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Original article

Endogenous carbon monoxide (CO) as a modulating factor of molecular biological clock in the hypothalamic structures involved in light transmission in pig and wild boar hybrid during long and short day season

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Abstract

Mature males of a wild boar-pig crossbreed, during the long and short day season, were used for the study which demonstrates that the chemical light carrier CO regulates the expression of biological clock genes in the hypothalamus 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. Molecular biology methods: qPCR and Western Blot were used to determine the expression of genes and biological clock proteins. The results showed that elevated endogenous CO levels, through blood irradiation, induces changes in genes expression involved in the functioning of the main biological clock located in suprachiasmatic nuclei. 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 *PER 1-2*, *CRY 1-2*, and *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 humoral signal received.

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

Keywords: carbon monoxide, humoral pathway, light, master biological clock, seasonal

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Introduction

Every living organism functions according to rhythms, both annual and circadian. The master biological clock is what makes organisms adjust to the 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 paralog NPAS2 in which an E-box-bound heterodimeric transcription factor drives the expression of *PER 1-2, CRY 1-2* and *REV-ERB* α-β (also known as a 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 the phasing of the core clock oscillation i.e. *REV-ERB* α or β and *ROR* β genes interloop (Wheaton et al. 2018, Ikeda et al. 2019). The expression of the core clock components, i.e. the genes *BMAL1, CLOCK*, and *NPAS2*, is constituted (Becker-Weimann et al. 2004) 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 indicates the important role of lags in the functioning of regulatory loops, thus determining the circadian cycle (Reppert and Weaver 2002).

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 non-forming 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 (Simonneaux and Ribelayga 2003, Fu et al. 2010). The SCN neuronal network gains important additional attributes through the plasticity within the network, including precision, robustness and the ability to code for day length. Electrophysiological studies have shown that a clustering of the phases of single cell oscillations (synchronization) leads to a narrow peak of the rhythm of the whole SCN network, representing short days, while the dispersal of activity patterns of SCN neurons (desynchronization) leads to a broader ensemble peak, representing long days. The electrical activity patterns of single SCN neurons are less affected by photoperiod, and the single-cell waveform changes reported are not sufficient to explain the adaptation of the ensemble output to day length. Similarly, some studies on clock gene expression have shown a change in the distribution of peak expression in response to different photoperiods, while others reveal spatial differences between dorsal and ventral SCN, and between caudal and rostral SCN. Thus, while the rhythm generating properties arise in individual cells, seasonal encoding depends on the integrity and plasticity of the SCN network (Michel and Meijer 2020). The amount of available endogenous CO whose formation and availability depends 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 independently of the neural signal, which may explain how single SCN cells can synchronize (Koziorowski et al. 2012, Gilun et al. 2013).

Functional clock gene proteins such as BMAL1, CLOCK, NPAS2 as well as PER2, REV-ERB α and β belong to a group of proteins called basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) containing PAS A and PAS B domains that form functional protein dimers with different biological functions. These domains are able to bind heme groups with Fe(II)-valent iron, which act as cofactors and influence the function of these proteins. Binding haem groups to Fe(II) at the active centre sensitizes these proteins to CO which, by binding to heme, exerts such biological effects as inhibiting the dimerization of BMAL1:N-PAS2 proteins (Gilun et al. 2021).

In recent years it has been demonstrated that light information can be transmitted to the hypothalamic structures via the humoral pathway by means of a gas transmitter – carbon monoxide (Gilun et al. 2013, Romerowicz-Misielak et al. 2016). 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 (Kutty et al. 1995, Oren 1996, Cao et al. 2000). The amount of CO in the blood flowing out of the eye has also been shown to depend on the amount of daylight which the retina receives (Koziorowski et al. 2012). The humoral phototransduction system, originally described by Oren et al., is a simpler and more inert system compared to 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 (Gilun et al. 2013).

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 diurnal. The fact that the animals are hybrids is also important for this study, 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 (Koziorowski et al. 2006). 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 study 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.

Materials and Methods

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 No. 8/2007.

Experiment design

Mature males of a wild boar-pig (Duroc) 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.

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.

Surgical procedures

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., 1.4 mm; i.d., 0.8 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.

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

Systemic venous blood was 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). 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 the dorsal 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 associated with elevated CO in ophthalmic venous blood in these animals. After two hours of bright light exposure the CO concentration was measured again. The autologous blood with an increased concentration

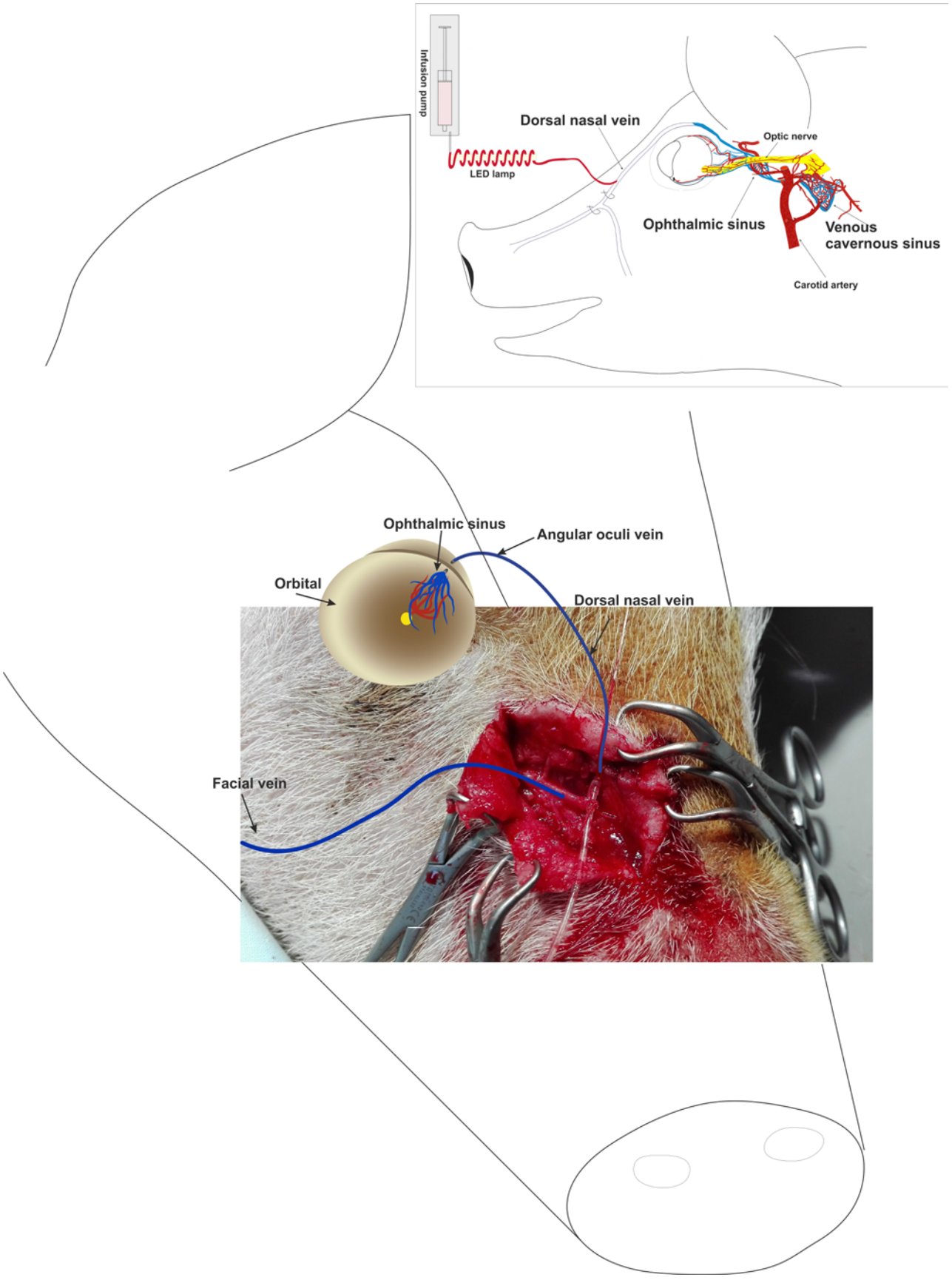

Fig. 1. Photograph (below) and schematic drawing (above) presenting catheter placement (into the dorsal nasal vein) allowed for the infusion of autologous blood with experimentally elevated concentrations of CO.

Table 1. Primers and TaqMan probes used in the 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

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 (Romerowicz-Misielak et al. 2016).

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

After the autologous blank or light treated blood were 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 using electrical stunning and exsanguination. The head was then immediately cut, the skull opened and the hypothalamus 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.

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 homogenisation, total RNA was isolated using the 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 using 2% agarose gel electrophoresis. The quality was expressed as the absorbance ratios at 260:280 nm, and the amount in the sample was measured with a Nano Drop 2000c (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Total protein isolation

After chloroform removal (see step RNA isolation), total protein was isolated according to Chomczynski's (1993) protocol.

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. The RNA samples were then 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 a 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. The RT products (cDNA) were then diluted 20-fold with nuclease-free water and used for real-time PCR analysis, as described below.

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 one consisting of heating at 95°C for 15 s and 60°C for 60 s. All primers and probes (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 (Zhao and Fernald 2005).

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 fractioned on 10% SDS-polyacrylamide gel (SDS-PAGE) and wet transferred to a nitrocellulose membrane (Bio-Rad, Germany). To block non-specific signals 1% blocking solution (BM Chemiluminescence Western Blotting Kit; Mouse/Rabbit, Roche, Switzerland) was used. The membranes were then 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 following 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. The blots were then washed 3 times for 10 min with TBS-T and developed using enhanced chemiluminescence reaction (Roche, Switzerland). The protein signals were detected with a Fusion FX7 camera. Optical density was calculated using 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. 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 using one-way ANOVA with Bonferroni's post test for the selected pairs of columns. The 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 expressions 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 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 ($p\leq0.01$-0.0001) (Fig. 2).

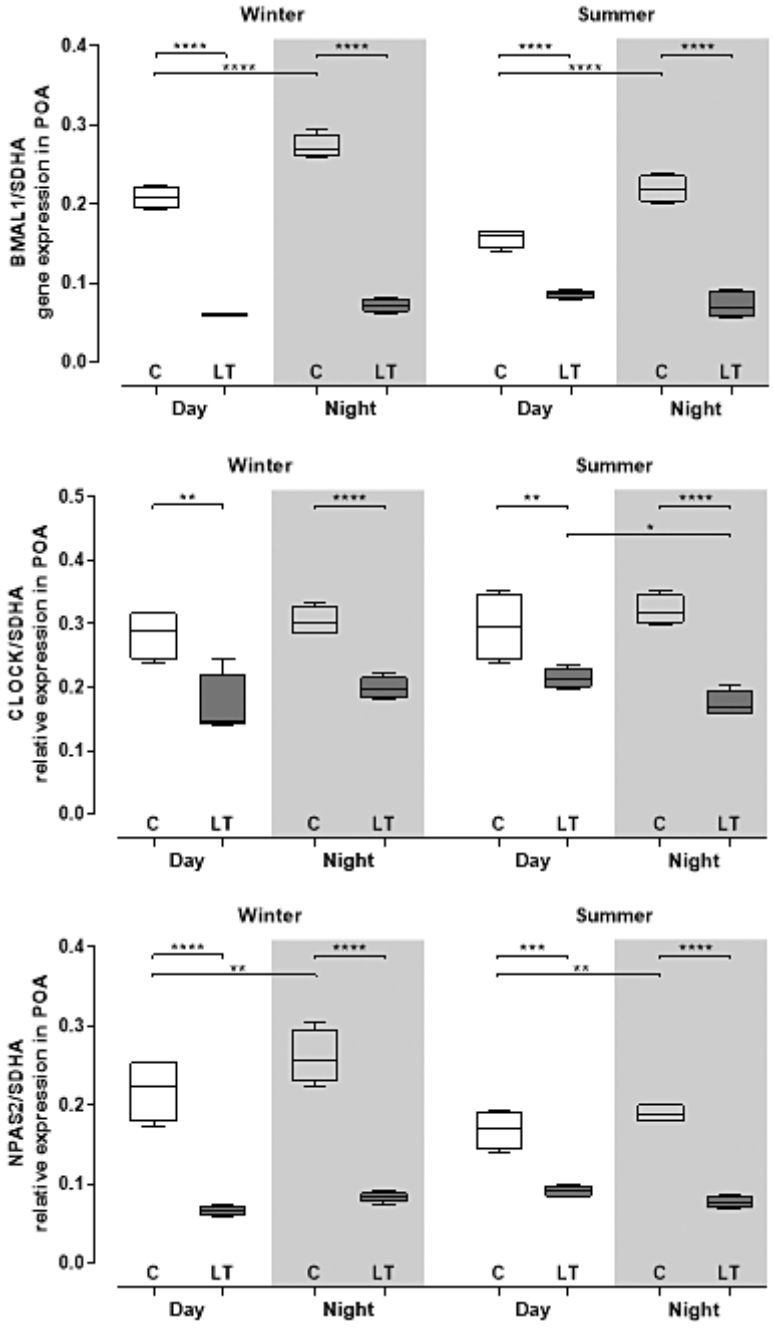

Fig. 2. Gene expression of biological clock transcription factors in POA (*Bmal1, Clock, Npas2*) in two examined groups; control (C) and light treated (LT) in two seasons, winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001).

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 LT than in the control group in both the summer and winter LT groups in, as well as during the day and at night. We observed a significant difference in the mRNA expression of *PER2* among LT group compared to the control group only in winter when the *PER* expression gene 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 a higher mRNA expression of these genes (p≤0.01) in winter in 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 that of the control group during in winter and summer during the day and at night. We also observed lower gene expression in the control groups

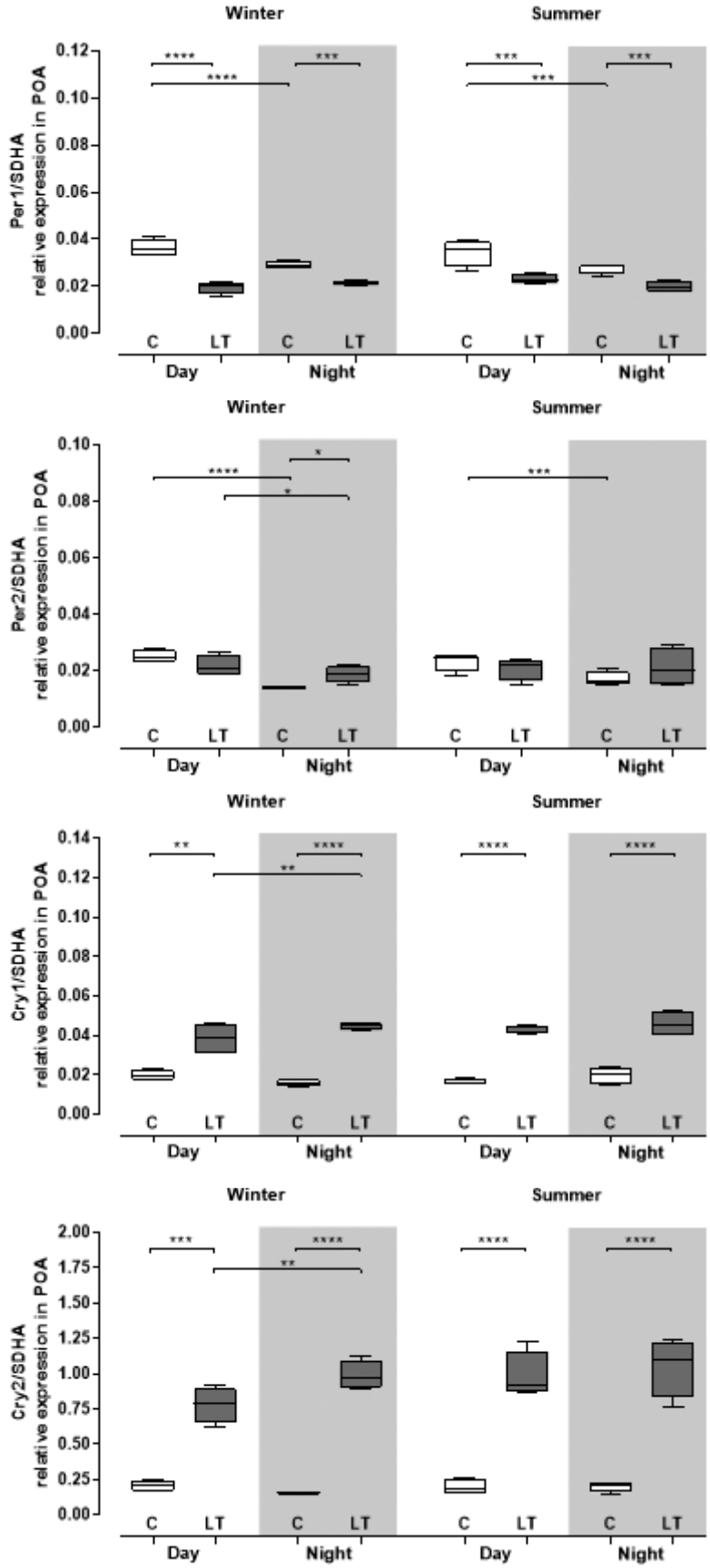

Fig. 3. Gene expression of biological clock genes in POA (*Per 1-2, Cry 1-2*) in two examined groups; control (C) and light treated (LT) in two seasons, winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001).

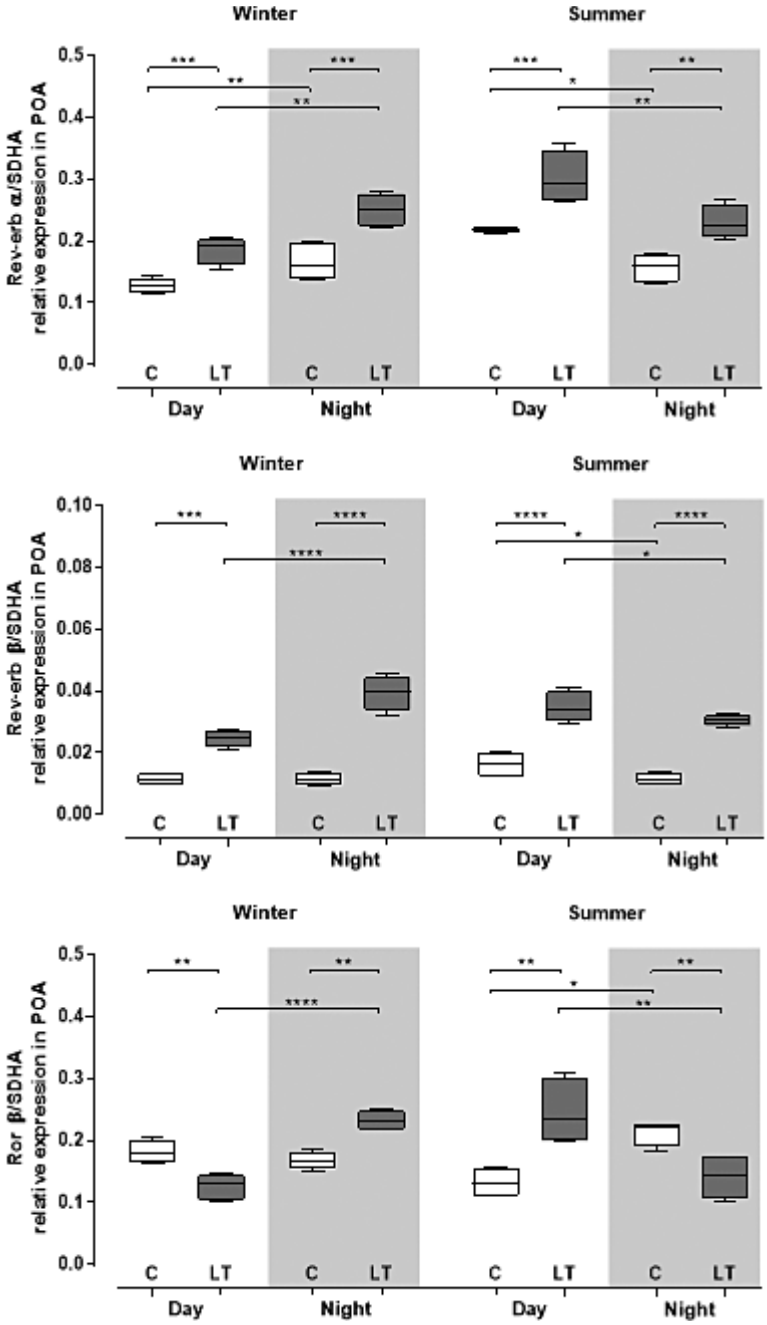

Fig. 4. Gene expression of biological clock genes in POA (*Rev-erb α-β and Ror β*) in two examined groups; control (C) and light treated (LT) in two seasons, winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001).

(P≤0.05) in summer at night than that during the day. The same relationship was observed concerning the LT group in summer. 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 LT groups than that of the control group during the day and higher at night (p≤0.01). On the other hand, this expression were 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 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 level was higher in the LT group than in the control group during the day and at night in

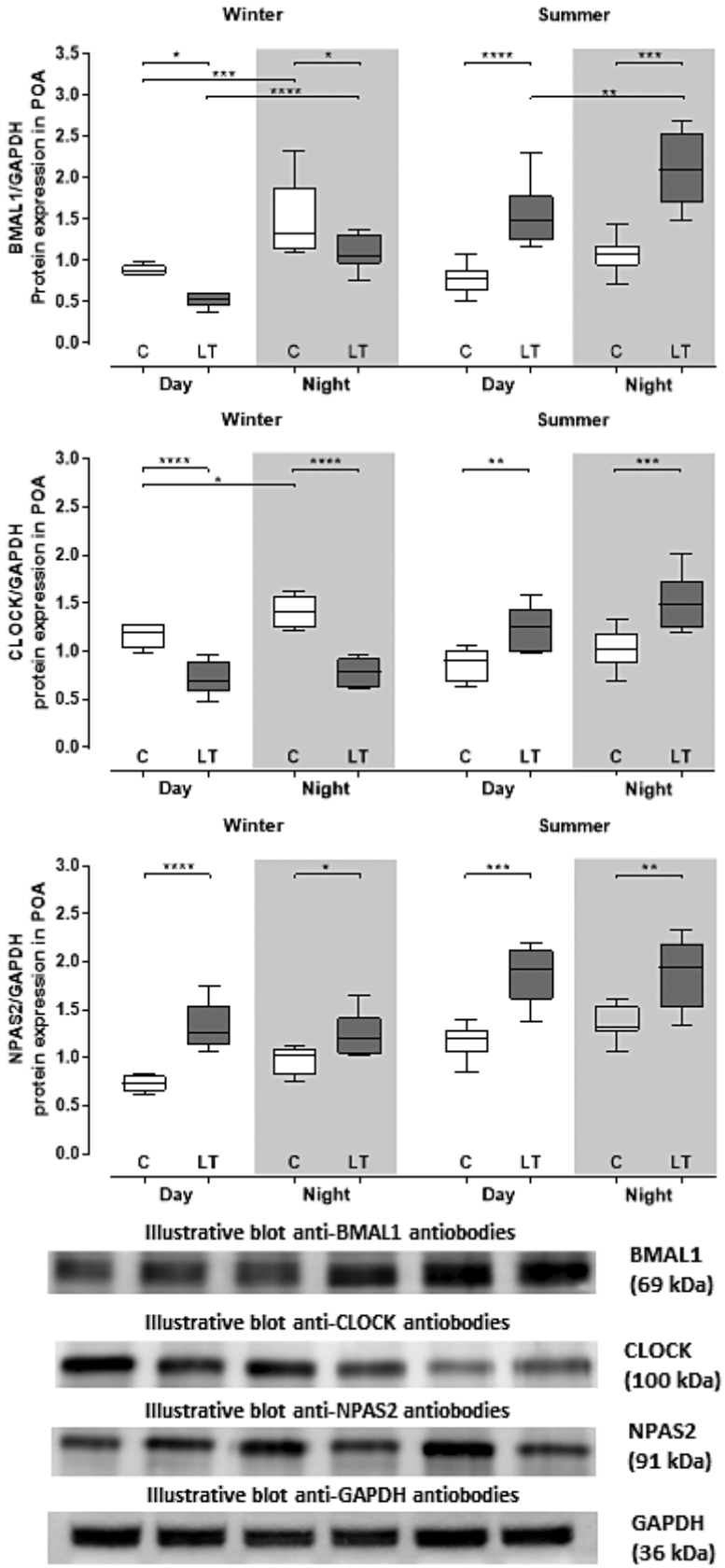

Fig. 5. Protein levels of biological clock transcription factors in POA (BMAL1, CLOCK, NPAS2) in two examined groups; control (C) and light treated (LT) in two seasons, winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

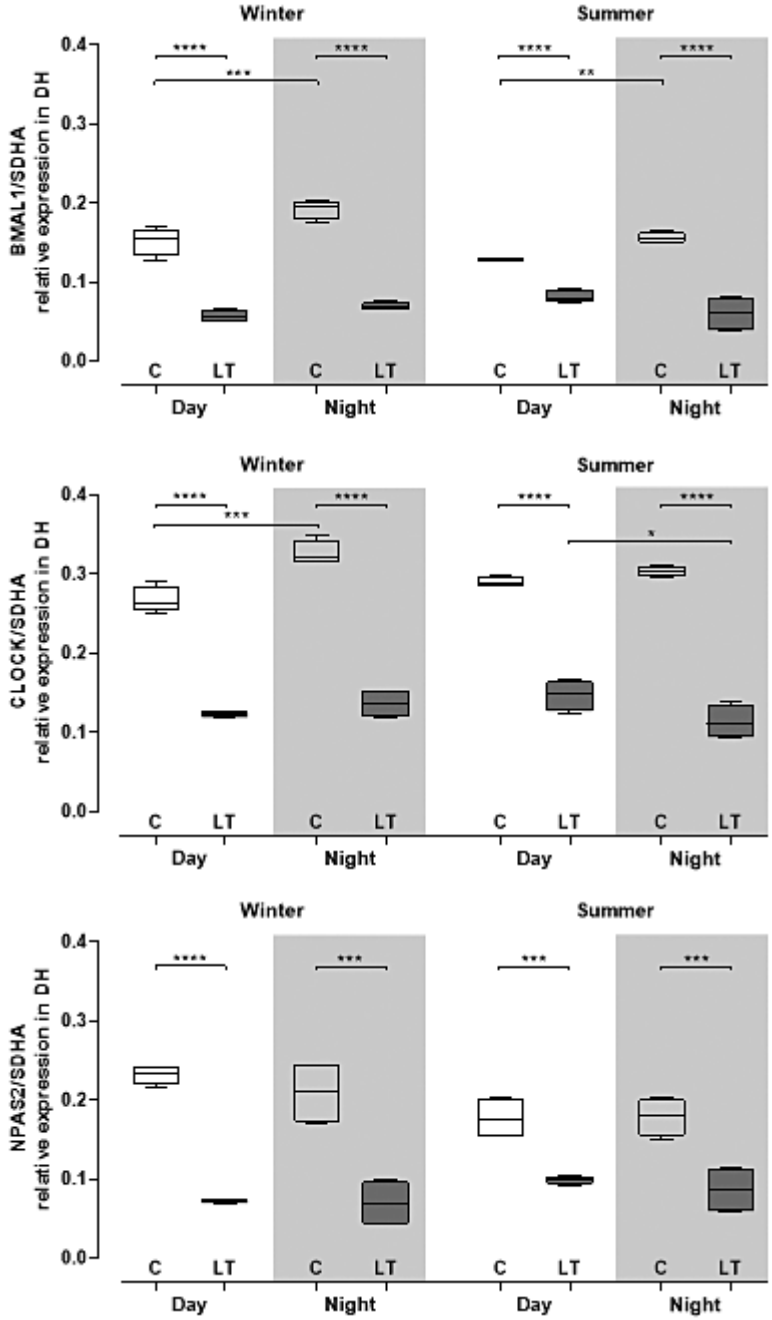

Fig. 6. Gene expression of biological clock transcription factors in DH (*Bmal1, Clock, Npas2*) in two examined groups; control (C) and light treated (LT) in two seasons, winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001).

winter (p≤0.05). Summer-time level of BMAL1, CLOCK and NPAS2 protein was observed to be higher in LT groups than in control groups, both during the day and at night (p≤0.001-0.0001). Winter-time BMAL1 and CLOCK level in control groups was higher at night than during the day (p≤0.05-0.001). BMAL1, CLOCK and NPAS2 level was at the same level in control groups during the day and at night in summer. NPAS2 protein level was higher in the LT group than in the control group in winter, in the day and night (p≤0.05-0.0001) (Fig. 5).

mRNA expression of clock genes and their TF's in dorsal hypothalamus (DA)

mRNA expression of clock specific TF's in DH

mRNA expression of *BMAL1, CLOCK and NPAS2* was lower in LT groups than in control groups during the day and at night, as well as in winter and summer. *BMAL1* expression in 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 nor winter and summer (Fig. 6).

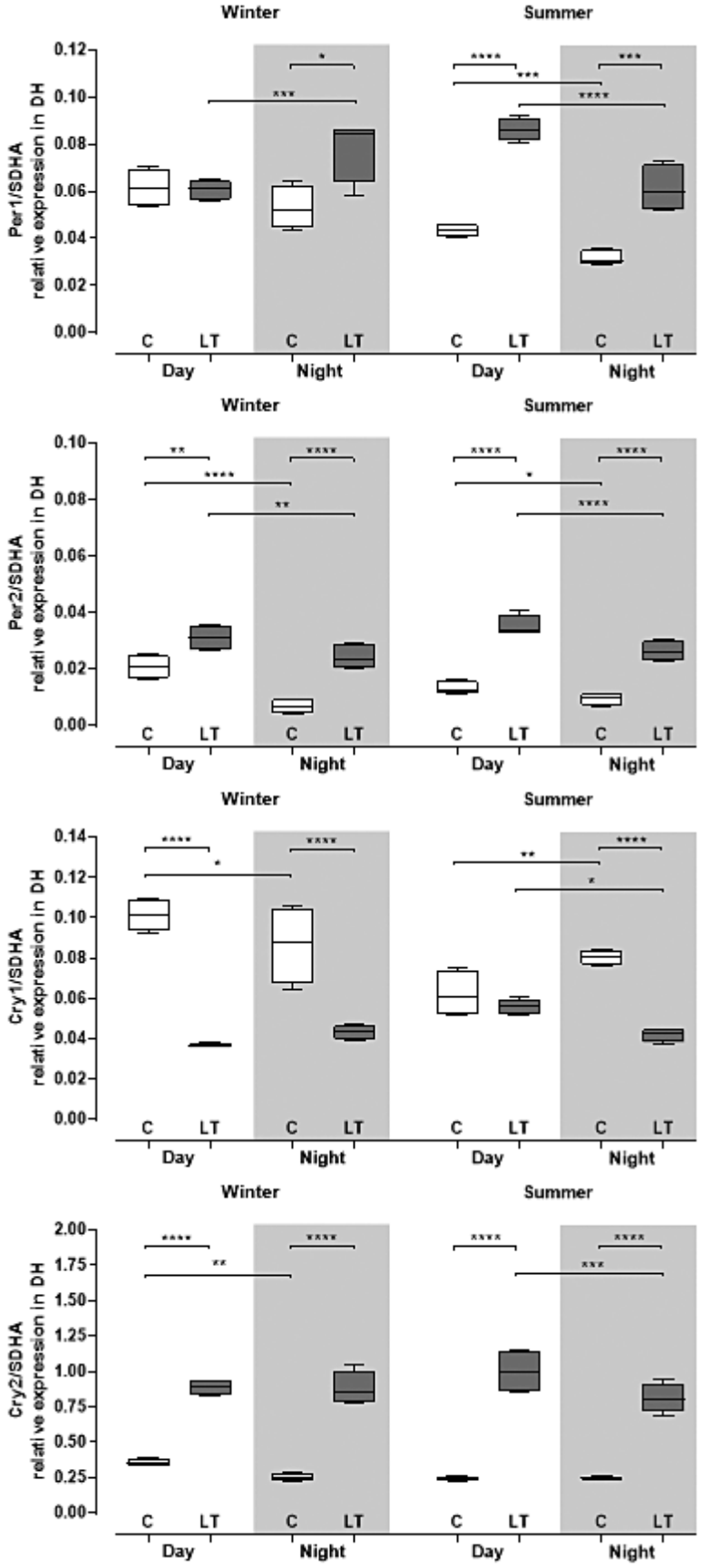

Fig. 7. Gene expression of biological clock genes in DH (*Per 1-2, Cry 1-2*) in two examined groups; control (C) and light treated (LT) in two seasons, winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001).

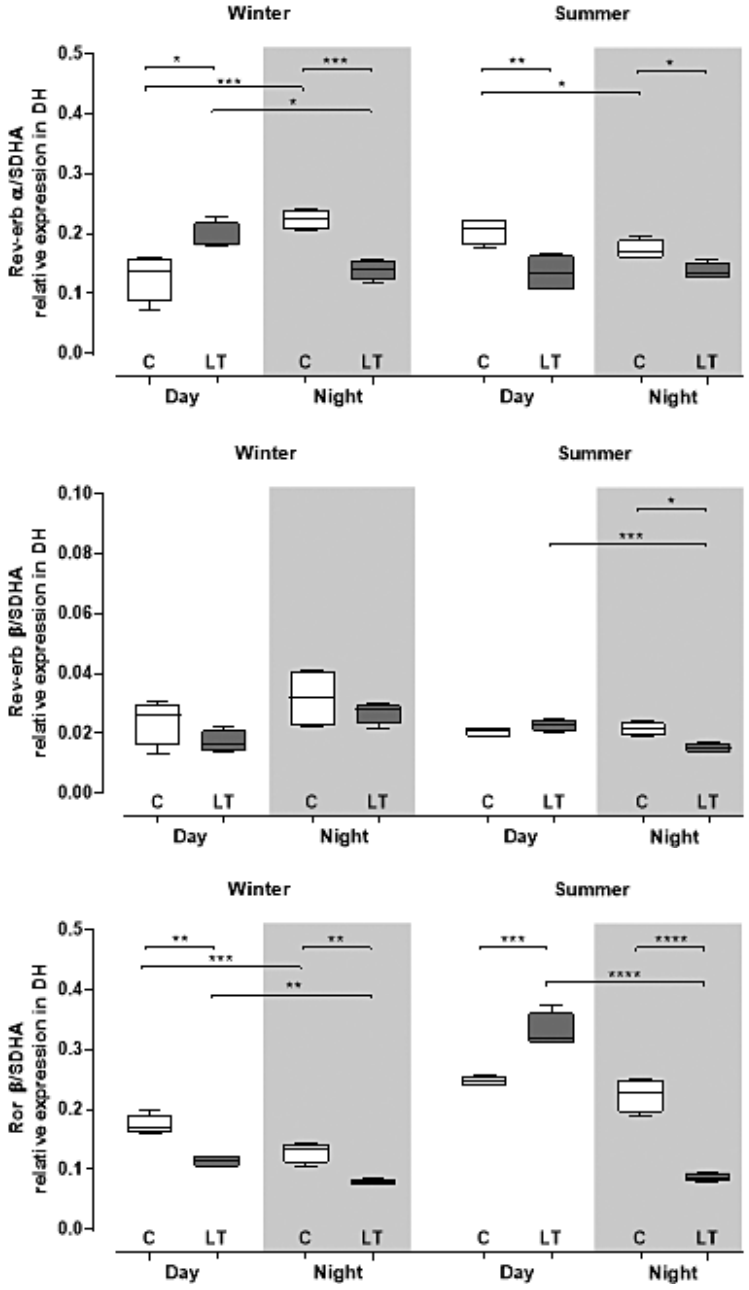

Fig. 8. Gene expression of biological clock genes in DH (*Rev-erb α-β and Ror β*) in two examined groups; control (C) and light treated (LT) in two seasons, winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001).

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

The mRNA expression of summer-time *PER1* or *PER2* was higher in LT groups than in control groups during the day and at night (p≤0.001-0.0001). We had the same results in winter except for day-time *PER1* expression when the levels were the same. The summer-time *PER1* and *PER2* expression between control groups was significantly (p≤0.05-0.001) lower at night than during the day, although we observed lower winter-time expression between 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 in the summer 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 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

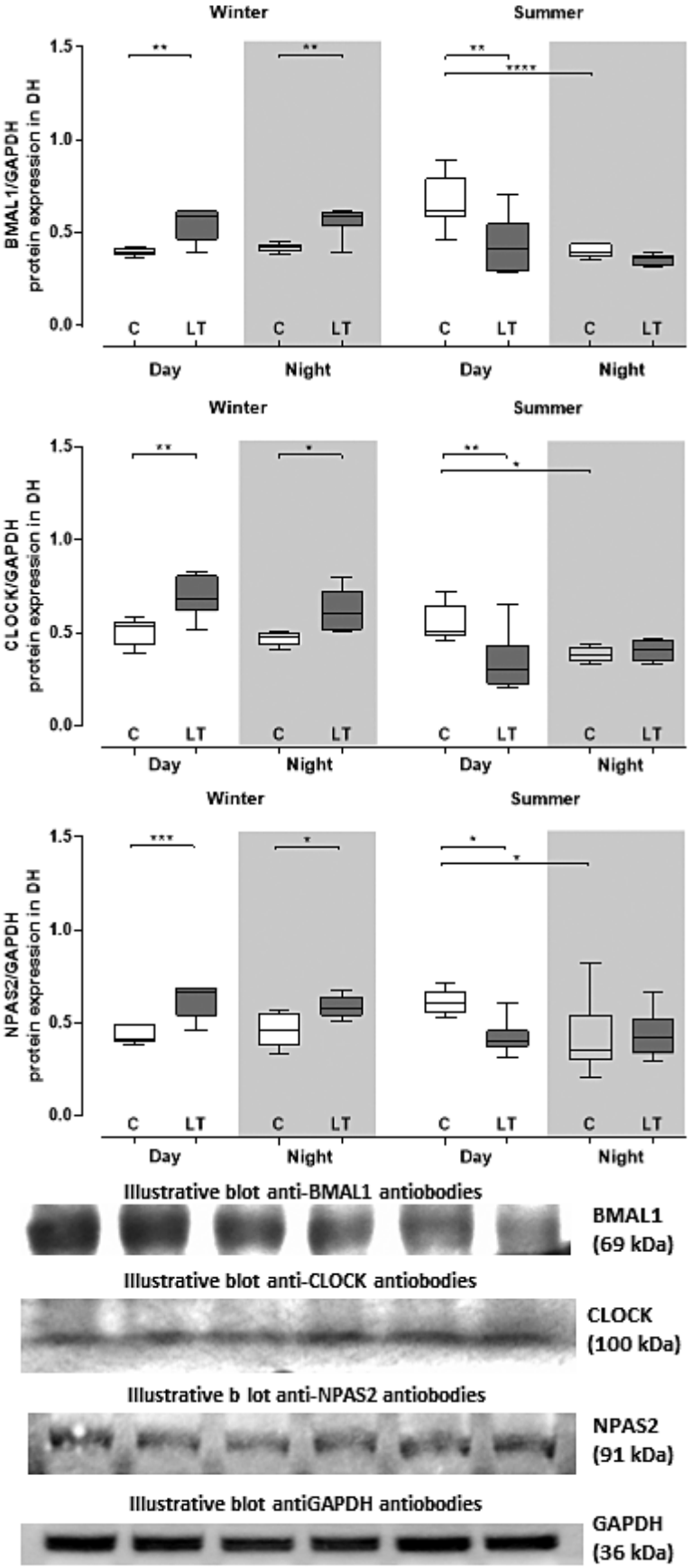

Fig. 9. Protein levels of biological clock transcription factors in DH (BMAL1, CLOCK, NPAS2) in two examined control (C) and light treated (LT) in two seasons, winter (SD) and summer (LD). Statistically significant results are marked with brackets and asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

at night in summer and at night in winter but higher than at day. *REV-ERB α* expression between control groups was lower during the day than at night ($p \leq 0.001$) in winter but lower at night in summer ($p \leq 0.05$) (Fig. 8).

REV-ERB β mRNA expression differed significantly only at night in summer when the expression in the LT group was lower than in the control group ($p \leq 0.05$) (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 ($p \leq 0.01$). However, lower expression was noted between these groups only at night in summer unlike during the day when higher expression was observed ($p \leq 0.001$) Fig. 8).

Protein level of clock specific transcription factors in DH

The protein level of BMAL1, CLOCK and NPAS2 was higher in the LT group than in the control group during the day and at night in winter ($p \leq 0.05$-0.001). In contrast to these results, the daytime level of BMAL1, CLOCK or NPAS2 was lower in summer ($p \leq 0.05$-0.01). We also noted that the protein level did not significantly differ at night in summer ($p \geq 0.05$) (Fig. 9).

Discussion

This study indicated the presence in the molecular elements of the biological clock of a domain capable of binding haem which, when combined with carbon monoxide gas, produces a biological effect. Additionally, carbon monoxide's possible participation 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 the diurnal and seasonal changes in environmental light (Koziorowski et al. 2012).

Analysis of the results showed that elevated endogenous CO levels through blood irradiation induces 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*, and *REV-ERB* α-β and *ROR* β are not the same for both POA and DH structures, indicating that each structures responds differently to the humoral signal received.

Additionally, the DH functioning (expression) pattern of clock elements should be considered as characteristic of the "peripheral clock" which tunes to the main pacemaker in POA. These factors are known to be elements of a positive regulatory loop which stimulates the expression of *PER* and *CRY* genes, the main clock components (Becker-Weimann et al. 2004). Under physiological conditions, their expression varies throughout the day (oscillation), particularly concerning the bmal1 gene (Becker-Weimann et al. 2004, Dardente et al. 2007). 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, but CO probably affected the PER2 protein. This protein has been shown to positively influence bmal1 expression and having PAS domains with a heme prosthetic group in its structure, reacts with carbon monoxide (Kaasik and Lee 2004, Schmutz et al. 2010, Airola et al. 2010).

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 data from cited literature (Shearman et al. 2000, Becker-Weimann et al. 2004, Yoo et al. 2004). 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 CLOCK:BMAL1 by displacing from promoters under physiological conditions (Chiou et al. 2016) but when PER proteins are blocked, the correct repressive complex may not form, displacement of 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 TFs 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* (Preitner et al. 2002, Teboul et al. 2009). However, silencing the expression of these nuclear receptors does not result in the disappearance of *BMAL1* and *PER* genes oscillations (Liu et al. 2008). In addition, such a situation would be unclear since NR1D1 also contain PAS domains and reacts with CO (Marvin et al. 2009, Pardee et al. 2009).

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/CLOCK dimer attached to the promoter. This occurs through the attachment of CRY-binding domains to CLOCK and also to BMAL1 (Parico et al. 2020). 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 to 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 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 it to be enhanced (Ukai-Tadenuma et al. 2011), 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 level of 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, since levels in winter are naturally three times lower than in summer when the length of the day and the intensity of additional light (Koziorowki et al. 2012) irradiation increases 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 has an influence at the level of translation. In summer, additional irradiation also increases the CO level about three times above the physiological level, and this amount 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 is down-regulated as above relative to 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 (Gilun et al. 2021). An increase in CO concentration over 1μM caused a breakdown of this complex (Gilles-Gonzales and Gonzales 2004). 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% of NO activity (Pardee et al. 2009). 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 (Pardee et al. 2009, Rey et al. 2011).

It is unclear why the expression of *PER* and *CRY* and NR1D genes increased when the expression level of transcription factors of these genes drastically decreased. Similarly, Zhang et al. (2009) and Baggs et al. (2009) had noticed an increase in the expression of *PER1, CRY1* and *CRY2* after *BMAL1* expression was silenced using siRNA. They suggest that the expression may be activation by *BMAL2* or the complexity of the post-translational 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 known

NR1D1 and 2) (Ikeda et al. 2019). Other authors demonstrate 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* (Okano et al. 2001), is also subject to transfection by the BMAL1/CLOCK dimer, and downregulation of *BMAL1* expression results in the downregulation of *BMAL2* expression (Shi et al. 2010). 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.

Conclusions

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.

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