JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2016, 67, 3, 431-442
www.jpp.krakow.pl

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CHANGES IN GONADOTROPIN-RELEASING HORMONE AND GONADOTROPIN-RELEASING HORMONE RECEPTOR GENE EXPRESSION AFTER AN INCREASE IN CARBON MONOXIDE CONCENTRATION IN THE CAVERNOUS SINUS OF MALE WILD BOAR AND PIG CROSSBREED

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Previous studies indicate that there are at least a few regulatory systems involved in photoperiodic synchronisation of reproductive activity, which starts with the retina and ends at the gonadotropin-releasing hormone (GnRH) pulse generator. Recently we have shown indicated that the amount of carbon monoxide (CO) released from the eye into the ophthalmic venous blood depends on the intensity of sunlight. The aim of this study was to test whether changes in the concentration of carbon monoxide in the ophthalmic venous blood may modulate reproductive activity, as measured by changes in GnRH and GnRH receptor gene expression. The animal model used was mature male swine crossbred from wild boars and domestic sows (n = 48). We conducted *in vivo* experiments to determine the effect of increased CO concentrations in the cavernous sinus of the mammalian perihypophyseal vascular complex on gene expression of GnRH and GnRH receptors as well as serum luteinizing hormone (LH) levels. The experiments were performed during long photoperiod days near the summer solstice (second half of June) and short photoperiod days near the winter solstice (second half of December). These crossbred swine demonstrated a seasonally-dependent marked variation in GnRH and GnRH receptor gene expression and systemic LH levels in response to changes in CO concentration in ophthalmic venous blood. These results seem to confirm the hypothesis of humoral phototransduction as a mechanism for some of bright light's effects in animal chronobiology and the effect of CO on GnRH and GnRH receptor gene expression.

Key words: *gonadotropin-releasing hormone, gonadotropin-releasing hormone receptor, luteinizing hormone, carbon monoxide, light, seasonal breeding*

INTRODUCTION

Photoperiod is the most reliable cue involved in regulation of reproduction in seasonal breeding animals (1, 2). Previous studies indicate that in photoperiodic synchronisation of reproductive activity there are some elements of pathway that starts with the retina and end at the gonadotropin-releasing hormone (GnRH) pulse generator (3, 4).

The GnRH neurons are distributed widely in the forebrain but most are found in the anterior hypothalamus and preoptic area. They terminate in the median eminence where GnRH enters the pituitary portal system and regulates the secretion of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Despite being widespread in the forebrain, the GnRH neurons are able to act in a synchronised manner, generating distinct pulses in GnRH release. In seasonal breeding animals the pulsatile GnRH and LH secretion is highly sensitive to the negative feedback effect of oestradiol. During the transition to anoestrus, GnRH and LH pulse frequencies decrease markedly (5-7). Recently it has been shown that gaseous molecules such as nitric oxide (NO) and carbon monoxide (CO) are involved in the regulation of reproductive processes (8-10). These molecules easily penetrate through cell membranes and participate in biologic processes. Gasotransmitters are produced endogenously. Carbon monoxide is formed during heme degradation *via* the enzyme heme oxygenase (HO); during this reaction, ferrous iron and biliverdin are generated in equimolar amounts to CO (11-13). Heme oxygenase exists as constitutive (HO-2, HO-3), and inducible (HO-1), isoforms, the latter of which responds to regulation by multiple stress stimuli (14-17). Carbon monoxide is produced in large quantities in areas of the hypothalamus involved in the central regulation of reproduction, including the ventromedial (VM) nucleus, paraventricular nucleus (PVN) and preoptic area (POA) (11, 18). Lamar *et al.* (19) demonstrated that CO may function as a neurotransmitter in the regulation of hypothalamic GnRH release (19).

Most seasonally breeding mammals have been shown to possess an endogenous rhythm of seasonal reproductive activity, which is either synchronised or entrained by photoperiod (20, 21). Daylight is perceived by the retina and neural information is transmitted to a biological clock that measures day length using a circadian mechanism (22).

In 1996, Oren published a hypothesis on humoral phototransduction, as an alternative or complementary explanation for the propagation of a light signal. He postulated that bilirubin, biliverdin, melatonin and/or photons may act as a signal of light *via* blood circulation (23). Such a model suggests a physiologic function for light's known capacities to stimulate haem-based enzymes to form reactive gases. These gases can then act upon the eye because of the special optical properties and the good vascularisation of the retina. In the retina and red blood cells, bright light stimulates heme oxygenase to produce CO and nitric oxide synthase to produce NO (24, 25). In the eye, CO is secreted into the ophthalmic venous blood and the intensity of CO secretion during the summertime is dependent on the light phase and nocturnal phase of the day; the amount of CO released from the eye into venous blood during summer days is several times higher than that during summer nights (and also higher than both winter days and nights) (26). Carbon monoxide and NO can be transported from the eye in the venous blood. Barone *et al.* were shown that blilirubin, which is formed during the processes leading to CO release, bind NO forming N-nitro-bilirubin (27). Haemoglobin participates in signalling and control of processes in which NO and CO are involved; NO binds to the haemoglobin molecule by the thiol group and forms S-nitrosohemoglobin (28, 29). S-nitrosohemoglobin, present in blood cells, becomes a carrier of CO, and is transported *via* ophthalmic venous blood into the cavernous sinus. Countercurrent substance exchange transfer into the arterial blood in the cavernous sinus could allow CO to reach the microvasculature of the brain and thus influence the neural centres (30-33).

Koziorowski *et al.* research conducted on the same animal model as was used in this study was show that during the summer days the concentration of CO in ophthalmic venous blood flowing out from the eye and collected from ophthalmic sinus was significantly higher compared to samples collected in parallel from control nasal venous blood. During the nocturnal phase, CO concentration in ophthalmic venous blood was approximately three times lower than the light phase of the day and did not differ from the CO concentrations in systemic arterial or venous blood or from nasal venous blood. During the winter CO concentration in OphVB collected during the day and night was almost three times lower than during the summer (26). The seasonal variation in CO concentration in brain regions involved in the central regulation of reproduction (26) suggests that CO may participate in regulation of seasonal breeding.

The aim of this study was to investigate the role of CO in the seasonal modulation of reproductive activity. To explore this potential mechanism, we conducted *in vivo* experiments to determine the effect of CO on serum LH levels and gene expression of GnRH and the gonadotropin-releasing hormone receptor (GnRH-R). Carbon monoxide was introduced *via* two experimental methods imitating the increase in CO concentration in the cavernous sinus of the mammalian perihypophyseal vascular complex. In the first experiment, blood plasma supplemented with chromatographically pure CO was infused into the ophthalmic venous sinus, and in the second experiment, blood with an increased CO concentration achieved by bright light exposure was infused. The concentration of CO in venous ophthalmic blood in the study animals was approximately three times higher compared with the control animals. The animal model we used was mature male swine crossbred from a wild boar (*Sus scrofa*) and a domestic sow (*Sus scrofa domestica*). The breeding season of the European wild boar occurs in early winter with piglets born once a year in late spring (34). Despite being capable of producing piglets throughout the year, the domestic sow shows a reduction in fertility in late summer and early autumn (34-36). The hybrid inherits strong seasonal behavioural and physiologic changes from the boar and relative docility from the pig, creating a workable model for studies of seasonality. The experiments were performed during long photoperiod days near the summer solstice (second half of June) and short photoperiod days near winter solstice (second half of December).

MATERIALS AND METHODS

Animals

All the procedures were carried out in compliance with Polish legal regulations (Act of January 21, 2005) that determine the terms and conditions for performing experiments on animals. The experimental conditions were approved by the Local Ethics Committee on Animal Experimentation in Lublin No. 8/2007.

Mature male crossbred swine (from a wild boar and domestic pig cross) (age 12 months, body mass ~100 – 120 kg) from the Experimental Farm Branch Campus of the Faculty of Biotechnology, University of Rzeszow, Kolbuszowa, Poland, were used. Condition of health and welfare of the animals was monitored daily by the researchers and animal care staff, and the veterinarian. Animals were kept under natural illumination, assigned to individual pens for accommodation for one week before experimental treatment, fed *ad libitum* and had free access to water.

During late June, the animals were maintained in an open-sided shed and exposed ad libitum to approximately 30,000 lx of daytime natural illumination. The mean ambient temperature was 24°C during the light phase and 12°C during the nocturnal phase. During late December, the animals were housed in a windowed room and exposed to between 40 and 50 lx of daytime natural illumination. Mean temperature during the day and night was 12°C.

Experimental schedule

Experiments were conducted according to previously described methods and procedures (67). Forty eight animals were randomly assigned to one of two experimental groups.

Experiment 1. Animals received autologous blood plasma infused into the ophthalmic venous sinus (OphVS) for 48 h at a rate of 8.3ml/h with an experimentally induced increase in CO concentration (see below). The identical procedure was performed during the longest days of summer (June; n = 6) and during the shortest days of winter (December; n = 6). The control group in this experiment were animals kept in natural photoperiodic conditions, one group (n = 6) during June and another (n = 6) during December.

Preparation and infusion of autologous plasma with elevated concentrations of carbon monoxide

Systemic venous blood was repeatedly collected under sterile conditions from each animal. The heparinized blood (heparin 10 IU/ml, Polfa, Poland) was centrifuged (1000 g, 20 min) and 50 ml plasma was transferred to a sealed glass container. Plasma concentration of CO was estimated (37). The average concentration was 1.2 nmol/ml and 0.9 nmol/ml in June and December, respectively. Plasma was supplemented

with chromatographically pure CO (0.8 cm^3 to each portion) and stirred with a roller for 30 min, and the concentration of CO was measured again. The autologous plasma, with the increased concentration of CO, up to 4.5 nmol/ml in June and 3.1 nmol/ml in December, was infused at a rate of 8.3 ml/h with the use of a pump (SEP 21S, Ascor, Poland) for 48 h into the ophthalmic sinus (OphS), from which the venous blood flowed into the venous cavernous sinus (VCS) of the perihypophyseal vascular complex (PVC). The autologous blood cells remaining after the collection of plasma were mixed with Ringer's solution in a volume equivalent to the collected plasma. The suspension was stirred with a roller for 30 min and then continuously infused into the external jugular vein (JV) as a protection against anaemia.

Experiment 2. The animals received autologous blood infused into the ophthalmic venous sinus for 48 h with an experimentally induced increase in the CO concentration, achieved by 2 h of bright light exposure (see below). This experimental treatment was repeated during the longest summer days (June; n = 6) and during the shortest winter days (December; n = 6). The control group in this experiment were animals kept in natural photoperiodic conditions, one group (n = 6) during June and another (n = 6) during December. The animals received autologous blood infused into the OphVS for 48 h at a rate of 8.3 ml/h.

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 condition from each animal and heparinised. Concentration of CO in blood was estimated using a standard addition method (26). The average concentration was 1.51 nmol/ml and 0.89 nmol/ml in June and December, respectively. This autologous blood was pumped at 8.8 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-T8 Standard). 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. 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 2 hours of bright light exposure the concentration of CO was measured again. The 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 rate of 8.3 ml/h with use of a pump (SEP 21S, Ascor, Poland) for 48 h into the OphVS.

Surgical procedures and blood sample collection

All solution infusions and blood sample collections were done by catheterisation. The animals were fasted for 12 h before surgery. They were pre-medicated with 0.05 mg/kg atropine (Biowet, Gorzow Wielkopolski, Poland) injected intramuscularly (IM) followed 10 min later by 2 mg/kg azaperone IM (Janssen Pharmaceutica, Beerse, Belgium). Once sedation occurred, anaesthesia was induced by intravenous administration of 10 mg/kg thiopental (Sandoz GmbH) into an ear vein. Silastic catheters (o.d., 2.4 mm; i.d., 1.8 mm) were inserted: (1) into the external jugular vein for thiopental administration to maintain a deep level of anaesthesia during surgery and for collection of systemic blood samples during the experimental procedure for analysis of luteinizing hormone (LH); (2) into the dorsal nasal vein in a cephalic direction through the angular vein of the eye to reach the OphS, from which the venous blood flowed into the VCS of the PVC, for infusion of autologous blood or autologous blood plasma. Catheters were fixed to the skin on the back of the animals to allow for blood sample collection with minimal animal stress. Once conscious after the surgical procedure, animals were housed in pens where they had free access to food and water. Antibiotics (Pen-Strep; ScanVet, Poland) and analgesics (Pyralgivet; Vet-Agro, Poland) were administered postoperatively. No unexpected death occurred during this study.

All animals were sampled at 2-hour intervals for 48 hours. Blood samples (10 ml) were obtained *via* external jugular venous catheters and collected into heparinised tubes. Samples were immediately centrifuged at room temperature and blood plasma was decanted and stored at –20°C until analysed for LH content. To facilitate sampling during the night, dim red penlights were used only during passing through the barn, and direct light exposure of the animals' eyes was avoided.

Hypothalamus and pituitary gland tissue

In order to analyse the effect of CO on GnRH and GnRH-R mRNA levels during the light and dark periods, animals in each group were sacrificed at midday or midnight after 48 hours infusion with the experimentally induced increased concentration of CO. The animals were sacrificed by electrical stunning and exsanguinations. Then the head was immediately cut and the skull was opened. The brains were removed from the skull. Tissue from the anterior pituitary gland was taken for GnRH-R mRNA analysis. Regions of brain encompassing the preoptic area/hypothalamus were sectioned sagittally and dissected from both sides into three parts: the preoptic area, anterior hypothalamus, and ventromedial hypothalamus, according to the stereotaxic atlas of the pig brain (38) for GnRH mRNA. Tissues were immediately dissected and frozen (in liquid nitrogen) prior to RNA isolation.

Hormone analyses

The plasma LH concentration was assayed by a double-antibody radioimmunoassay according to Ziecik *et al.* using rabbit antibodies against porcine LH conjugated with ovalbumin (39). The sensitivity of the assay and intra-assay coefficient of variation was 0.07 ng/ml and 9 %, respectively. Serum LH concentrations were transformed into the area under the curve (AUC).

Total RNA extraction and reverse transcription

Total RNA was extracted from samples (anterior pituitary gland, preoptic area of hypothalamus, anterior hypothalamus, ventromedial hypothalamus) using the TRI Reagent (Invitrogen) procedure (40), and precipitated with ethanol. Each RNA pellet was dissolved in RNase-free water and the quantity and quality of RNA were assessed spectrophotometrically at 260 and 260/280 nm, respectively (NanoDrop, Thermo Scientific). RNA integrity was electrophoretically verified in a 1.5% agarose gel stained with ethidium bromide.

For elimination of probe contamination by genomic DNA, the total RNA was treated with RNase-free DNase 1 (Sigma-Aldrich). One microgramme of RNA was treated with 1U of DNase 1 for 15 min at room temperature. The reaction was stopped by addition of stop solution and DNase was inactivated at 70°C for 10 min. Extracted RNA (1 µg) was reverse-

transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Real time RT-PCR

Messenger RNA (mRNA) levels of GnRH and GnRH-R were determined by real-time quantitative PCR using the TaqMan assay-based real-time PCR performed using TaqMan Gene Expression Assays (GnRH-1 Ss03394548_m1; GnRH-R Ss03394546_m1; GAPDH Ss03375435; Applied Biosystems). The results for the genes were analysed on the StepOnePlus System (Applied Biosystems) and normalised against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (41). Relative gene expression was expressed in arbitrary units.

Statistical analysis

Data were submitted to analysis of variance (ANOVA) and Bonferroni post-test for paired comparison of means, where appropriate. Statistics were calculated using Prism 5

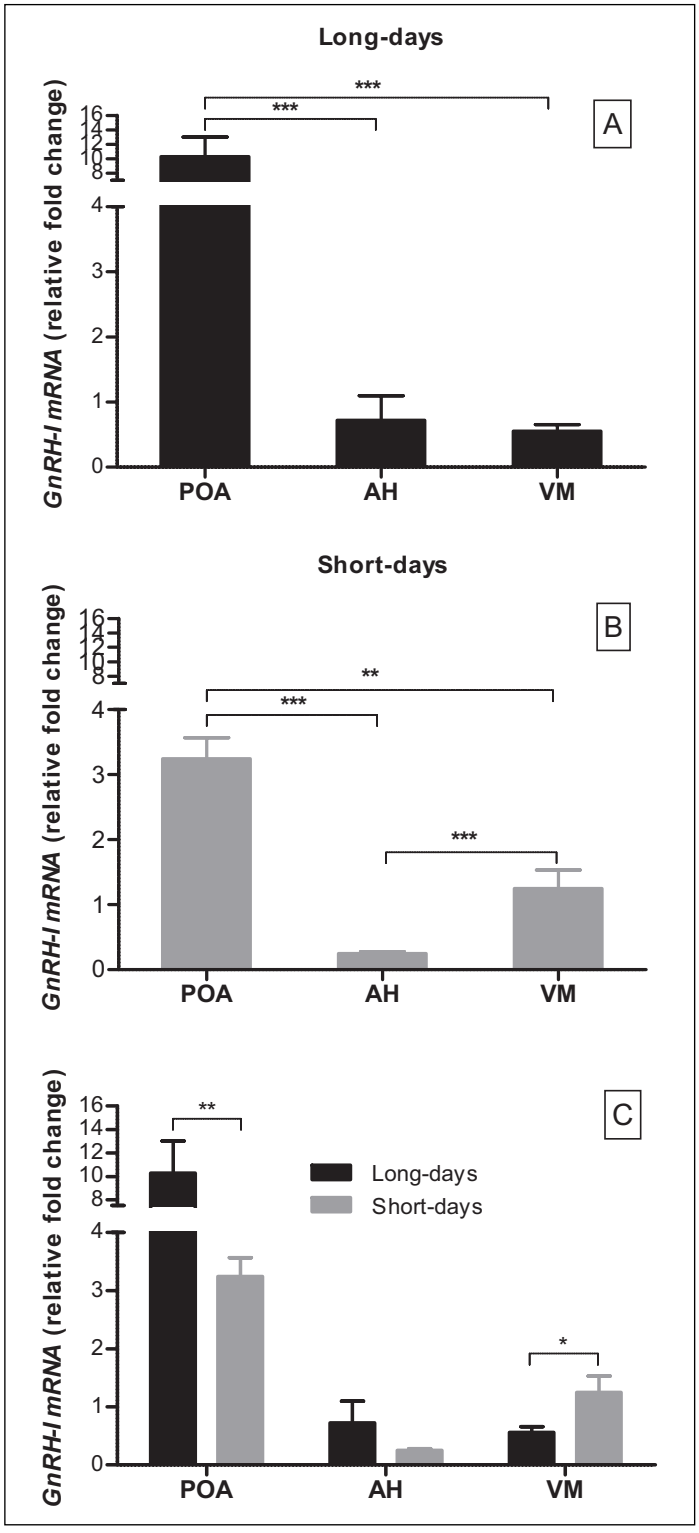

Fig. 1. GnRH-I mRNA concentration in the preoptic area (POA), anterior hypothalamus (AH) and ventromedial hypothalamus (VM) of control animals during the long (n = 12) [A, C] and short (n = 12) [B, C] days, mean ± S.E.M., *P ≤ 0.05; **P ≤0.01; ***P ≤ 0.001.

(GraphPad). Each assay was run in triplicate; each experiment was repeated at least once. P values less than 0.05 were considered statistically significant. Values are mean ± S.E.M.

RESULTS

The effects of autologous blood plasma or autologous blood with increased carbon monoxide concentration infused into the ophthalmic venous sinus on GnRH-I mRNA in the preoptic area, anterior hypothalamus and ventromedial hypothalamus

Gonadotropin-releasing hormone mRNA was found in structures throughout the preoptic area (POA), anterior (AH) and ventromedial (VM) hypothalamus. The highest concentration of GnRH mRNA was found in the POA (*Fig. 1*). The GnRH-I mRNA level in the POA during the long days (LD) was significantly higher ($P \leq 0.001$) than in the AH and VM (*Fig. 1A*). Also during the short days (SD) the concentration of GnRH-I mRNA in the POA was significantly higher ($P \leq 0.001$) than in the AH and VM (*Fig. 1B*). The concentration of GnRH-I mRNA in the hypothalamic tissue differed significantly depending on length of the day (summer/winter). In the POA during SD the GnRH-I mRNA level was significantly decreased ($P \leq 0.01$) as compared with LD. In the VM, the GnRH-I mRNA concentration was significantly higher during SD than during LD ($P \leq 0.05$) (*Fig. 1C*).

During LD, in experiment 2 animals the concentration of GnRH-I mRNA in the AH was significantly decreased ($P \leq 0.05$) as compared with the control. In the VM, the concentration of GnRH-I mRNA was also significantly increased ($P \leq 0.001$) in experiment 1 and experiment 2 animals as compared with the control (*Fig. 2*).

During SD, the concentrations of GnRH-I mRNA in the POA and VM were significantly higher in experiment 1 animals and experiment 2 animals as compared with the control. In the AH, the concentration of GnRH-I mRNA was significantly ($P \leq 0.01$) increased only in experiment 2 animals as compared with the control (*Fig. 3*).

The effects of autologous blood plasma or autologous blood with increased carbon monoxide concentration infused into the ophthalmic venous sinus on GnRH-R mRNA in the hypothalamus and in the anterior pituitary gland

Gonadotropin-releasing hormone receptor mRNA was found in structures throughout the POA, AH and VM, and in the anterior pituitary gland (AP) (*Fig. 4*). Only during SD was the GnRH-R gene expressed at different levels in the analysed tissues of control animals; the highest concentration of GnRH-R mRNA was found in the AP. In the AH, the GnRH-R mRNA concentration was significantly higher than in the POA and VM (*Fig. 4B*). In the AP, the amount of GnRH-R mRNA varied with

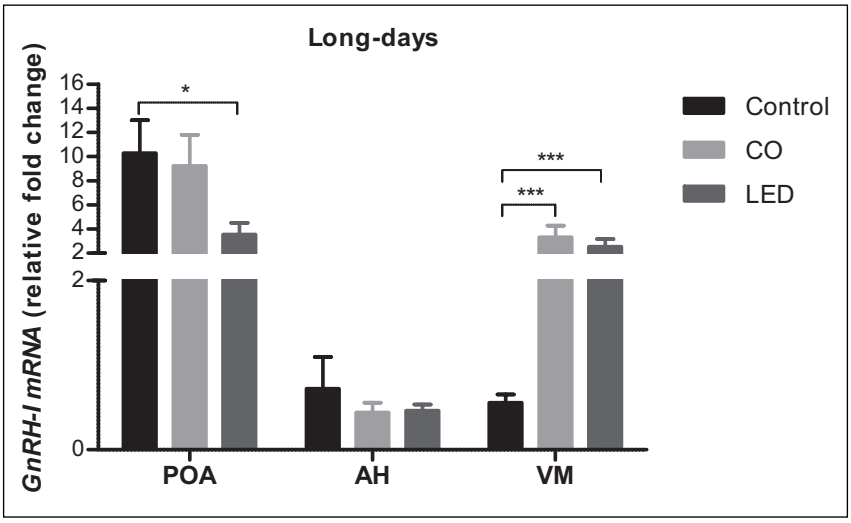

Fig. 2. Changes in GnRH-I mRNA concentration in the preoptic area (POA), anterior hypothalamus (AH) and ventromedial hypothalamus (VM) in the control (n = 6), CO (n = 6) and LED (n = 6) groups animals, during the long days, mean ± S.E.M., *P ≤ 0.05; ***P ≤ 0.001.

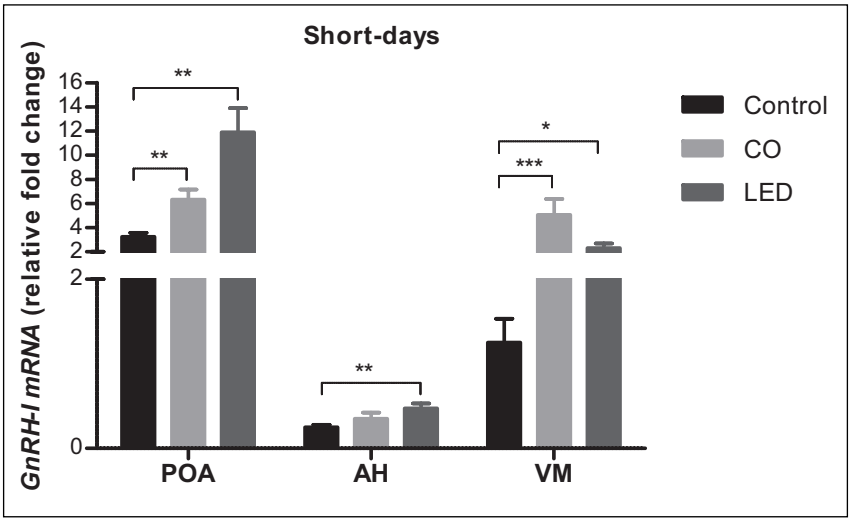

Fig. 3. Changes in GnRH-I mRNA concentration in the preoptic area (POA), anterior hypothalamus (AH) and ventromedial hypothalamus (VM) in the control (n = 6), CO (n = 6) and LED (n = 6) groups animals, during the short days, mean ± S.E.M., *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

the seasons. During SD, GnRH-R mRNA concentration in the AP was higher than during LD (*Fig. 4C*).

During LD, marked augmentation of mRNA encoding GnRH-R was detected in the AP of experiment 1 animals and experiment 2 animals as compared with the control (*Fig. 5*). During SD, the concentration of GnRH-R mRNA in the AP was decreased in experiment 1 animals and experiment 2 animals as compared with the control. Also in the POA, the concentration of GnRH-R mRNA was significantly decreased ($P \leq 0.01$) after infusion of autologous blood plasma with increased CO concentration (experiment 1) as compared with the control (*Fig. 6*).

The effects of autologous blood plasma or autologous blood with increased carbon monoxide concentration infused into the ophthalmic venous sinus on luteinizing hormone secretion

Mean serum LH concentration in the control animals during SD was approximately three times higher ($P \leq 0.01$) than during LD. During LD, blood plasma LH concentration in experiment 1 animals was increased over 23 times ($P \leq 0.01$) when compared with the control group. Experiment 2 animals had an approximately 6-fold increase ($P \leq 0.01$) in LH concentration compared to the control (*Fig. 7A*). During SD, blood plasma LH

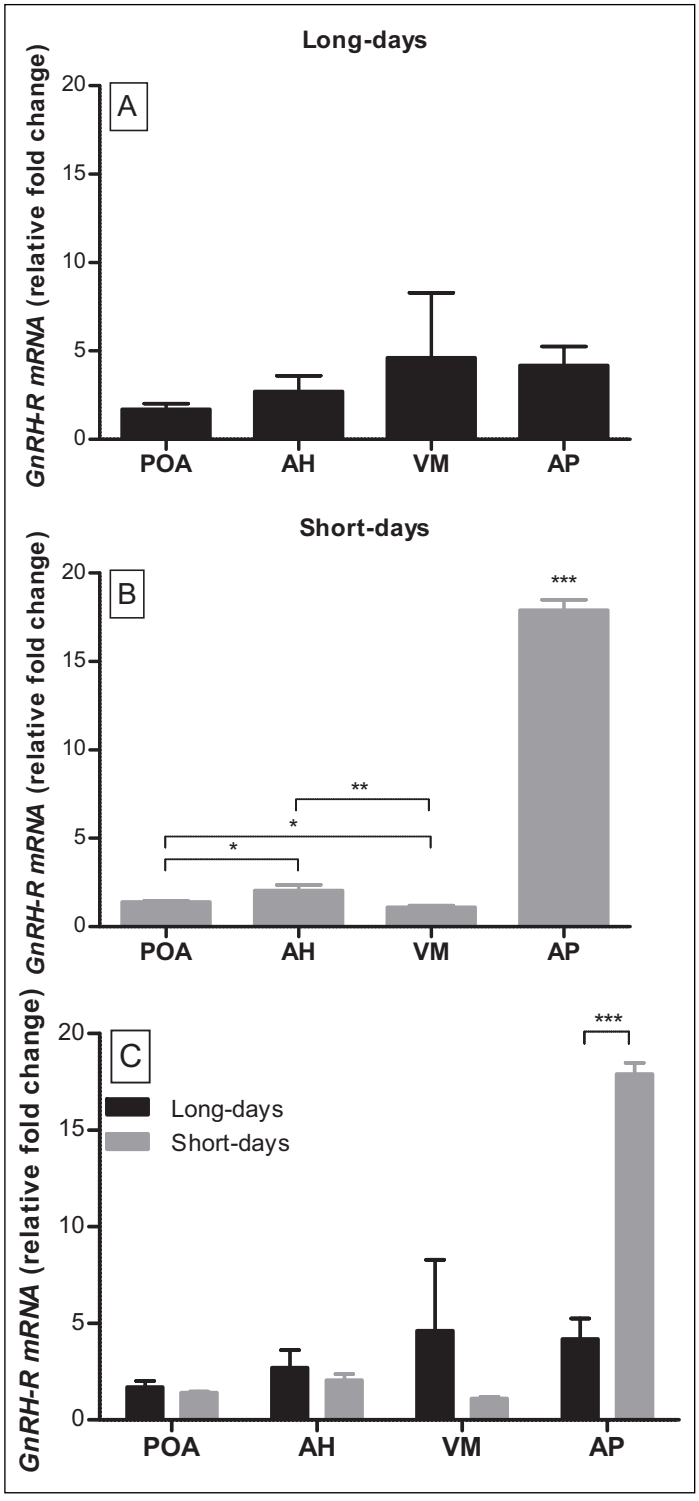

Fig. 4. GnRH-R mRNA concentration in the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VM) and anterior pituitary gland (AP) of control animals during the long (n = 12) ; [A, C] and short (n = 12) [B, C] days, mean ± S.E.M., *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

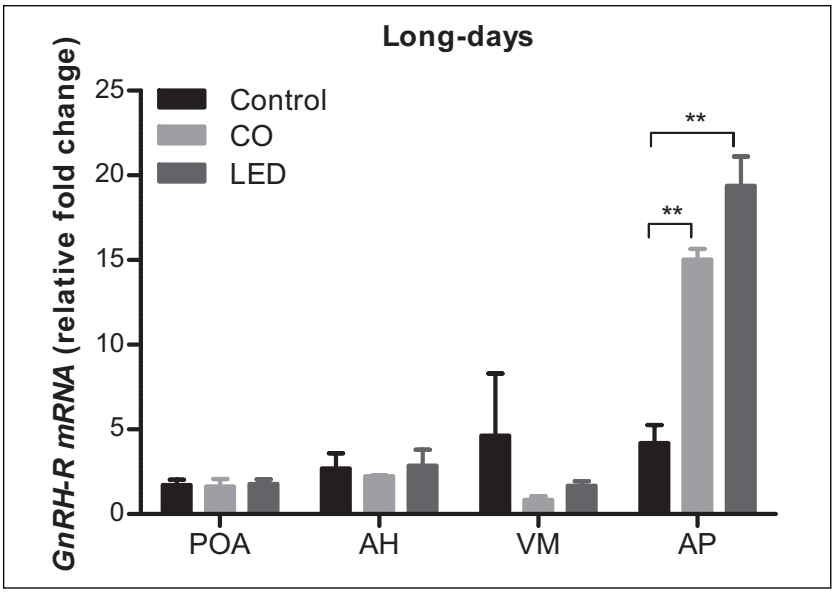

Fig. 5. Changes in GnRH-R mRNA concentration in the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VM) and anterior pituitary gland (AP) in the control (n = 6), CO (n = 6) and LED (n = 6) groups animals, during the long days, mean ± S.E.M., **P ≤ 0.01.

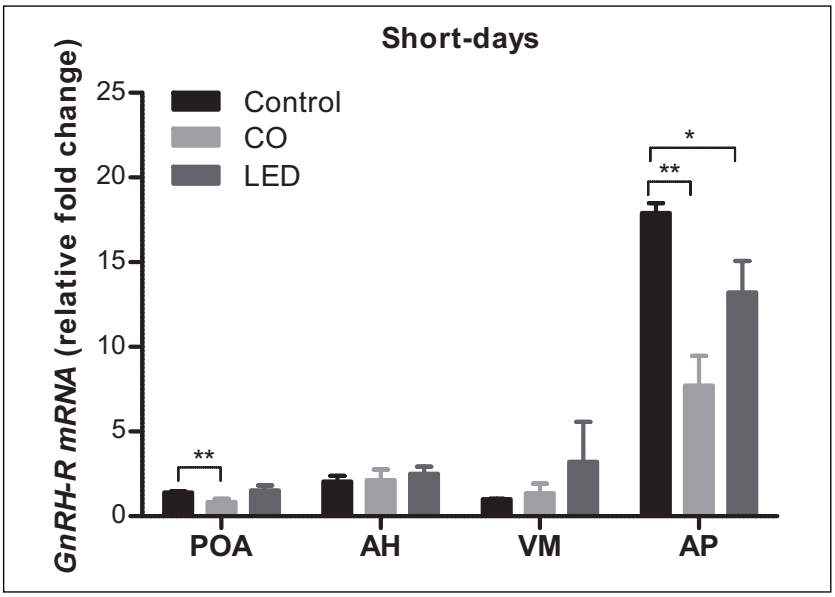

Fig. 6. Changes in GnRH-R mRNA concentration in the preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VM) and anterior pituitary gland (AP) in the control (n = 6), CO (n = 6) and LED (n = 6) groups animals, during the short days, mean ± S.E.M., *P ≤ 0.05; **P ≤ 0.01.

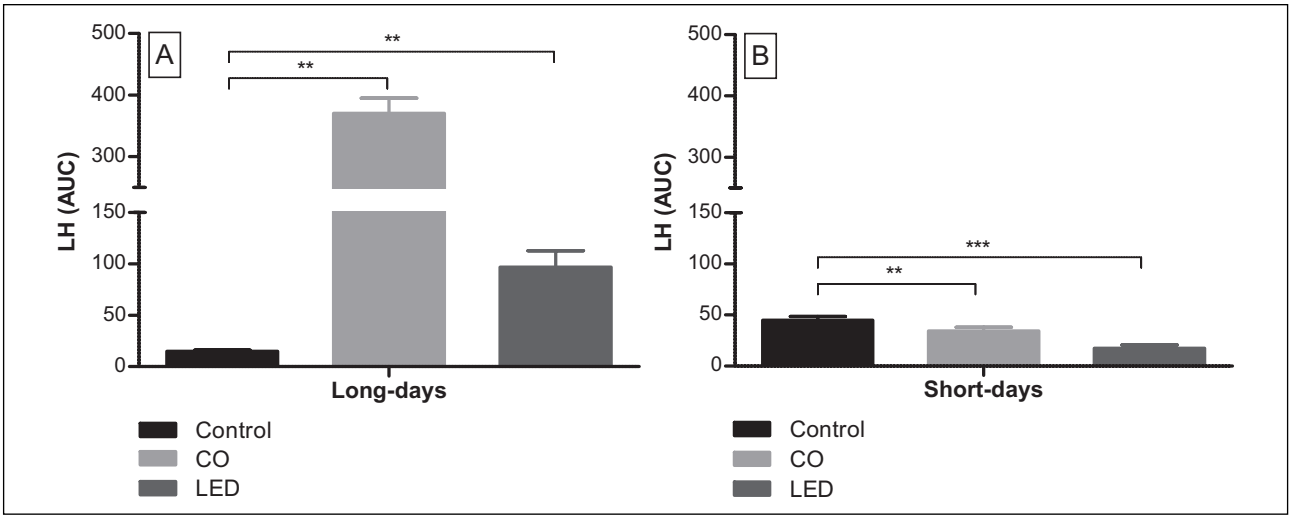

Fig. 7. Plasma LH concentration (AUC) in the control (n = 6), CO (n = 6) and LED (n = 6) groups animals, during the long [A] and short [B] days, mean ± S.E.M., **P ≤ 0.01; ***P ≤ 0.001.

concentration of experiment 1 animals was significantly decreased ($P \leq 0.05$) when compared with the control group. Experiment 2 animals also had a significantly ($P \leq 0.001$) decreased LH concentration compared to the control group (*Fig. 7B*).

DISCUSSION

Prior studies indicate that the gasotransmitter CO is released from the eye into ophthalmic venous blood depending on the intensity of sunlight (26). This study was designed to determine whether changes in the concentration of CO in the OphVB may modulate the reproductive axis and therefore, whether CO released from the eye under ambient light can be a carrier of light intensity information in regulation of seasonal breeding. As the experimental infusions were administered for 48 hours continuously and did not vary over 24-h periods as do normal environmental light conditions, the effects that we have demonstrated represent physical changes observed, but are not necessarily representative of normal physiologic changes. We decide to use crossbreed swine maintained behavioural reaction and seasonal reproduction. The size of their blood vessels allows for the implantation of the polyethylene catheters allows for repeated collection of systemic blood and for infusion of autologous blood or autologous blood plasma. Domoki *et al.* research was shown that acute reversible bilateral carotid artery occlusion does not reduce cerebrocortical blood flow in the piglet (42).

In all analysed experimental groups, the highest concentration of GnRH mRNA was noted in the preoptic area of the hypothalamus where the GnRH neurons reside (43-46). In the control groups, hypothalamic GnRH gene expression showed a significant seasonal variation (*Fig. 1*). Gonadotropin-releasing hormone mRNA levels in the preoptic area were significantly higher during the short days than during the long days. In the ventromedial hypothalamus, GnRH mRNA levels were significantly higher during the winter compared to the summer (*Fig. 1*). LH surge was associated with a decrease in GnRH mRNA expression in the preoptic area of hypothalamus (*Figs. 1* and *7*). It would appear that GnRH release in these crossbred swine is regulated *via* mechanisms other than those that control GnRH mRNA production. This is supported by the finding that modulation of GnRH release in sheep by both oestrogen and testosterone is not associated with marked alterations in GnRH gene expression (47, 48). Also, the study conducted by Robinson *et al.* (49) showed that the participation of the progesterone in the mechanism of the control of the ovine oestrous cycle is not connected with regulation of GnRH biosynthesis but with its secretion (49). In our study, unlike the preoptic area, GnRH gene expression in the ventromedial hypothalamus was significantly increased during the winter compared to the summer (*Fig. 1C*). Research conducted by Lopot *et al.* (50) showed that exogenous GnRH infused to the third cerebral ventricle in anaestrous ewes stimulated transcriptional activity of GnRH but does not alter GnRH synthesis. The authors suggested that GnRH can increase the transcriptional activity of the GnRH gene or it's transcript stability in ventromedial hypothalamus (50).

Analysis of GnRH-R mRNA in the control group showed a significant increase in GnRH-R gene expression in the anterior pituitary gland during the short days compared to the long days (*Fig. 4C*). Gonadotropin-releasing hormone receptor mRNA content in the anterior pituitary gland during the winter was also significantly higher than other structures analysed (*Fig. 4*). Increase in the GnRH-R mRNA concentration in the anterior pituitary gland during the winter is probably affected by GnRH, which can regulate gene expression (51-55) and activity (56-58) of GnRH-R on its own.

Analysis of GnRH mRNA levels after infusion of autologous blood or autologous blood plasma with experimentally increased CO concentrations demonstrated different effects of CO on GnRH mRNA depending on the photoperiod. During the short days, an elevated concentration of CO in the OphVS increased GnRH gene expression in all analysed structures of the hypothalamus (*Fig. 3*). During the long days this same treatment increased GnRH mRNA levels in the ventromedial hypothalamus, but in the preoptic area decreased GnRH mRNA levels were observed after infusion of illuminated blood (*Fig. 2*).

Lamar *et al.* (19) suggested that CO may function in the hypothalamus as a transmitter involved in regulating the neurosecretory activity of GnRH neurons. They demonstrated that heme molecules can stimulate GnRH release from the medial basal hypothalamus (MBH) fragments incubated *in vitro*. As CO-scavenger molecule, hemoglobin, completely reversed the effect of hematin the authors suggest that CO, mediates hematin's effect on GnRH release (19). Research conducted by Errico *et al.* (59) showed that CO stimulates a GnRH release in GnRH-secreting hypothalamic neurons GT1-7 (59). On the other hand, in the rat, CO decrease the hypothalamic release of both corticotrophin-releasing hormone and arginine-vasopressin (60-63). It can by assumed that the CO helps to protect the reproductive processes by decreasing the exaggerated activation of the hypothalamic-pituitary-adrenal axis (64). Mancuso *et al.* were shown the possibility of a significant role for carbon monoxide/cyclooxygenase (COX) pathway to regulate GnRH release; carbon monoxide increased COX activity and PGE_2 production in rat hypothalamic explants (65). In the brain heme oxygenase can regulates COX activity by reducing the intracellular heme content or by generation CO, which stimulates PGE_2 release. Prostaglandin E_2 (PGE_2), a phospholipid-derived signaling molecule, plays a fundamental role in modulating the gonadotropin-releasing hormone neuroendocrine system (66). Cyclooxygenase/prostaglandin system is a transduction mechanism through which CO can modulates neuropeptides release, including GnRH (59, 67, 68). In seasonal breeding animals the annual reproductive cycle is induced by photoperiodic-mediated changes that control GnRH release from the hypothalamus (69, 70). Photoperiodic information is encoded in the melatonin secretory rhythm; rhythmic release of melatonin regulates the rhythm in dopamine. The role of dopamine in the regulation of GnRH secretion is well documented, and numerous pharmacologic and anatomic data as well as surgical disruption of dopaminergic structures (71-77) suggest that dopaminergic neurones have a predominant inhibitory effect on GnRH release in the non-breeding season but not in the breeding season (78-81). A study performed in an identical model to this work demonstrated that elevated concentrations of CO in the OphVS had an acute impact on systemic melatonin levels (82). Carbon monoxide exerted opposite effects on melatonin concentration depending on the photoperiod. During the short days, elevated concentrations of CO in the OphVS limited the nocturnal melatonin rise. During the long days, this same treatment enhanced the nocturnal melatonin rise. Changes in GnRH mRNA levels after autologous blood or autologous blood plasma with increased CO concentrations was infused into the OphVS were also seasonally variable. It can be assumed that the mechanism of action of CO in regulation of GnRH gene expression involves dopaminergic systems. Taskiran *et al.* (83) demonstrated that in the rat striatum and hippocampus, CO inhibited dopamine reuptake in a time-, dose-, and temperature-dependent manner, independent of the nitric oxide synthase (NOS) activity. This suggested that CO may act as a modulator of synaptic transmission (83). CO appears also to modulate the local and systemic effect and expression of opioid receptors in a NO-depend fashion (84, 85). The research conducted by Wylot *et al.*

indicate that the opioid peptides, acting through *kappa*- and *delta*-opioid receptors, may participate in the modulation of LH and FSH secretion at the pituitary level in pigs (86).

Regulation of the GnRH-R is a key aspect of normal reproductive function. Changes in GnRH-R mRNA levels were demonstrated to be one of the mechanisms by which GnRH up-regulates its own receptor (87, 88). Analysis of GnRH-R mRNA levels in the anterior pituitary after infusion of autologous blood or autologous blood plasma with experimentally increased CO concentrations showed an opposite effect of CO on GnRH-R mRNA depending on the photoperiod. During the short days, elevated concentrations of CO in the OphVS decreased GnRH-R gene expression (*Fig. 6*). During the long days, however, this same treatment increased GnRH-R mRNA levels (*Fig. 5*). Based on these results it seems that GnRH-R mRNA was affected by the frequency and amplitude of GnRH pulses secreted by the hypothalamus. The physiological significance of GnRH-R mRNA in the hypothalamus for GnRH gene expression and GnRH release still remains not well understood. The results obtained on rats (89, 90) and sheep (91) suggest that GnRH-R mRNA in the hypothalamus may be involved in the control of GnRH release from the GnRH nerve terminals as well as GnRH gene expression in the preoptic area (92). Analysis of GnRH-R gene expression in the hypothalamus showed that only during the short days after infusion of autologous blood plasma with experimentally increased CO concentration the level of GnRH-R mRNA was lower as compared with the control (*Fig. 6*). The physiological importance of GnRH-R mRNA in different parts of the hypothalamus and carbon monoxide role in its regulation waits to be established.

Gilun *et al.* (93) demonstrated the influence of elevated concentrations of CO in the OphVB on the expression level of internal clock genes in the preoptic area and dorsal hypothalamus in the same type of crossbred swine. After CO treatment, the experimental animals experienced deregulation of their master clock machinery which could cause chronodisruption (93). A circannual clock drives expression of genes central for seasonal reproduction (94). Both the circannual and melatonin signals converge on the pars tuberalis of the pituitary gland to regulate beta subunits of thyroid-stimulating hormone (TSHβ) expression. In turn, TSH, *via* the regulation of type 2 thyroid hormone deiodinase (Dio2) gene expression in the tenocytes, most likely controls hypothalamic levels of triiodothyronine (T3). Changes in T3 may be relayed *via* RF-amide-related peptide (RFRP) and kisspeptin neurones to influence GnRH expression of pituitary gonadotrophins and thus gonadal activity (94). The alpha and beta subunits of LH and GnRH-R mRNA levels are stimulated to the greatest extend by this same (high) pulse frequency of GnRH; LHβ mRNA levels are maximally stimulated when GnRH-R concentrations are relatively high (55). In this study, increased concentrations of CO in the OphVS had a photoperiod-dependant influence on LH release which correlated with GnRH-R mRNA levels in the anterior pituitary gland (*Figs 7*, *5* and *6*). It is likely that the mechanism for this regulation occurs *via* the pattern of GnRH pulse frequency.

In conclusion, this study demonstrated the influence of elevated concentrations of CO in the OphVB on the expression level of GnRH and GnRH-R genes and systemic LH levels. In order to determine the physiologic relevance of this finding, it would be useful to study the effect of normal variations in OphVB CO levels on the HPA axis in different seasons. This study would support the concept that the effect of light in regulation of seasonal breeding is mediated, at least in part, *via* CO.

Abbreviations: AH, anterior part of the hypothalamus; AP, anterior pituitary gland; CO, carbon monoxide; GnRH, gonadotropin-releasing hormone; GnRH-R, gonadotropin-releasing hormone receptor; HO, heme oxygenase; LH, luteinizing hormone; OphS, ophthalmic sinus; OphVB, ophthalmic venous blood; OphVS, ophthalmic venous sinus; POA, preoptic area of the hypothalamus; PVC, perihypophyseal vascular complex; TSH, thyroid-stimulating hormone; VCS, venous cavernous sinus; VM, ventromedial part of the hypothalamus

Acknowledgments: This work was supported by the Polish State Committee for Scientific Research (N N 311 1001 33).

Data was presented previously as a poster during 11th International Symposium on GnRH (2014) The Hypothalamic-Pituitary-Gonadal Axis in Cancer and Reproduction: Romerowicz-Misielak M, Koziorowski MJ 'Expression analysis of GnRH and GnRH-R genes after increase of the carbon monoxide concentration in the ophthalmic venous blood'.

Conflict of interests: None declared.

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Received: February 2, 2016
Accepted: May 30, 2016

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