated antibodies (Santa Cruz Biotechnology, USA) diluted 1:25,000 in 0.5% blocking solution. After that, the blots were washed 3 times for 10 min with TBS-T and developed by enhanced chemiluminescence reaction (Roche, Switzerland). The protein signals were detected with a Fusion FX7 camera. Optical density was calculated by Bio1D + + software (Vilber Lourmat, France). As a control for transfer and loading, GAPDH was assessed on each membrane. For this, anti-GAPDH mouse IgG antibody (Santa Cruz Biotechnology, USA; dilution 1:200) and goat anti-mouse IgG-HRP conjugated antibody (Bio-Rad, Germany; dilution 1: 20,000) were used. The final results represent the ratio of the optical density of a particular protein to the optical density of GAPDH present in the same sample.

Statistical analysis

Statistical analysis was performed by one-way ANOVA with Bonferroni post's test for the selected pairs of columns. A critical value for significance in all experiments was $P < 0.05$. For statistical analysis, GraphPad PRISM software version 9.2 for Windows (San Diego, CA, USA) was used. All data are shown as a box and whiskers from minimum to maximum. Values are presented as means ± S.E.M.

Results

mRNA expression of clock genes and their transcription factors (TFs) in

preoptic area (POA)

mRNA expression of clock-specific transcription factors (TFs) in POA

The mRNA expression levels of *BMAL1, CLOCK* and *NPAS2* were significantly lower in the LT group than in the control group both during the day and at night as well as in winter and summer. We also observed higher *BMAL1* and *NPAS2* expression between the control groups at night compared to day as well as in winter and summer ($P < 0,01 - 0,0001$; Fig. 2).

mRNA expression of genes activated by clock-specific TFs in POA

The mRNA expression of *PER1* was significantly lower (P < 0,001 – 0,0001) in the LT group than in the control group in both the summer and winter LT groups during the day and at night. We observed a significant difference in the mRNA expression of *PER2* among the LT group compared to the control group only in winter, when the *PER* gene expression was higher at night (P < 0,05). The mRNA expression of *CRY1* and *CRY2* was significantly higher (P < 0,01 – 0,0001) in the LT group than in the control group during the day and at night as well as in winter and summer. We also observed higher mRNA expression of these genes (P < 0,01) in winter in the LT groups at night than during the day (Fig. 3).

The mRNA expression of *REV-ERB α* and *REV-ERB β* was significantly higher (P < 0,01 – 0,0001) in the LT group than in the control group during winter and summer during the day and at night. We also observed lower gene expression in the control groups (P < 0,05) in summer at night than during the day. The same relationship was observed concerning the LT group in this season. In contrast to these observations, we noted that the mRNA expression of *REV-ERB α* or *REV-ERB β* in the LT groups was higher (P < 0,01 – 0,0001) in winter at night than during the day (Fig. 4).

Winter-time *ROR β* gene expression was lower in the LT groups than in the control group during the day and higher at night (P < 0.01). On the other hand, this expression was reversed in the summer, higher during the day and lower at night. We also noted that the mRNA expression of this gene in the LT group was significantly (P < 0,0001) higher at night than during the day in winter and significantly longer at night than during the day in summer (P < 0,01). We noted a statistical significance between the control groups of this gene expression only in summer, when it was higher at night than during the day (P < 0,05; Fig. 4)

Protein level of clock specific TFs in POA

BMAL1 protein levels were higher in the LT group than in the control group during the day and at night in winter (P < 0,05). Summer-time levels of BMAL1, CLOCK and NPAS2 protein were observed to be higher in the LT groups than in the control groups, both during the day and at night (P < 0,001 – 0,0001). Winter-time BMAL1 and CLOCK levels in the control groups were higher at night than during the day (P < 0,05 – 0,001). BMAL1, CLOCK and NPAS2 levels were at the same level in the control groups during the day and at night in summer. The NPAS2 protein level was higher in the LT group than in the control group in winter, day and night (P < 0,05 – 0,0001; Fig. 5).

mRNA expression of clock genes and their TFs in the dorsal hypothalamus (DA)

mRNA expression of clock specific TFs in DH

mRNA expression of *BMAL1, CLOCK and NPAS2* was lower in the LT groups than in the control groups during the day and at night, as well as in winter and summer. *BMAL1* expression in the control groups in both winter and summer was higher during the day than at night (P < 0,01 – 0,001**).** *NPAS2* expression in the control groups did not differ day or night during either winter or summer (Fig. 6).

mRNA expression of clock genes activated by clock-specific TFs in DH

The mRNA expression of summer-time *PER1* or *PER2* was higher in the LT groups than in the control groups during the day and at night (P < 0,001 − 0,0001). We had the same results in winter except for daytime *PER1* expression when the levels were the same. The summer-time *PER1* and *PER2* expression between the control groups was significantly (P < 0,05 − 0,001) lower at night than during the day, although we observed lower winter-time expression between the control groups for only *PER2* (P < 0,0001; Fig. 7).

mRNA *CRY1* expression in LT groups was lower (P < 0,0001) than that in the control group both during the day and at night as well as in winter and summer except for on summer was during when the expression levels were the same. In contrast to these results, winter and summer *CRY2* expression was higher in the LT group than in the control group (P < 0,01 − 0,0001**).** *CRY1* expression between the control groups was noted to be lower at night than during the day in winter but higher in summer (P < 0,05 − 0,01; Fig. 7).

mRNA *REV-ERB α* expression was lower in the LT group than in the control group during the day and at night in summer and at night in winter but higher than at day. Rev-erb α expression between the control groups was lower during the day than at night (P < 0,001) in winter but lower at night in summer (P < 0,05; Fig. 8).

REV-ERB β mRNA expression differed significantly only at night in summer when the expression in the LT group was lower than that in the control group (P < 0,05; Fig. 8).

ROR β mRNA expression was lower in the LT group than in the control group, both during the day and at night in winter (P < 0,01). However, lower expression was noted between these groups only at night in summer, unlike during the day when higher expression was observed (P < 0,001; Fig. 8).

Protein level of clock-specific TFs in DH

The protein levels of BMAL1, CLOCK and NPAS2 were higher in the LT group than in the control group during the day and at night in winter (P < 0,05 − 0,001). In contrast to these results, the daytime levels of BMAL1, CLOCK and NPAS2 were lower in summer (P < 0,05 − 0,01). We also noted that the protein level did not significantly differ at night in summer (P > 0,05; Fig. 9).

Discussion

This study indicated the presence in the molecular elements of the biological clock of a domain capable of binding heme, which when combined with carbon monoxide gas produces a biological effect. Additionally, the possible participation of carbon monoxide in regulating the cyclic functions of the biological clock mechanism demonstrated that carbon monoxide is released into venous blood flowing away from the eye in amounts dependent on diurnal and seasonal changes in environmental light [8].

Analysis of the results showed that elevated endogenous CO levels through blood irradiation induce numerous changes in the functioning of the main biological clock in the POA, as well as in parts of the DH. Changes in the expression of the transcription factors *BMAL1, CLOCK* and *NPAS2* have a similar pattern in both structures, where a very large decrease in gene expression was shown after exposure to elevated endogenous CO levels. The changes in the gene expression of *PER1-2*, *CRY1-2*, *REV-ERB α-β* and *ROR β* are not the same for both POA and DH structures, indicating that both structures respond differently to the received humoral signal.

Additionally, the DH functioning (expression) pattern of clock elements should be considered a characteristic of the "peripheral clock", which tunes to the main pacemaker in POA. The aforementioned factors are known to be elements of a positive regulatory loop that stimulate the expression of *PER* and *CRY* genes, the main clock components [3]. Under physiological conditions, their expression varies throughout the day (oscillation), particularly concerning the bmal1 gene [3, 18]. The reductions in the expression levels of the transcription factors in question in both experimental groups obtained in the present study indicate the influence of CO on this process. It is difficult to indicate which element of the regulatory loop was blocked, and CO probably affected the PER2 protein. This protein has been shown to positively influence bmal1 expression, and PAS domains with heme prosthetic groups in its structure react with carbon monoxide [19–21].

Although our study showed no significant changes in the expression of the *PER2* gene under the influence of CO and only some samples showed significant changes in this expression, it cannot be excluded that the resulting PER2 protein could bind to the CO molecule via the heme group and exert a physiological effect in the form of a lack of positive effects on the expression of the bmal1 gene contrary to the cited literature data [3, 22, 23]. Furthermore, the presence of *CRY1* and *2* overexpression had some importance. The CRY protein, through the photolyase homology domain, binds to the CRY-binding domain on the PER2 protein to form a repressive complex that inhibits CLOK:BMAL1 by displacing from promoters under physiological conditions [24], but when PER proteins are blocked, the correct repressive complex may not form. Displacement of the TF dimer from promoters does not occur, and expression is induced; hence, the emerging overexpression of clock elements regulated by BMAL1/CLOCK or BMAL1/NPAS2 TF heterodimers.

Another explanation could be the increased NR1D2 expression compared to controls observed in both experimental groups. NR1D and especially NR1D1 are known to negatively regulate the expression of *BMAL1* and *CLOCK* [25, 26]. However, silencing the expression of these nuclear receptors does not result in the disappearance of bmal1 or per gene oscillations [27]. In addition, such a situation would be unclear because NR1D1 also contains PAS domains and reacts with CO [28, 29].

Experimental group animals showed high *CRY1* and *CRY2* expression levels. The protein product of the *CRY* gene is known to be the only factor able to inhibit the function of the BMAL1/CLOK dimer attached to the promoter. This occurs through the attachment of CRY-binding domains to CLOCK and to BMAL1 [30]. In the animals of this group, the CO levels were elevated through the action of natural light on the

blood, and CO is assumed to have been produced endogenously. The effect of hyperstimulation of *PER1* expression was probably already inhibited by CRY proteins. Additionally, the expression of both nuclear receptors whose proteins inhibit the expression of the mentioned transcription factors increased in group II. Increased expression levels of both *CRY1* and *CRY2* in the animals of this group confirm a more rapid response of the prelimbic part of the hypothalamus to the experimental conditions in comparison with the control group. Due to the specificity of *CRY1* gene expression, it always occurs later than other clock genes with a twelve-hour delay after transactivation with the BMAL1/CLOCK dimer. Thus, the elevated expression of *CRY1* in POA and DH and *CRY2* only in POA indicates an advanced response of clock elements to carbon monoxide. High *CRY1* expression levels are important for maintaining the oscillation of clock elements and allowing them to be enhanced [31], which may have been the case in our experiment. After analysing the protein expression of transcription factors, we noticed that the gene expression of *BMAL1*, *CLOCK* and *NPAS2* in both examined structures decreased in comparison to the control group. However, the analysis of the protein levels in the same samples showed that in POA, the levels of the constitutive transcription factors *BMAL1* and *CLOCK* decreased in winter but increased in summer in comparison to the control group. This situation may be related to CO availability because levels in winter are naturally three times lower than those in summer when the length of the day and the intensity of additional light [8] irradiation increase the amount of CO (but only to the physiological level obtained in summer). However, such an amount accompanied by a general deficit inhibits not only gene expression but also translation. In summer, additional irradiation also increases the CO level approximately three times above the physiological level, which probably blocks the possibility of dimerization and the dissociation of dimers from the promoter, additionally increasing the amount of detected CLOCK and BMAL1 proteins. In contrast, we observed an increase in the amount of NPAS2 protein in both winter and summer, but gene expression was downregulated relative to the controls. This pattern of expression is difficult to explain, but all the presented transcription factors are likely blocked from dimerization by CO through haem groups contained in their structure. For example, CO concentration in the cellular environment has been shown to affect the formation of the NPAS2:BMAL1 protein dimer, which acts as a transcription factor in the master biological clock mechanism located in the prelimbic area of the hypothalamus (cited in [10]). An increase in CO concentration over 1 μM caused a breakdown of this complex [32]. In the case of the results from the dorsal part of the hypothalamus, there was also a clear decrease in the expression of the clock transcription factor genes, whereas in the case of the protein, the changes in expression were irregular and the opposite of those obtained in the prelimbic part. Higher protein expression was observed in winter in all the factors studied (BMAL1, CLOCK, NPAS2). In contrast, the expression was statistically significantly lower in summer. This situation may be influenced by the CO-altered output from the master clock from the POA and the influence of CO itself, which reached these structures via the humoral pathway and directly affected transcription and translation.

The mechanism of the regulatory effect of carbon monoxide on biological clock components is unclear. NR1D receptor activity is known to be more strongly inhibited by NO than by CO, whereas CO exhibits only 15% NO activity [29]. Thus, it seems impossible for CO to significantly affect the function of NR1D

proteins. Our results support this concept, as NR1D expression levels were higher compared to controls, assuming that the body, in an attempt to compensate for the effect of CO blocking these receptors, increased their expression. In group II, where CO was assumed to be more available, there was an increase in the expression of both nuclear receptors, and the effect of their blocking transcription factors was also evident. This is favoured by the appearance of mRNA for both NR1Ds immediately after their expression was activated by the BMAL1/CLOCK dimer [29, 33].

It is unclear why the expression of *Per* and *Cry* and NR1D genes increased when the expression level of transcription factors of the mentioned genes drastically decreased. Similarly, Zhang et al. and Baggs et al. [34, 35] noticed an increase in the expression of *PER1, CRY1* and *CRY2* after *BMAL1* expression was silenced using siRNA. They suggested that the expression may be activated by *BMAL2* or the complexity of the posttranslational processing step of the *BMAL1, CLOCK* or *NPAS2* gene products, as their expression was not silenced completely but drastically reduced. The same is also true for the *PER2* gene, as its expression was unaffected despite the disruption of *BMAL1* gene expression by *REV-ERB α* and *β* (also called NR1D1 and 2) [1]. Other authors have demonstrated that *BMAL1* can be effectively replaced by *BMAL2*. However, the constitutive expression of *BMAL2*, which is constant over the course of a day compared to *BMAL1* [36], is also subject to transfection by the BMAL1/CLOCK dimer, and downregulation of *BMAL1* expression results in the downregulation of *BMAL2* expression [37]. This situation suggests that as gene expression decreases, especially for such essential genes, assuming no mutation is present that precludes the correct sequence of events (transcription/translation), translation efficiency increases or protein ubiquitination levels decrease to maintain system function. The short lifespan of mRNA also suggests that a reduction in expression levels may be compensated for by an increase in translational efficiency.

Conclusion

- An adequate amount of light is an essential factor for the proper functioning of the internal biological clock, which is a regulator of many important physiological processes.
- Carbon monoxide is a chemical light molecule whose production depends on the amount of light. Disturbance of the synthesis can have a deregulatory effect on the functioning of diurnal and circadian cycles affecting homeostasis of the whole organism.

Declarations

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Author contributions

Animal management: P.G., K.K., M.R-M., M.K., Supervision: M.K. Conception and design of the experiments: P.G. and M.K. Investigation: P.G., K.K., M.R-M., M. K, Data analysis and interpretation: P.G., B.W., M. S-K., M.K. Writing – original draft, writing review & editing: P.G., B.W., M. K-G. Revising it critically for important intellectual content: P.G., B.W., M.S-K., K.K., M.R-M., M.K., M.K-G.

Declaration of interest

No conflict of interest

Data and model availability statement

The model and data are available in the corresponding author, and raw Western blot data are available in M.S.K.

Ethics approval

All procedures were carried out in compliance with Polish legal regulations (act of 21 January 2005), which determined the terms and conditions for experiments on animals and were in accordance with the protocol of the Local Ethics Commission for Animal Experiments in Lublin, Poland; Consent No. 8/2007.

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Tables

Table 1. Primers and TaqMan probes used in experiment.

Gene name	Sequences of used primers and probes	Accesion numer of template sequence
Per 1	Forward: TCCACAGCATCGACCTTGTC Reverse: GGGACTCGGGTCCATTGG Probe: [6FAM]CTGATGACACGGATGCCAACAGCA [TAM]	AJ277735.1
Per 2	Forward: AGCATCGGCCTCTAAGATGAAC Reverse: CATCTTCCACCGTCTCAAGCT Probe: [6FAM]ACGTTAGCACGCACTTGACCTCGCTG[TAM]	XM_003483786.1
Cry 1	Forward: ACACCATCCGCTGCGTCTAC Reverse: TGAAAAGCCTGGGAAACACAT Probe: [6FAM]CTGGTTCGCCGGCTCTTCCAATGT[TAM]	XM_003126079.2
Cry 2	Forward: TTTGATGAGCTGCTCCTGGAT Reverse: GTGCGGCGGCCAAAG Probe: [6FAM]TGTCCTGCAGTGCTTTCTTCCAACAGTTCT[TAM]	XM_003353887.1
Bmal 1	Forward: GGTGTAATCTCAGCTGCCTTGTC Reverse: TTTCCCCGTTCACTGGTTGT Probe: [6FAM]CAATCGGACGACTGCACTCGCACAT[TAM]	EF216896.1
Clock	Forward: GCACACAATGGTTTTGAAGGAAT Reverse: CAGTGCACATTTCCTTGATGAAC Probe: [6FAM]ACACCACGCACACACAGGCCTTCTT[TAM]	XM_003356944.2
Npas2	Forward: ACCGCGGGCCTTAACAC Reverse: CTGTGCGCGAGGATTTGTG Probe: [6FAM]CCACTCACCATCGGCATCCTCAAGA[TAM]	XM_003124898.3
Rev-erb α (NR1D1)	Forward: GCCCCCAATGACAACAACAT Reverse: GGAAGGAGCCTGGCGTAAA Probe: [6FAM]TGCCCAGCGTCATAACGAGGCC[TAM]	DQ120775.1
Rev-erb β (NR1D2)	Forward: TCTCTGGGACGTTGTCTGCTT Reverse: CACCTATGCGCCCAGAAAAG Probe: [6FAM]TCCTCCACCTTGGTTCCCGAGGC[TAM]	XM_003358340.2

Ror β	Forward: GAAGCCCTCGCCAGAGTGTA Reverse: GGTCGATGACGTGTCCATTG Probe: [6FAM]CAGCAGCATCAGTAATGGCCTCAGCA[TAM]	XM_003480537.1
Sdha	Forward: CTCTCTGAGGCCGGGTTTAA Reverse: CCAGTTGTCCTCCTCCATGTT Probe: [6FAM]TTACGAAGCTCTTTCCCACCAGATCACACA[TAM]	XM_003362140.1

Figures

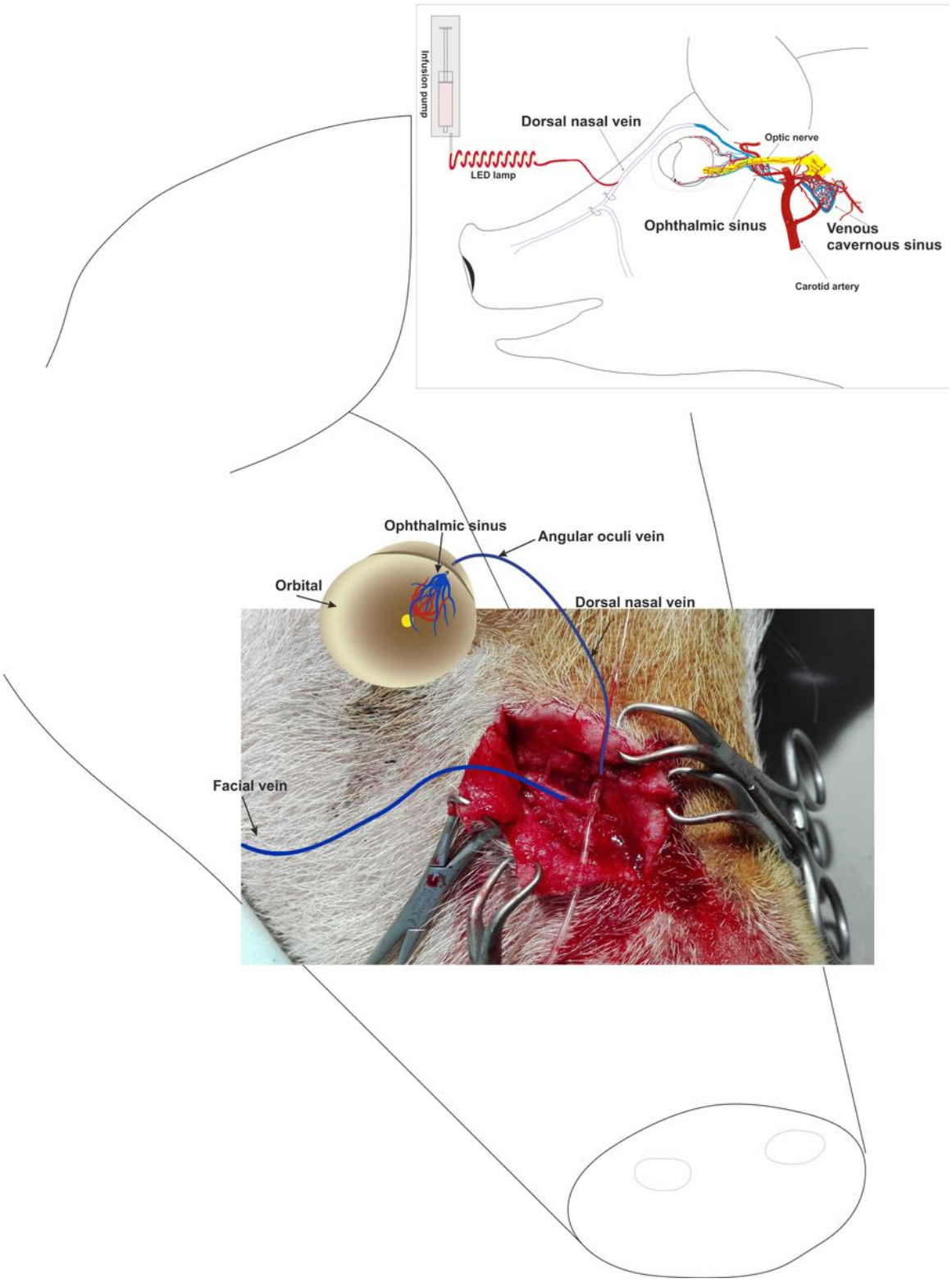

Figure 1

Photograph (below) and schematic draw (above) presenting catheter placement (into the dorsal nasal vein) allowed for the infusion of autologous blood with experimentally elevated concentrations of CO.

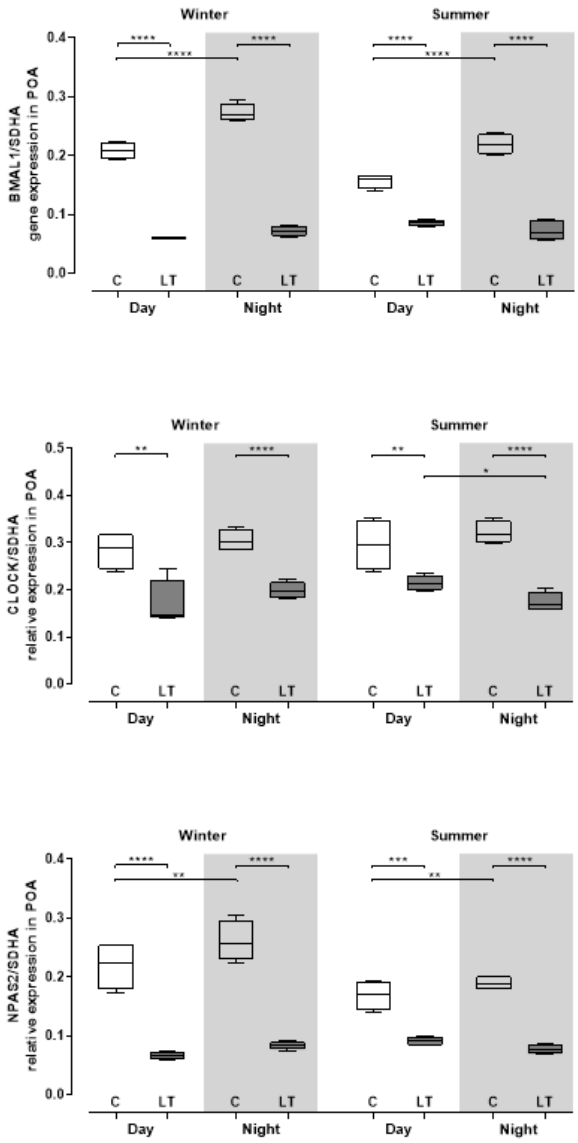

Figure 2

Gene expression of biological clock transcription factors in POA (Bmal1, Clock, Npas2) in two examined groups: control (C) and light treated (LT) in two seasons winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* - p≤0.05, ** - p≤0.01, *** - p≤0.001, **** - p≤0.0001).

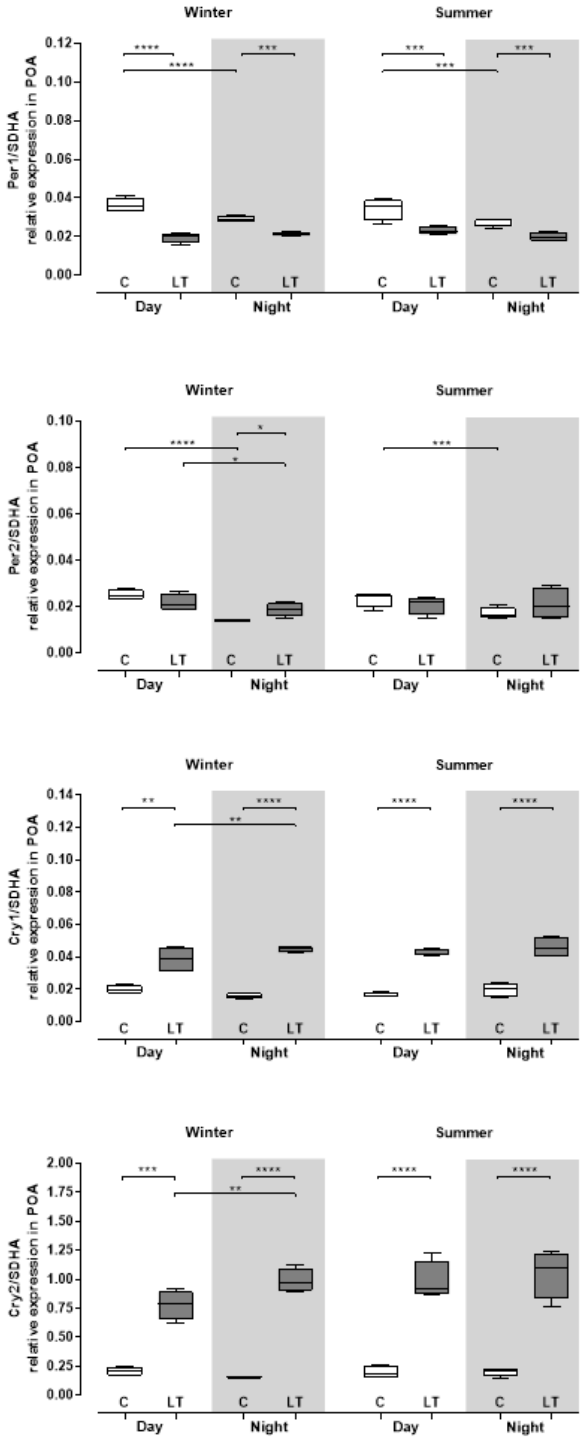

Figure 3

Gene expression of biological clock genes in POA (Per 1-2, Cry 1-2) in two examined groups: control (C) and light treated (LT) in two seasons winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* - p≤0.05, ** - p≤0.01, *** - p≤0.001, **** - p≤0.0001).

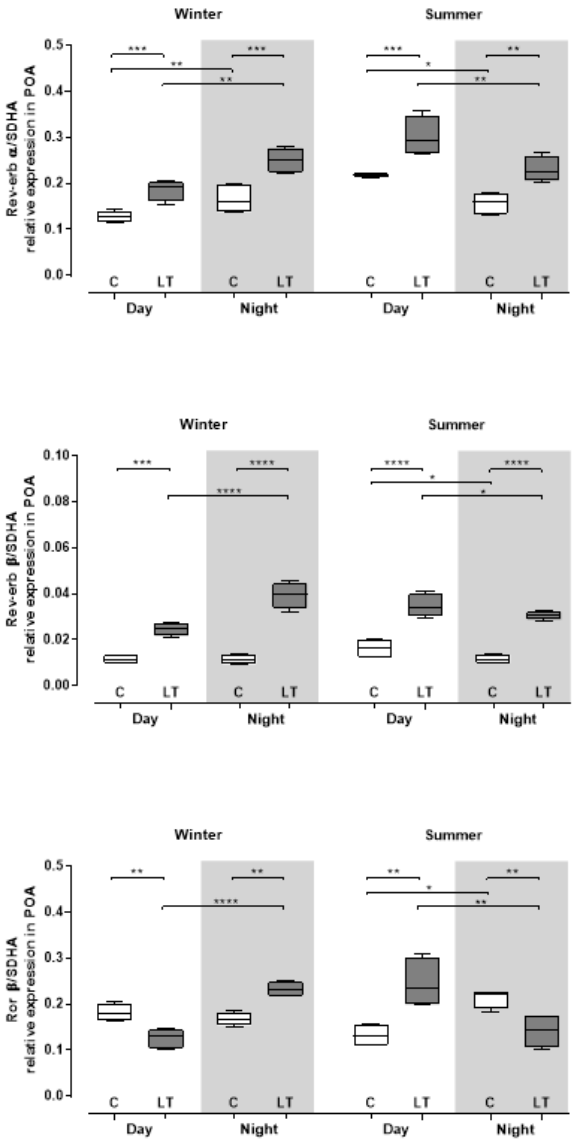

Figure 4

Gene expression of biological clock genes in POA (Rev-erb α-β and Ror β) in two examined groups: control (C) and light treated (LT) in two seasons winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* - p≤0.05, ** - p≤0.01, *** - p≤0.001, **** - p≤0.0001).

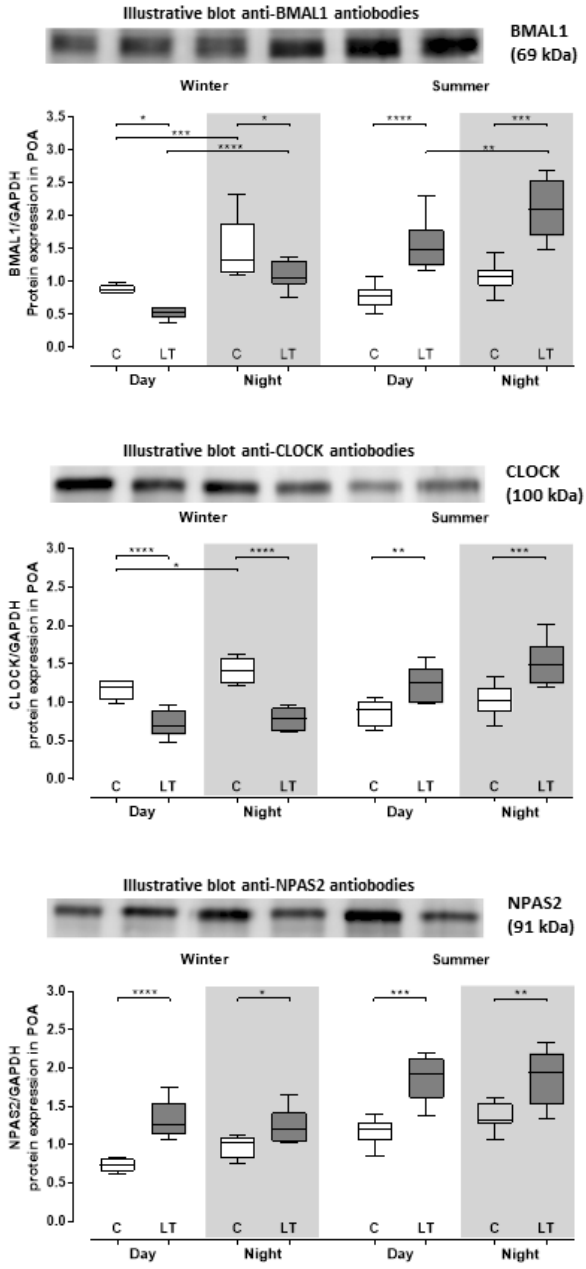

Figure 5

Protein levels of biological clock transcription factors in POA (BMAL1, CLOCK, NPAS2) in two examined groups: control (C) and light treated (LT) in two seasons winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* - p≤0.05, ** - p≤0.01, *** - p≤0.001, **** - p≤0.0001). An illustrative blot of the analysed protein is included for each graph.

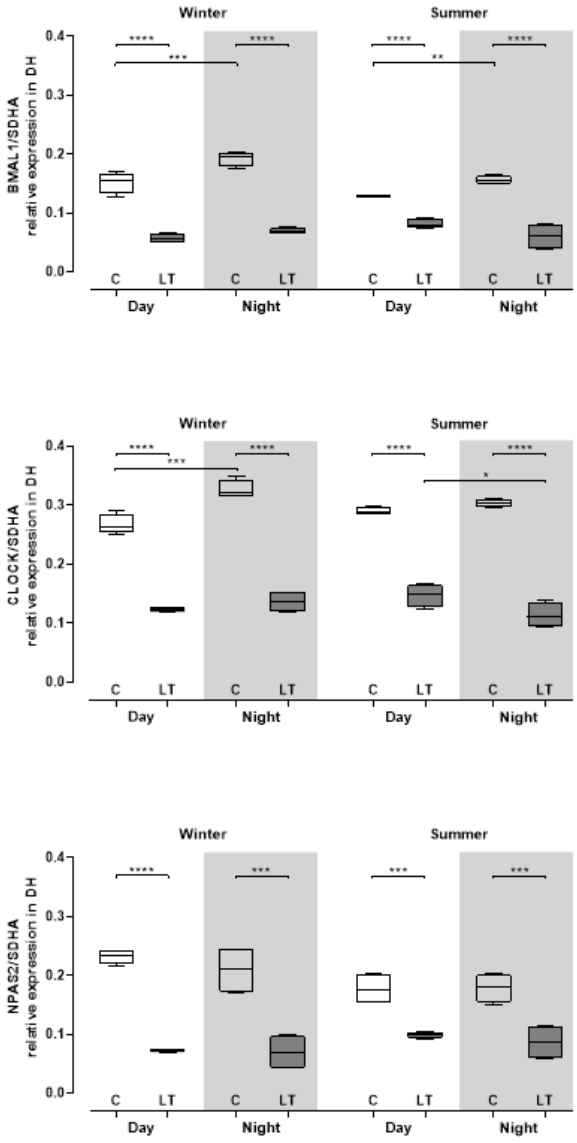

Figure 6

Gene expression of biological clock transcription factors in DH (Bmal1, Clock, Npas2) in two examined groups: control (C) and light treated (LT) in two seasons winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$, **** - $p \leq 0.0001$).

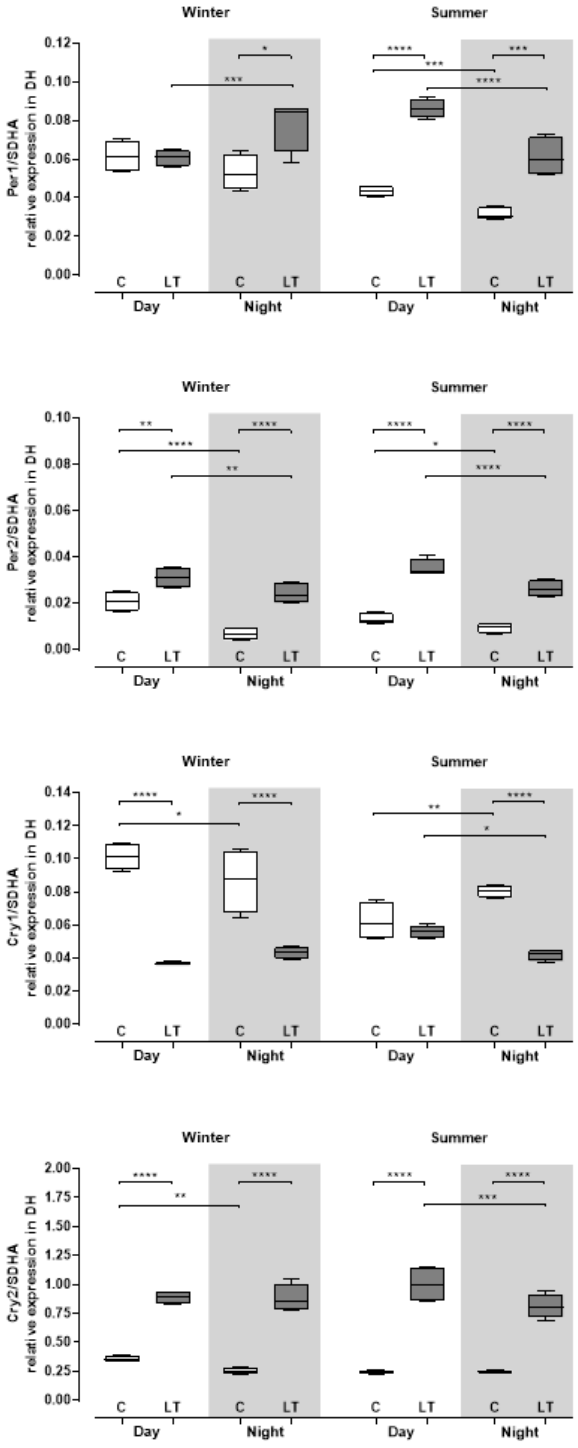

Figure 7

Gene expression of biological clock genes in DH (Per 1-2, Cry 1-2) in two examined groups: control (C) and light treated (LT) in two seasons winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* - p≤0.05, ** - p≤0.01, *** - p≤0.001, **** - p≤0.0001).

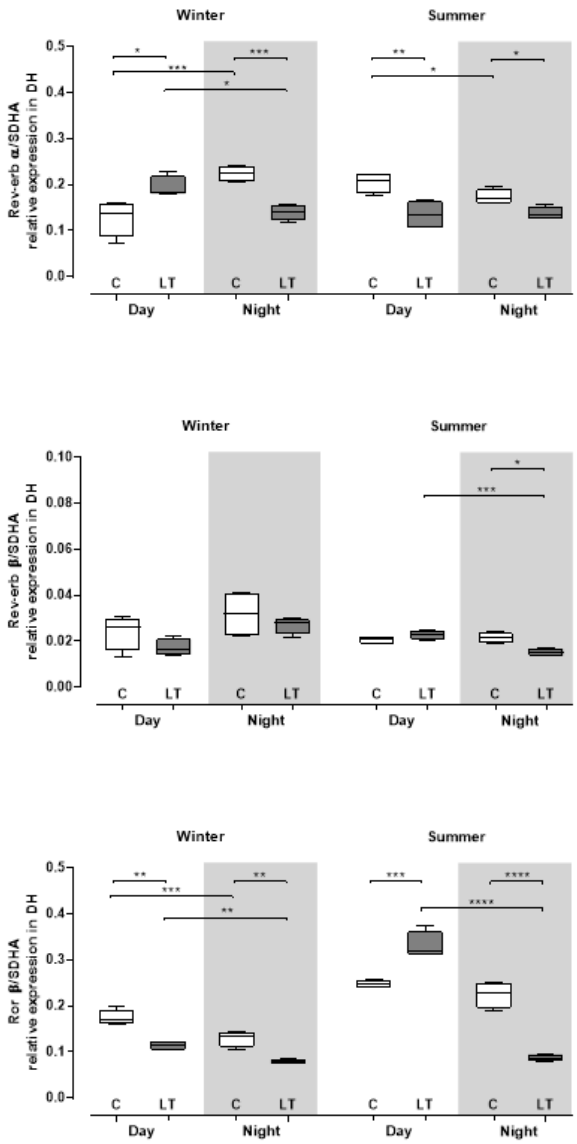

Figure 8

Gene expression of biological clock genes in DH (Rev-erb α-β and Ror β) in two examined groups: control (C) and light treated (LT) in two seasons winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* - p≤0.05, ** - p≤0.01, *** - p≤0.001, **** - p≤0.0001).

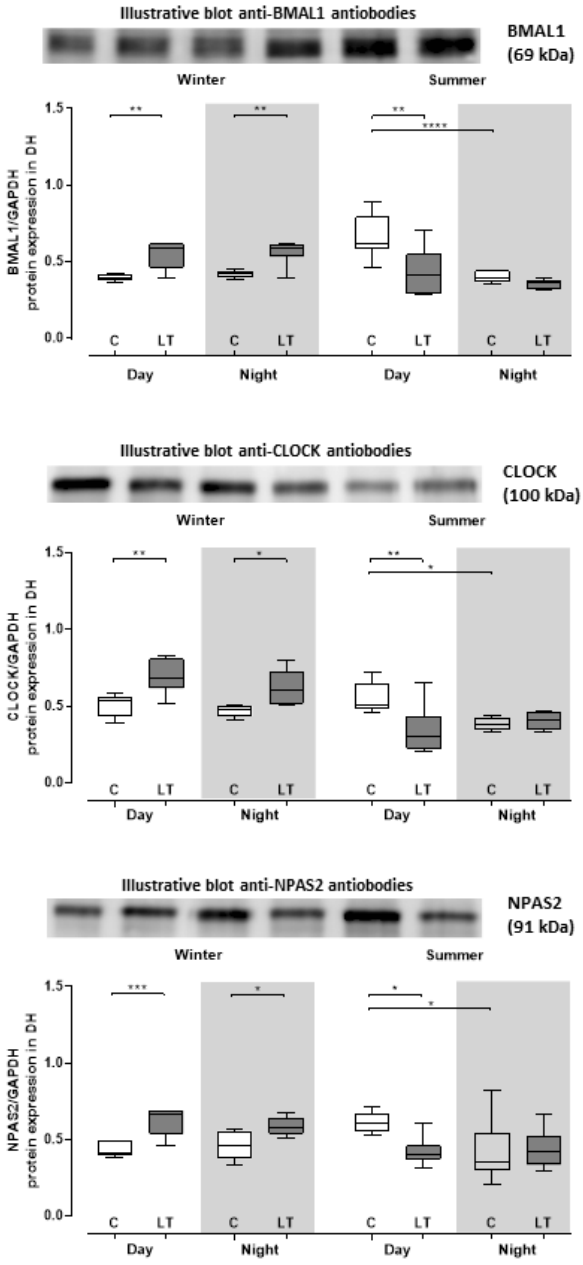

Figure 9

Protein levels of biological clock transcription factors in DH (BMAL1, CLOCK, NPAS2) in two examined controls (C) and light-treated plants (LT) in two seasons: winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* - p≤0.05, ** - p≤0.01, *** - p≤0.001, **** - p≤0.0001). An illustrative blot of the analysed protein is included for each graph.