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Role of AA-NAT and TPH1 in the Ghrelin-Dependent Regulation of Melatonin Secretion in Sheep during Different Seasons: An *In Vitro* Study

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Abstract

Several studies suggests that ghrelin (GHRL) has neurobiological effects that extend beyond control of food intake. Our previous results confirmed that GHRL modulates the secretory activity of the pineal gland (PG) through nocturnal melatonin (MEL) secretion in sheep, the seasonally reproductive animals. Here we investigated the effects of GHRL (10 ng/ml) on the expression of enzymes limiting synthesis of MEL, including tryptophan 5-hydroxylase 1 (TPH1), serotonin N-acetyltransferase (AA-NAT) and its phosphorylated form p31T-AA-NAT in sheep PG explants (n = 72) during the 4-hour incubation in a gas-liquid interface, at a short (SD) and long (LD) photoperiods. After each hour of incubation selected explants were frozen in liquid nitrogen and stored at -80°C for subsequent analysis (real-time PCR, western-blotting, ELISA). Results show that GHRL regulates nightly MEL secretion in a TPH1-independent manner. The factor modulating GHRL activity was photoperiod. During SD photoperiod GHRL significantly reduced the expression of p31T-AA-NAT, AA-NAT and inhibited MEL secretion from PG explants. Whereas, during LD photoperiod no effect of GHRL on MEL secretion and expression of examined enzymes was noted. Studies indicate that GHRL directly affects PG under *in vitro* conditions and causes MEL secretion in animals which exhibit seasonality in reproductive and metabolic processes.

Keywords: ghrelin; serotonin N-acetyltransferase; tryptophan 5-hydroxylase 1; melatonin; pineal gland; photoperiod; sheep

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1. Introduction

Ghrelin (GHRL), which is mainly produced in the stomach, is one of the most important hormones engaged in maintaining the energy balance of the body at a constant level. A preprandial increase in GHRL secretion activates mechanisms that counter energy deficiencies, stimulating food searching and intake [1]. The circadian system also plays a pivotal role in determining plasma GHRL levels by fine-tuning the timing of GHRL release throughout the 24-hour cycle, as has been shown in humans and rodents [2]. Studies with a GHRL analogue (GHRP-6) have confirmed the influence of GHRL on neuron activity in the mammalian "master clock" (suprachiasmatic nuclei, SCN) and period circadian clock 2 gene expression [3]. Electron microscopic analysis has revealed that the SCN and intergeniculate leaflet efferents establish symmetrical synaptic contacts on GHRL-immunoreactive cell bodies and dendrites [4]. In seasonal animals, such as sheep [5-10], the blue fox (*Alopex lagopus*) [11], the raccoon dog (*Nyctereutes procyonoides*) [12] and the golden-mantled ground squirrel (*Spermophilus lateralis*) [13], season-dependent changes in the secretion and endocrine activity of GHRL have also been observed.

It is a relatively recent finding that GHRL in sheep is involved in the regulation of pineal gland (PG) activity [6,8]. The PG, together with the retina and the SCN, constitutes a part of the central system that controls the rhythms of physiological processes. The mammalian PG disseminates information about circadian time to peripheral tissues through the synthesis and release of melatonin (MEL), which is restricted to nighttime. The initiation of MEL synthesis begins within the well-known sympathetic pathways [14]. MEL is formed by a four-stage reaction. The first two stages are associated with the conversion of tryptophan into serotonin (5-HT), which undergoes acetylation by serotonin N-acetyltransferase (AA-NAT) into N-

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acetylserotonin (NAS). Then, NAS is methylated by hydroxyindole O-methyltransferase (HIOMT) to generate the final biologically functional N-acetyl-5-methoxytryptamine [14]. Intracerebroventricular infusions of GHRL have been shown to significantly alter nightly MEL secretion in sheep depending on the season [8]. *In vitro* studies of PG sheep explants [6] and rat pinealocytes [15] have confirmed the direct effect of GHRL or its analogues on the PG and the GHRL receptor (*growth hormone secretagogue receptor type 1a;* GHSR-1a) has been identified in goldfish pinealocytes [16]. However, the mechanism through which GHRL acts on the biological clock of seasonal mammals, such as sheep, including its effects on the regulation of MEL biosynthesis and secretion, has not been well explained.

In studies on sheep [17] and rat [18] PGs, *in vitro* MEL synthesis was found to require *de novo* 5-HT synthesis, suggesting an essential role of tryptophan 5-hydroxylase 1 (TPH1) in that process. Therefore, the first objective of our study was to evaluate the action of GHRL on TPH1 mRNA expression and protein concentration in ovine PG explants. The second objective was to determine the role of GHRL in the nightly changes in phosphorylated AA-NAT (p31T-AA-NAT) protein expression and concentration in the PG of sheep. The posttranscriptional control of this enzyme plays a dominant role in regulating MEL synthesis in sheep [19,20], cattle [21,22] and primates [23]. Phosphorylated AA-NAT binds to 14-3-3 proteins to maintain its catalytic activity. The last objective of the study was to resolve how GHRL-induced changes in TPH1 and AA-NAT expression levels affect the nighttime profile of MEL secretion from ovine PG explants. Considering the seasonal dependence of changes in the concentration and activity of GHRL and MEL in sheep [5,8-10,24], all experiments were carried out during the short-day (SD) and long-day (LD) photoperiods testing the hypothesis that the influence of GHRL on the PG is related to the time of year. The results of the present study will improve our understanding

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of the PG as a potential site to integrate metabolic signals sparked by feeding with light information, producing the most favorable circadian rhythm and circannual physiological and behavioral activities under prevalent environmental conditions.

2. Results

2.1. GHRL increases TPH1 mRNA expression during the SD season but has no effect on TPH1 protein concentration in ovine PG explants

To clarify the potential mechanisms of action of GHRL on PG activity at night, we investigated whether GHRL affects TPH1 mRNA expression and protein concentration in ovine PG explants collected during different seasons. We performed real-time PCR and enzyme-linked immunosorbent assays (ELISAs) to determine how TPH1 transcript and protein levels in PG explants incubated with GHRL change at various time intervals (1, 2, 3 and 4 hrs). As shown in Figure 1A, the effect of GHRL on TPH1 mRNA expression in ovine PG explants was dependent on the season. During the SD season, TPH1 transcript levels were significantly higher in the GHRL-treated PGs than in the control PG explants ($P < 0.01$). During the LD season, TPH1 mRNA levels were similar in both control explants and samples incubated in the presence of GHRL (Figure 1A). However, after analyzing the time profile of the experiment, the effect of GHRL was found to be dependent on the duration of incubation. During the SD season, we noticed a stimulatory effect of GHRL on TPH1 mRNA expression levels in explants after 2 and 4 hrs of incubation (Figure 1B). During the LD season, GHRL was observed to initially, at 1 hr of incubation, significantly reduce TPH1 mRNA expression ($P < 0.001$). However, after this time point, we did not observe any significant differences in TPH1 transcript levels relative to the control samples (Figure 1C). There were no significant differences in the basal endogenous TPH1 expression levels of PGs isolated during the SD and LD seasons (Figure 1D).

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As shown in Figure 2A, there were no significant ($P > 0.05$) effects of GHRL on TPH1 protein concentrations compared with concentrations in the control group in both seasons. However, within the GHRL group, the mean TPH1 concentration was greater ($P < 0.05$) for PG explants collected from ewes during the SD season than for those collected from ewes during the LD season (Figure 2A). The time charts (Figures 2B and 2C) show a decrease ($P< 0.05$) in TPH1 protein concentration in comparison with concentrations in the control groups after incubating PG explants with GHRL for 1 hr. This effect was similar during the SD (Figure 2B) and LD (Figure 2C) seasons. For the remaining incubation time intervals, there was no effect of GHRL ($P > 0.05$) on the levels of examined proteins (Figures 2B and 2C).

2.2.The effect of GHRL on p31T-AA-NAT protein expression and AA-NAT protein concentration in ovine PG explants is dependent on the season

To further understand the molecular mechanism of nocturnal MEL synthesis, we analyzed p31T-AA-NAT protein expression via western blotting and measured AA-NAT protein concentrations via ELISA in ovine PGs collected during different photoperiods and incubated with GHRL for 1, 2, 3 and 4 hrs. The immunoblot analysis demonstrated that p31T-AA-NAT protein levels in the PG explants treated with GHRL for 3 and 4 hrs were lower ($P < 0.05$ and $P < 0,01$, respectively) than those in the control PG explants collected from sheep during the SD season (Figure 3A). Compared with expression in the controls, a decrease ($P < 0.05$) in p31T-AA-NAT protein expression was observed after 4 hrs of GHRL exposure for PG explants obtained from ewes in the LD season (Figure 3B). Within the control group, the mean AA-NAT concentration was greater ($P < 0.01$) for PG explants collected during the SD season than for those collected during the LD season (Figure 4A). GHRL treatments decreased ($P < 0.01$) AA-

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NAT concentrations in the PG explants obtained from sheep in the SD season compared with concentrations in the untreated controls (Figure 4A). For PGs collected from sheep during the SD season, AA-NAT protein concentrations in PG explants treated with GHRL for 1, 2, and 3 hrs were decreased ($P < 0.01$) compared with concentrations in the controls (Figure 4B). In contrast, there was no effect of GHRL ($P > 0.05$) on AA-NAT concentrations in the PG explants collected from ewes during the LD season compared with concentrations in the untreated controls (Figure 4C).

2.3. GHRL decreases MEL concentration in the culture medium during the SD season but has no effect on MEL secretion during the LD season

To determine whether the exogenous GHRL is involved in the nightly regulation of PG activity, we measured the concentration of MEL secreted into the culture medium using radioimmunoassay (RIA). Within the control group, the mean MEL concentration was greater ($P < 0.05$) for PG explants collected during the SD season than for those collected during the LD season (Figure 5). GHRL treatment decreased ($P < 0.001$) MEL concentrations in the cultures of PG explants obtained from ewes in the SD season compared with concentrations in the controls. A decline ($P < 0.05$) in MEL secretion in PG explants collected from sheep during the SD season was observed within the GHRL treatment group (Figure 5).

3. Discussion

Our results confirm the direct effect of GHRL on the secretory activity of the PG and the process of MEL biosynthesis in sheep. Ghrelin inhibited PG hormone secretion, melatonin, and its effect was observed during the SD photoperiod. Differences in MEL secretion have also been reported during *in vitro* study of ovine pineal explants treated with GHRL, regardless of the

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applied dose [6] and in *in vivo* study after infusions of GHRL into the third ventricle of sheep brain at or after the sunset [8]. However, in rats no changes were observed in either MEL concentration or AA-NAT and HIOMT activity levels in the PG after the subcutaneous injection of GHRL before the middle of the dark phase [25]. The aforementioned studies suggest that the species, route of administration of GHRL and the pathway through which GHRL acts may be important in the regulation of PG activity.

The further factor modulating action of GHRL on PG activity has been shown to be a photoperiod. During an SD, GHRL inhibited MEL secretion and had no significant effect on MEL concentration during an LD. In GHRL-treated explants the level of TPH1 transcripts increased during an SD, whereas it did not change during an LD. These differences were not reflected in the protein concentration of TPH1. An increase in TPH1 concentration was only observed within the group of GHRL-treated PG explants during the SD season compared with concentrations during the LD season. The results suggest that TPH1 may not play a key role in the GHRL-dependent regulation of MEL secretion in sheep. Because neither the TPH1 mRNA expression profile nor the TPH1 protein concentration profile correspond to the previously described changes in MEL secretion from the PG, it cannot be determined which mechanism of TPH1 regulation (transcriptional or posttranscriptional) is predominant in MEL biosynthesis in sheep. The results of Privat et al. [17] and Namboodiri et al. [26] partially confirmed those findings. They showed that the required TPH1 mRNAs and proteins are available at the beginning of the dark period in both the PG in sheep and in cultured pinealocytes. The fast availability of TPH1 decides about the rapid (~30 min) increase in MEL secretion after light removal in sheep [26], while several hours are required in the case of rats, possibly reflecting the time necessary for *de novo* synthesis and accumulation of TPH1 and AA-NAT messengers [27].

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Huang et al. [28] also suggest that, in rats, pineal TPH1 protein expression displays marked diurnal rhythms, and cAMP-dependent TPH1 phosphorylation is responsible for daily changes in 5-HT availability, on which further stages of MEL biosynthesis are dependent.

The GHRL-induced increase in TPH1 mRNA expression during the SD season is surprising in the context of the concurrent inhibitory effect of GHRL on MEL secretion. After the theory of a constitutive level of 5-HT existing in the PG was undermined, TPH1 has been considered to be the main enzyme determining the availability of the substrate (5-HT) for MEL production [29]. Data of *in vivo* experiments in sheep indicated that administration of TPH1 caused an increase in the levels of 5-HT, NAS and MEL in the PG, as well as MEL in the blood. However, the activity of AA-NAT and HIOMT did not change, indicating that the augmentation in MEL was due to the increase 5-HT acetylation resulting from the rise in 5-HT concentration [26]. Privat et al. [17] showed, that while working on *in vitro* culture of ovine pinealocytes an inhibitor of TPH1 activity (*para*chlorophenylalanine, pCPA) almost totally suppressed the induction of MEL synthesis. Similarly, Miguez et al. [18] observed the inhibition of MEL secretion from rat pinealocytes in *in vitro* conditions after supplementation with culture medium with pCPA added.

Ghrelin-induced changes in the level of TPH1 transcripts may indicate that there is a relationship between GHRL and the serotoninergic system at the level of the PG that, however, it does not directly affect MEL secretion. So far, such interactions have been demonstrated in the hypothalamus [30], hippocampus [31], dorsal raphe and amygdala [32]. They primarily involve the regulation of energy homeostasis, emotional behavior and the ability to remember and learn. In these nerve centers, GHRL inhibits [30,31] or boosts [32] 5-HT activity, biosynthesis and receptor expression. Moreover, in studies by Schellekens et al. [33,34], a novel heterodimer

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involving GHSR-1a and the 5-HT receptor 5-HT2C was identified in the hypothalamus and hippocampus. Moreover, experiments on rodents [35] and ruminants [36] have shown that 5-HT2C and/or 5-HT2B receptors are directly involved in regulating MEL biosynthesis and secretion. In light of those results a further analysis is needed to determine the effect of GHRL on the level of expression of 5-HT, its metabolites and its receptors in the PGs of sheep.

Regulation of the biosynthesis and secretion of MEL from the PG is a complex process, primarily due to species differences. Between species such as rodents and sheep or cattle and primates, there are differences in the specific way norepinephrine affects α- and β-adrenergic receptors, as well as in the activation of an intracellular regulatory pathway dependent on cAMP, cGMP and Ca^{2+} [37]. However, the most attention has been paid to the species-specific regulation of AA-NAT synthesis. The nocturnal elevation of AA-NAT activity is dependent on AA-NAT gene transcription [38] and the role of the RNA-binding protein heterogeneous ribonucleoprotein R (hnRNP R) in the translation of AA-NAT in rats [39]. However, in sheep [14,19,20], cattle [21] and humans [14], it mainly depends on changes in AA-NAT phosphorylation and post-translational modifications of the AA-NAT protein, such as proteasomal degradation. In present study, GHRL decreases p31T-AA-NAT expression and AA-NAT protein concentration in the ovine PG, and the effect of its activity was the strongest during the SD season. A decrease in AA-NAT protein phosphorylation at threonine-31 of the N-terminus of the polypeptide chain is important for the biosynthesis and secretion of MEL in sheep [19] and primates [23]. Phosphorylated AA-NAT together with 14-3-3 proteins forms a complex in which AA-NAT retains its catalytic activity and is protected against dephosphorylation and enzymatic degradation. Light causes the immediate destruction of this complex and proteolytic degradation of AA-NAT, resulting in a immediate suppression of MEL

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secretion in sheep [40]. Present studies indicate that the decrease in AA-NAT protein concentration and p31T-AA-NAT expression after GHRH treatment corresponded to a simultaneous reduction in MEL secretion from the PG. This result reveals that GHRL regulates the secretory activity of the PG of sheep by influencing AA-NAT protein synthesis. The next step will be to examine the impact of GHRL on the c-AMP-dependent, protein kinase A-mediated phosphorylation mechanism involving 14-3-3 proteins.

Ghrelin may affect the PG through several pathways, including via the SCN, which is reached by efferent nerve fibers from the arcuate, paraventricular and dorsomedial hypothalamic nuclei, where GHRL and its receptor have been identified [1,41,42]. Information from the abovementioned centers is amplified and synchronized in the SCN with light, which enters through the retinohypothalamic tract, and then, "ready nerve instructions" are sent via multi-neuronal tracks from the SCN to the PG [1]. In the second case, the GHRL produced in the central nervous system (CNS) or in the periphery reaches the PG through the cerebrospinal fluid (CSF). The PG is in direct contact with the CSF due to the incomplete ependymal lining in the pineal recess, an extension of the third ventricle [43]. Studies carried out on sheep [29], humans [44] and rodents [45] have confirmed the presence of GHRL in the CSF, through which, it is able to reach most of the forebrain, midbrain and hindbrain areas where GHSR-1a gene expression has been reported. Intracerebroventricular infusions of GHRL into the CSF allows GHRL to influence the PG directly or indirectly through the nerve centers of the brain, such as the mediobasal hypothalamus, that are functionally and anatomically connected with the PG, in which GHSR-1a has been identified [1]. Cabral et al. [45] also detected GHRL uptake in the ependymal cells of the ventricular system of the brain, where GHSR-1a is presumably not present. The PG is rich in fenestrated capillaries and canaliculi, through which, even large

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molecules such as peptides and proteins, including GHRL, can probably penetrate from the CSF into the gland [43]. To reach the majority of neural structures connected to the PG, peripherally administered GHRL must first cross the blood-brain barrier (BBB). This is crucial, especially in sheep, in which GHRL administered intravenously crosses the BBB to a very small (< 1:1000) extent, and the concentration of this hormone in the CSF is about one thousand times lower than it is in the blood plasma [46].

In addition to the sympathetic pathways that initiate MEL synthesis, there are nerve projections of the central areas of the brain that modulate the activity of the PG. There are several neurotransmitters and neuropeptides that are involved in this process, including glutamate, γ-aminobutyric acid, acetylcholine, neuropeptide Y, somatostatin, vasopressin, substance P, calcitonin gene-related peptide, oxytocin, histamine and the neuronal isoform of nitric oxide synthase [47,48]. In sheep, the above list is complemented by GHRL, leptin (a hormonal indicator of energy metabolism) and orexin B (which controls the rhythm of sleep and wakefulness, as well as energy homeostasis) [6,8]. Interestingly, well-described interactions between GHRL and leptin [49] also occur in sheep at the level of the PG [6]. Among these ruminants, photoperiod is an additional factor that interferes with these interactions, as demonstrated by the fact that GHRL has been shown to inhibit the leptin-stimulated increase in MEL secretion during an SD and strengthen the impact of leptin on PG during an LD [6].

To sum up, the results presented here have substantially contributed in the latest research regarding the role of GHRL as an important neurobiological factor that regulates numerous processes in animals. Our results indicate that GHRL, so far known mainly as a hormonal indicator of energy metabolism, influences the secretory activity of the PG and MEL biosynthesis, which demonstrates that the mechanism underlying MEL release is not completely

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understood. These results may also bring us closer to understanding the function of the PG in humans. Between human and sheep PGs, there is anatomical homology of gland vascularization and its distribution in relation to brain ventricles, particularly the position of the PG relative to the third ventricle [50] is comparable, which makes study using ovine PG valuable to carry on.

4. Material and methods

All animal-related procedures used in these studies were approved by the Local Agricultural Animal Care and Use Committee of Krakow (protocol #80/2012). The study was carried out at the Experiment Station in the Department of Animal Biotechnology, Agricultural University of Krakow (longitude, 19°57_E; latitude, 50°04_N).

Sixteen Polish Longwool ovariectomized ewes (a breed with pronounced reproductive seasonality; 4-5 y of age; mean body weight of 70 ± 5 kg), each bearing a subcutaneous estradiol implant, were used. In the present study, mean concentrations of estradiol in implanted ewes were 2-4 pg/ml as previously reported [8]. Hormonal implants provide a constant level of estradiol negative feedback without the complications associated with ovarian cyclicity. Ovariectomized, steroid-treated females and castrated, steroid treated males have proven to be good models for studying the effects of nutrition on the neuroendocrine axis [8-10].

All ewes were euthanized humanely via exsanguination following captive bolt stunning after sunset. The PGs were removed aseptically from ewes within 10-15 min of death. Glands were collected from 8 ewes selected randomly during the LD season in May and from an additional 8 ewes during the SD season in November. The glands were placed in Dulbecco's modified Eagle's medium (Biomed, Lublin, Poland) and transported to the laboratory. All subsequent procedures

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were performed under sterile conditions and a dim red light. This light was insufficiently bright (< 3 lux) and at an improper wavelength (622 - 780 nm) to influence MEL production [51].

4.1.Pineal gland explant culture

Isolation, processing, and culture of PG explants were performed as previously described [6,52]. Briefly, PGs collected from each ewe (n = 8/season) were dissected and sliced sagittally into 0.5- to 2-mm thick strips (n = 36/season). Each PG explant was selected randomly, placed on a stainless steel grid covered with lens paper and incubated in a gas-liquid interface in 1 ml of Dulbecco's modified Eagle's medium (Biomed, Lublin, Poland). The explants were incubated in a 12-well tissue culture dish (Corning Netwell Insert, 15-mm membrane diameter, New York, NY) with 95% humidified air and 5% CO_2 at 37°C for a total of 5.0 hours. After a 60 min period of equilibration, PG explants were challenged with either 0 (control) or 10 ng/ml of ovine GHRL (PolyPeptide Labs, Strasburg, France), and incubated for another 4 hrs. The dose of GHRL was based on previous studies [4]. Sixty min after GHRL treatment 50 µl of medium was harvested from each well. Samples were stored at −20°C until RIA for melatonin.

Not treated explants (n = 4), collected from PGs immediately after euthanization and explants from the controls and the GHRL-treated groups, harvested from culture every hour of 4 hrs incubation (4 explants per group per hour), were frozen in liquid nitrogen and then stored at -80°C for subsequent analysis. Analysis include the determination of TPH1 mRNA expression by Real-time PCR, measurement of p31T-AA-NAT protein expression via western blotting, and determination of TPH1 and AA-NAT protein concentrations via ELISA.

4.2. Isolation of total RNA and protein from pineal samples

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Pineal RNA and protein extracts from sheep PGs were obtained using a commercial NucleoSpin ® RNA/Protein kit (Marchery-Nagel, Oensingen, Switzerland), which allows for the isolation of total RNA (approx. 70 µg) and protein (approx. 1200 µg) from the same sample. According to the instructions provided by the manufacturer of the NucleoSpin® RNA/Protein kit, the frozen tissue was first mechanically fragmented using a rotor stator homogenizer (Omni TH™; Omni International Inc., Kennesaw, GA, USA) equipped with an Omni Soft Tissue Tip™. After isolating total RNA, its quality was verified by determining the ratio of 28S subunits to 18S subunits after separating the RNA samples on an agarose gel with ethidium bromide. The purity of the total RNA was measured spectrophotometrically using a NanoDrop 2000/2000c device (Thermo Scientific, Waltham, MA, USA). The concentration of isolated protein was measured using bicinchonic acid (BCA) (Sigma Aldrich, Poznan, Poland).

4.3.Real-time PCR analysis of pineal samples

Real-time PCR was used to measure TPH1 mRNA levels. The RNA (0.5 µg) was reverse transcribed to cDNA using Quantiscript reverse transcriptase and RT primer mix (QuantiTect® Reverse Transcription Kit; Qiagen, Hilden, Germany) by incubating the samples at 42°C for 15 min. The reaction was terminated by heating samples to 94°C for 3 min. Genomic DNA was removed by incubating the samples at 42°C for 2 min in the presence of gDNA Wipeout Buffer (QuantiTect® Reverse Transcription Kit). Amplification of cDNA was performed using TaqMan® Gene Expression Master Mix (Life Technologies, Foster City, CA, USA) and an Applied Biosystems 7300 Real-Time PCR System. Primers and probes were designed using Primer Express software v.2.0 (Applied Biosystems, Foster City, CA, USA). The products were amplified using the following primers, each at a final concentration of 900 nM: 5'-CCCTCTTTTGGCTGAACCTAGTT-3' and 5'-AGAGAAGCCAGGCCAATTTCT-3'

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(corresponding to the bovine TPH1 gene, Ensembl accession number ENSBTAT00000007030; Sequence Detection Primers, Life Technologies, Foster City, CA, USA) or 5'-CCTGCGGCATTCACGAA-3' and 5'-GCGGATGTCGACGTCACA-3' (corresponding to ovine β-actin, GenBank accession number U39357; Sequence Detection Primers, Life Technologies, Foster City, CA, USA). The probe sequences were FAM™-TGCTCAGTTCTCCC-MGB (corresponding to the TPH1 gene; TaqMan MGB Probes; Life Technologies, Foster City, CA, USA) and FAM™-CTACCTTCAATTCCATCATG-MGB (corresponding to the β-actin gene; TaqMan MGB Probes; Life Technologies, Foster City, CA, USA). The final concentration of each probe was 250 nM. Each gene assay was run in a singleplex reaction in triplicate for each cDNA sample. Amplification was carried out under the following conditions: 1) initial incubation at 50°C for 2 min, 2) polymerase activation at 95°C for 10 min, and 3) 40 cycles of denaturation (95°C for 15 sec) and annealing/elongation (60°C for 60 sec). The data were collected and recorded using the Applied Biosystems 7300 Real-Time PCR System with SDS Software and are expressed as a function of the threshold cycle (C_t). Using diluted samples, the amplification efficacies for the TPH1 (target gene) and β-actin (reference gene) amplimers were found to be identical. There was no significant variation in the C_t values for β-actin among the treatment groups.

4.4.Enzyme-linked immunosorbent assay of TPH1 and AA-NAT protein concentrations

The concentrations of the TPH1, AA-NAT proteins in the ovine PG explants and the concentrations of estradiol in plasma were determined via enzyme immunoassay using commercially available kits (rat TPH1 ELISA kit, Wuhan Fine Biological Technology Co., Ltd., Wuhan, China; sheep AA-NAT ELISA kit, Cusabio, Wuhan, China; Sheep E2 Estradiol Elisa kit, MyBioSource, San Diego, USA) according to the manufacturer's specifications. All samples

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(20 µg of total protein) were analyzed in duplicate in a single assay. The sensitivity of the TPH1 assay was < 0.188 ng/ml, and the intra- and inter-assay coefficients of variation (CVs) were < 8% and < 10%, respectively. The sensitivity of the AA-NAT assay was < 19.5 pg/ml, and the intra- and inter-assay CVs were < 8% and < 10%, respectively. The sensitivity of the estradiol assay was 1.0 pg/ml, and the intra- and inter-assay CVs were < 5.6%. The sensitivity of the estradiol assay was 1.0 pg/ml, and the intra-assay and inter-assay CVs were < 5.6 %.

4.5.Western blot analysis of pineal samples

For western blot analysis, 20 µg of protein were separated using gradient gels (4-12%) and transferred onto a polyvinylidene (PVDF) membrane (Merck Millipore, Darmstadt, Germany). After blocking nonspecific sites (Rotiblock; Roth, Karlsruhe, Germany), membranes were incubated overnight at 4°C with a primary rabbit anti-p31T-AA-NAT (1:500 dilution; Merck Millipore, Darmstadt, Germany, AB-5467) or anti-β-actin (1:400 dilution; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA, sc-1616) antibody. Following incubation for 1 h at room temperature with a secondary anti-rabbit IgG HRP antibody (1:10000 dilution; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA, sc-2004), signals were detected using chemiluminescence (Pierce ECL Plus Western Blotting Substrate, Thermo Scientific, Waltham, MA, USA). The intensities of individual protein bands were digitized and quantified using GeneTools capture and image analysis software (Syngen, Cambridge, UK). The values of the protein signal were normalized against the β-actin signal.

4.6.Radioimmunoassay for melatonin

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MEL concentrations were assayed in 10 µl of medium according to the method of Fraser et al. [53] as modified by Misztal et al. [54] and measured per 1 g of tissue. Ovine anti-melatonin serum (AB/S/01) was obtained from Stockgrand Ltd. (Guildford, Surrey, UK). Synthetic MEL (Sigma Aldrich, Poznan, Poland) was used for standards, and [*O*-methyl-3H] melatonin (Amersham Biosciences, Buckinghamshire, UK) was used as the radiolabeled tracer. The range of the calibration curve was 0.4 to 50 ng/mL, and the working dilution of the antiserum was 1:4000. Bound and free fractions were separated using dextran-coated charcoal after an overnight incubation at 4°C. The sensitivity of the assay was 14.6 ± 2.0 pg/ml, and the intra- and inter-assay CVs were 7.8% and 9.1%, respectively.

4.7.Statistical Analysis

TPH1 mRNA expression levels were calculated using a relative quantification (RQ) analysis. In brief, the amplification plot consisted of the plot of fluorescence versus PCR cycle number. The C_t value was the fractional PCR cycle number at which the fluorescent signal reached the detection threshold. Therefore, the input cDNA copy number and C_t value were inversely related. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method, and C_t values were converted to fold change RQ values as follows: fold change (RQ) = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_t$ sample - ΔC_t calibrator = [($Ct_{\text{target gene}} - Ct_{\text{reference gene}}$) sample - ($Ct_{\text{target gene}} - Ct_{\text{reference gene}}$) calibrator]. The RQ values from each gene were then used to compare the target gene expression across all groups. The mean TPH1 mRNA expression in each sample was normalized against the expression of a reference gene (β-actin) and expressed relative to the calibrator. To compare changes in expression levels at selected incubation time points, we used the mean ΔC_t value of the control group for the indicated time of incubation as a calibrator. To determine the basal TPH1 expression levels, depending on the season, we

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considered the ΔC_t values of the samples frozen immediately after tissue isolation and used the mean ΔC_t value for non-incubated samples isolated during the LD as a calibrator.

The real-time PCR, western blot and ELISA data were analyzed via one-way analysis of variance (ANOVA) using SigmaPlot statistical software (version 11.0; Systat Software Inc., Richmond, CA) followed by Tukey's multiple comparison test. MEL concentrations in the culture medium were analyzed by a series of two-way ANOVAs. All datasets that failed normality and/or equal variance tests were natural-log transformed. If the main effects or their interactions were significant, Tukey's test was used as a post-hoc test to compare individual means. A P-value of <0.05 was considered statistically significant. The results are presented as the mean ± standard error of the mean.

Supplementary Materials:

Figure 1A. Mean (± SEM) TPH1 mRNA expression in ovine PG explants incubated in the presence of GHRL (10 ng/ml) for experiments carried out during the SD and LD seasons. TPH1 mRNA expression is reported in arbitrary units relative to β-actin mRNA expression. The mean ΔC_t value calculated for the explants not exposed to exogenous hormone (control) during the appropriate season was used as a calibrator. ** denote significant differences from the control ($P < 0.01$).

Figure 1B. Mean (± SEM) TPH1 mRNA expression in ovine PG explants incubated for the indicated time in the presence of GHRL (10 ng/ml) for experiments carried out during the SD season. TPH1 mRNA expression is reported in arbitrary units relative to β-actin mRNA expression. The mean ΔC_t value calculated for the explants not exposed to exogenous hormone

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(control) at the appropriate time point was used as a calibrator. ** denote significant differences from the control ($P < 0.01$).

Figure 1C. Mean (± SEM) TPH1 mRNA expression in ovine PG explants incubated for the indicated time in the presence of GHRL (10 ng/ml) for experiments carried out during the LD season. TPH1 mRNA expression is reported in arbitrary units relative to β-actin mRNA expression. The mean ΔC_t value calculated for the explants not exposed to exogenous hormone (control) at the appropriate time point was used as a calibrator. ** denote significant differences from the control ($P < 0.001$).

Figure 1D. Mean (± SEM) basal endogenous TPH1 mRNA expression in ovine PGs collected during the SD and LD seasons and frozen immediately after tissue isolation. TPH1 mRNA expression is reported in arbitrary units relative to β-actin mRNA expression. The mean ΔC_t value calculated for PGs isolated in the LD season was used as a calibrator.

Figure 2A. Mean (± SEM) TPH1 protein concentrations in ovine PG explants incubated in the presence of GHRL (10 ng/ml) for experiments carried out during the SD and LD seasons. Values with asterisks are significantly different: * ($P < 0.05$).

Figure 2B. Mean (± SEM) TPH1 protein concentrations in ovine PG explants obtained during the SD season. After 60 min of equilibration, PG explants were incubated with medium alone (control) or with medium containing GHRL (10 ng/ml) for 1, 2, 3 and 4 hrs. Values with asterisks are significantly different: * ($P < 0.05$).

Figure 2C. Mean (± SEM) TPH1 protein concentrations in ovine PG explants obtained during the LD season. After 60 min of equilibration, PG explants were incubated with medium alone

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(control) or with medium containing GHRL (10 ng/ml) for 1, 2, 3 and 4 hrs. Values with asterisks are significantly different: * ($P < 0.05$).

Figure 3A. Representative western blots showing pT31-AA-NAT protein levels in ovine PG explants collected during the SD season and treated with 10 ng/ml of GHRL for 1, 2, 3 and 4 hrs. Protein bands were quantified via densitometry. The results are shown as the percentage of pT31-AA-NAT protein relative to the control protein levels. The blots were stripped and reprobed with an anti-actin antibody to control for the amounts of protein loaded onto the gel. Each column represents the mean (± SEM). * $P < 0.05$ and ** $P < 0.01$ indicate significant differences relative to the control.

Figure 3B. Representative western blots showing pT31-AA-NAT protein levels in ovine PG explants collected during the LD season and treated with 10 ng/ml of GHRL for 1, 2, 3 and 4 hrs. Protein bands were quantified via densitometry. The results are shown as the percentage of pT31-AA-NAT protein relative to the control protein levels. The blots were stripped and reprobed with an anti-actin antibody to control for the amounts of protein loaded onto the gel. Each column represents the mean (± SEM). * $P < 0.05$ indicates significant differences relative to the control.

Figure 4A. Mean (± SEM) AA-NAT protein concentrations in ovine PG explants incubated in the presence of GHRL (10 ng/ml) for experiments carried out during the SD and LD seasons. Values with asterisks are significantly different: ** ($P < 0.01$).

Figure 4B. Mean (± SEM) AA-NAT protein concentrations in the ovine PG explants obtained during the SD season. After 60 min of equilibration, PG explants were incubated with medium

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alone (control) or with medium containing GHRL (10 ng/ml) for 1, 2, 3 and 4 hrs. Values with asterisks are significantly different: ** ($P < 0.01$).

Figure 4C. Mean (± SEM) AA-NAT protein concentrations in ovine PG explants obtained during the LD season. After 60 min of equilibration, PG explants were incubated with medium alone (control) or with medium containing GHRL (10 ng/ml) for 1, 2, 3 and 4 hrs.

Figure 5. Mean (± SEM) MEL concentrations in media collected from cultures of PG explants obtained during the SD and LD seasons. After 60 min of equilibration, PG explants were incubated with medium alone (control) or with medium containing GHRL (10 ng/ml) for 1 hr. Values with asterisks are significantly different: * ($P < 0.05$), ** ($P < 0.01$).

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Author Contributions: Katarzyna Kirsz designed and performed the experiments, analyzed the data and wrote the paper. Małgorzata Szczesna performed the experiments. Dorota A. Zieba participated in the experiments and revised manuscript.

Abbreviations

5-HT	Serotonin
5-HT2B	Serotonin Receptor Type 2C
5-HT2C	Serotonin Receptor Type 2B
AA-NAT	Serotonin N-acetyltransferase

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BBB	Blood Brain Barrier
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
ELISA	Enzyme-linked Immunosorbent Assay
GHRL	Ghrelin
GHSR-1a	Growth Hormone Secretagogue Receptor Type 1a
HIOMT	Hydroxyindole O-methyltransferase
LD	Long-Day Photoperiod, Long-Day Season
MEL	Melatonin
NAS	N-acetylserotonin
pCPA	*Para*chlorophenylalanine
PG	Pineal Gland
RIA	Radioimmunoassay
SCN	Suprachiasmatic Nucleus
SD	Short-Day Photoperiod, Short-Day Season
TPH1	Tryptophan 5-hydroxylase 1

References

1. Kirsz, K.; Zieba, D. A., Ghrelin-mediated appetite regulation in the central nervous system. *Peptides* **2011,** 32, (11), 2256-64.
2. Laermans, J.; Vancleef, L.; Tack, J.; Depoortere, I., Role of the clock gene Bmal1 and the gastric ghrelin-secreting cell in the circadian regulation of the ghrelin-GOAT system. *Scientific reports* **2015,** 5, 16748.
3. Yi, C. X.; Challet, E.; Pevet, P.; Kalsbeek, A.; Escobar, C.; Buijs, R. M., A circulating ghrelin mimetic attenuates light-induced phase delay of mice and light-

 doi:10.20944/preprints201702.0101.v1

induced Fos expression in the suprachiasmatic nucleus of rats. *The European journal of neuroscience* **2008,** 27, (8), 1965-72.

4. Horvath, T. L.; Abizaid, A.; Dietrich, M. O.; Li, Y.; Takahashi, J. S.; Bass, J., Ghrelin-immunopositive hypothalamic neurons tie the circadian clock and visual system to the lateral hypothalamic arousal center. *Molecular metabolism* **2012,** 1, (1-2), 79-85.
5. Harrison, J. L.; Miller, D. W.; Findlay, P. A.; Adam, C. L., Photoperiod influences the central effects of ghrelin on food intake, GH and LH secretion in sheep. *Neuroendocrinology* **2008,** 87, (3), 182-92.
6. Zieba, D. A.; Kirsz, K.; Molik, E.; Romanowicz, K.; Wojtowicz, A. K., Effects of orexigenic peptides and leptin on melatonin secretion during different photoperiods in seasonal breeding ewes: an *in vitro* study. *Domestic animal endocrinology* **2011,** 40, (3), 139-46.
7. Zieba, D. A.; Kirsz, K.; Szczesna, M.; Molik, E.; Romanowicz, K.; Misztal, T., Photoperiod influences the effects of ghrelin and serotonin receptor agonist on growth hormone and prolactin secretion in sheep. *Journal of neurology and neurophysiology* **2015,** 6:4.
8. Kirsz, K.; Szczesna, M.; Molik, E.; Misztal, T.; Wojtowicz, A. K.; Zieba, D. A., Seasonal changes in the interactions among leptin, ghrelin, and orexin in sheep. *Journal of animal science* **2012,** 90, (8), 2524-31.
9. Kirsz, K.; Szczesna, M.; Dudek, K.; Bartlewski, P. M.; Zieba, D. A., Influence of season and nutritional status on the direct effects of leptin, orexin-A and ghrelin on

luteinizing hormone and growth hormone secretion in the ovine pituitary explant model. *Domestic animal endocrinology* **2014,** 48, 69-76.

10. Kirsz, K.; Szczesna, M.; Molik, E.; Misztal, T.; Zieba, D. A., Induction of the secretion of LH and GH by orexin A and ghrelin is controlled *in vivo* by leptin and photoperiod in sheep. *Annals of animal science* **2017,** 17, 155–168.
11. Mustonen, A. M.; Pyykonen, T.; Asikainen, J.; Hanninen, S.; Mononen, J.; Nieminen, P., Circannual leptin and ghrelin levels of the blue fox (Alopex lagopus) in reference to seasonal rhythms of body mass, adiposity, and food intake. *Journal of experimental zoology. Part A, Comparative experimental biology* **2005,** 303, (1), 26-36.
12. Nieminen, P.; Mustonen, A. M.; Asikainen, J.; Hyvarinen, H., Seasonal weight regulation of the raccoon dog (Nyctereutes procyonoides): interactions between melatonin, leptin, ghrelin, and growth hormone. *Journal of biological rhythms* **2002,** 17, (2), 155-63.
13. Healy, J. E.; Ostrom, C. E.; Wilkerson, G. K.; Florant, G. L., Plasma ghrelin concentrations change with physiological state in a sciurid hibernator (Spermophilus lateralis). *General and comparative endocrinology* **2010,** 166, (2), 372-8.
14. Maronde, E.; Saade, A.; Ackermann, K.; Goubran-Botros, H.; Pagan, C.; Bux, R.; Bourgeron, T.; Dehghani, F.; Stehle, J. H., Dynamics in enzymatic protein complexes offer a novel principle for the regulation of melatonin synthesis in the human pineal gland. *Journal of pineal research* **2011,** 51, (1), 145-55.
15. Rekasi, Z.; Varga, J. L.; Schally, A. V.; Halmos, G.; Groot, K.; Czompoly, T., Antagonistic actions of analogs related to growth hormone-releasing hormone

(GHRH) on receptors for GHRH and vasoactive intestinal peptide on rat pituitary and pineal cells *in vitro*. *Proceedings of the national academy of sciences USA* **2000,** 97, (3), 1218–1223.

16. Sanchez-Bretano, A.; Blanco, A. M.; Unniappan, S.; Kah, O.; Gueguen, M. M.; Bertucci, J. I.; Alonso-Gomez, A. L.; Valenciano, A. I.; Isorna, E.; Delgado, M. J., In Situ Localization and Rhythmic Expression of Ghrelin and ghs-r1 Ghrelin Receptor in the Brain and Gastrointestinal Tract of Goldfish (Carassius auratus). *PloS one* **2015,** 10, (10), e0141043.
17. Privat, K.; Brisson, C.; Jouvet, A.; Chesneau, D.; Ravault, J. P.; Fevre-Montange, M., Evidence for implication of tryptophan hydroxylase in the regulation of melatonin synthesis in ovine pinealocytes in culture. *Cellular and molecular neurobiology* **2002,** 22, (4), 417-29.
18. Miguez, J. M.; Simonneaux, V.; Pevet, P., The role of the intracellular and extracellular serotonin in the regulation of melatonin production in rat pinealocytes. *Journal of pineal research* **1997,** 23, (2), 63-71.
19. Ganguly, S.; Gastel, J. A.; Weller, J. L.; Schwartz, C.; Jaffe, H.; Namboodiri, M. A.; Coon, S. L.; Hickman, A. B.; Rollag, M.; Obsil, T.; Beauverger, P.; Ferry, G.; Boutin, J. A.; Klein, D. C., Role of a pineal cAMP-operated arylalkylamine N-acetyltransferase/14-3-3-binding switch in melatonin synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **2001,** 98, (14), 8083-8.
20. Lewczuk, B.; Ziolkowska, N.; Prusik, M.; Przybylska-Gornowicz, B., Adrenergic activation of melatonin secretion in ovine pineal explants in short-term superfusion

culture occurs via protein synthesis independent and dependent phenomena. *BioMed research international* **2014,** 2014, 715708.

21. Schomerus, C.; Korf, H. W.; Laedtke, E.; Weller, J. L.; Klein, D. C., Selective adrenergic/cyclic AMP-dependent switch-off of proteasomal proteolysis alone switches on neural signal transduction: an example from the pineal gland. *Journal of neurochemistry* **2000,** 75, (5), 2123-32.
22. Schomerus, C.; Laedtke, E.; Korf, H. W., Activation of arylalkylamine N-acetyltransferase by phorbol esters in bovine pinealocytes suggests a novel regulatory pathway in melatonin synthesis. *Journal of neuroendocrinology* **2004,** 16, (9), 741-9.
23. Coon, S. L.; Del Olmo, E.; Young, W. S., 3rd; Klein, D. C., Melatonin synthesis enzymes in Macaca mulatta: focus on arylalkylamine N-acetyltransferase (EC 2.3.1.87). *The Journal of clinical endocrinology and metabolism* **2002,** 87, (10), 4699-706.
24. Misztal, T.; Romanowicz, K.; Barcikowski, B., Melatonin--a modulator of the GnRH/LH axis in sheep. *Reproductive biology* **2002,** 2, (3), 267-75.
25. Djeridane, Y.; Touitou, Y., Lack of effect of ghrelin treatment on melatonin production in rat pineal and Harderian glands. *Life sciences* **2005,** 76, (20), 2393-401.
26. Namboodiri, M.; Valivuallah, H.; Moffett, J., Regulation of melatonin synthesis in the ovine pineal gland. *Advances in Experimental Medicine and Biology* **1991**, 294:137-48.
27. Reiter, R. J., Pineal melatonin: Cell biology of its synthesis and of its physiological interactions. *Endocrine Reviews* **1991,** 12:151-180.

28. Huang, Z.; Liu, T.; Chattoraj, A.; Ahmed, S.; Wang, M. M.; Deng, J.; Sun, X.; Borjigin, J., Posttranslational regulation of TPH1 is responsible for the nightly surge of 5-HT output in the rat pineal gland. *Journal of pineal research* **2008,** 45, (4), 506-14.

29. Schellekens, H.; van Oeffelen, W. E.; Dinan, T. G.; Cryan, J. F., Promiscuous dimerization of the growth hormone secretagogue receptor (GHS-R1a) attenuates ghrelin-mediated signaling. *The Journal of biological chemistry* **2013,** 288, (1), 181-191.

30. Brunetti, L.; Recinella, L.; Orlando, G.; Michelotto, B.; Di Nisio, C.; Vacca, M., Effects of ghrelin and amylin on dopamine, norepinephrine and serotonin release in the hypothalamus. *European journal of pharmacology* **2002,** 454, (2-3), 189-92.

31. Ghersi, M. S.; Casas, S. M.; Escudero, C.; Carlini, V. P.; Buteler, F.; Cabrera, R. J.; Schioth, H. B.; de Barioglio, S. R., Ghrelin inhibited serotonin release from hippocampal slices. *Peptides* **2011,** 32, (11), 2367-71.

32. Hansson, C.; Alvarez-Crespo, M.; Taube, M.; Skibicka, K. P.; Schmidt, L.; Karlsson-Lindahl, L.; Egecioglu, E.; Nissbrandt, H.; Dickson, S. L., Influence of ghrelin on the central serotonergic signaling system in mice. *Neuropharmacology* **2014,** 79, 498-505.

33. Schellekens, H.; De Francesco, P. N.; Kandil, D.; Theeuwes, W. F.; McCarthy, T.; van Oeffelen, W. E.; Perello, M.; Giblin, L.; Dinan, T. G.; Cryan, J. F., Ghrelin's Orexigenic Effect Is Modulated via a Serotonin 2C Receptor Interaction. *ACS chemical neuroscience* **2015,** 6, (7):1186-1197.

 doi:10.20944/preprints201702.0101.v1

34. Sugden, D., Comparison of circadian expression of tryptophan hydroxylase isoform mRNAs in the rat pineal gland using real-time PCR. *Journal of neurochemistry* **2003,** 86, (5), 1308-11.

35. Steardo, L.; Monteleone, P.; Trabace, L.; Cannizzaro, C.; Maj, M.; Cuomo, V., Serotonergic modulation of rat pineal gland activity: *in vivo* evidence for a 5-Hydroxytryptamine(2C) receptor involvement. *The Journal of pharmacology and experimental therapeutics* **2000,** 295, (1), 266-73.

36. Govitrapong, P.; Prapapanich, V.; Ebadi, M., Identification of serotonin 5HT2 receptors in bovine pineal gland. *Journal of pineal research* **1991,** 11, (3-4), 182-7.

37. Schomerus, C.; Laedtke, E.; Olcese, J.; Weller, J. L.; Klein, D. C.; Korf, H. W., Signal transduction and regulation of melatonin synthesis in bovine pinealocytes: impact of adrenergic, peptidergic and cholinergic stimuli. *Cell and tissue research* **2002,** 309, (3), 417-28.

38. Foulkes, N. S.; Borjigin, J.; Snyder, S. H.; Sassone-Corsi, P., Rhythmic transcription: the molecular basis of circadian melatonin synthesis. *Trends in neurosciences* **1997,** 20, (10), 487-92.

39. Lee, H. R.; Kim, T. D.; Kim, H. J.; Jung, Y.; Lee, D.; Lee, K. H.; Kim, D. Y.; Woo, K. C.; Kim, K. T., Heterogeneous ribonucleoprotein R regulates arylalkylamine N-acetyltransferase synthesis via internal ribosomal entry site-mediated translation in a circadian manner. *Journal of pineal research* **2015,** 59, (4), 518-29.

40. Pozdeyev, N.; Taylor, C.; Haque, R.; Chaurasia, S. S.; Visser, A.; Thazyeen, A.; Du, Y.; Fu, H.; Weller, J.; Klein, D. C.; Iuvone, P. M., Photic regulation of arylalkylamine

N-acetyltransferase binding to 14-3-3 proteins in retinal photoreceptor cells. Journal of Neuroscience **2006,** 26, (36), 9153-61.

41. Miller, D. W.; Harrison, J. L.; Brown, Y. A.; Doyle, U.; Lindsay, A.; Adam, C. L.; Lea, R. G., Immunohistochemical evidence for an endocrine/paracrine role for ghrelin in the reproductive tissues of sheep. *Reproductive biology and endocrinology : RB&E* **2005,** 3, 60.

42. Huang, Z. G.; Xiong, L.; Liu, Z. S.; Qiao, Y.; Dai, R.; Xie, Z.; Liu, S. R.; Shi, G. Q.; Liu, G. Q., The tissue distribution and developmental changes of ghrelin mRNA expression in sheep. *Yi chuan xue bao = Acta genetica Sinica* **2006,** 33, (9), 808-13.

43. Tan, D. X.; Manchester, L. C.; Reiter, R. J., CSF generation by pineal gland results in a robust melatonin circadian rhythm in the third ventricle as an unique light/dark signal. *Medical hypotheses* **2016,** 86, 3-9.

44. Tritos, N. A.; Kokkinos, A.; Lampadariou, E.; Alexiou, E.; Katsilambros, N.; Maratos-Flier, E., Cerebrospinal fluid ghrelin is negatively associated with body mass index. *Journal of clinical endocrinology and metabolism* 2003, 88,(6), 2943-6.

45. Cabral, A.; Fernandez, G.; Perello, M., Analysis of brain nuclei accessible to ghrelin present in the cerebrospinal fluid. *Neuroscience* **2013,** 253, 406-15.

46. Grouselle, D.; Chaillou, E.; Caraty, A.; Bluet-Pajot, M. T.; Zizzari, P.; Tillet, Y.; Epelbaum, J., Pulsatile cerebrospinal fluid and plasma ghrelin in relation to growth hormone secretion and food intake in the sheep. *Journal of neuroendocrinology* **2008,** 20, (10), 1138-46.

47. Nowicki, M.; Wojtkiewicz, J.; Lewczuk, B.; Kosacka, J.; Majewski, M.; Przybylska-Gornowicz, B., Peptidergic and nitrergic innervation of the pineal gland in the

domestic pig: an immunohistochemical study. *Anatomia, histologia, embryologia* **2007,** 36, (4), 311-20.

48. Simonneaux, V.; Ribelayga, C., Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. *Pharmacological reviews* **2003,** 55, (2), 325-95.

49. Kirsz, K.; Szczesna, M.; Borsuk, A.; Zieba D. A., Cross-talk between leptin, ghrelin and orexins in the central nervous system of seasonal animals – a review. *Annals of animal science* **2017,** DOI: 10.1515/aoas-2016-0070.

50. Legros, C.; Chesneau, D.; Boutin, J. A.; Barc, C.; Malpaux, B., Melatonin from cerebrospinal fluid but not from blood reaches sheep cerebral tissues under physiological conditions. *Journal of neuroendocrinology* **2014,** 26, (3), 151-63.

51. Reiter, R. J., Action spectra, dose-response relationships, and temporal aspects of light's effects on the pineal gland. *Annals of the New York Academy of Sciences* **1985,** 453, 215-30.

52. Zieba, D. A.; Klocek, B.; Williams, G. L.; Romanowicz, K.; Boliglowa, L.; Wozniak, M., In vitro evidence that leptin suppresses melatonin secretion during long days and stimulates its secretion during short days in seasonal breeding ewes. *Domestic animal endocrinology* **2007,** 33, (3), 358-65.

53. Fraser, S.; Cowen, P.; Franklin, M.; Franey, C.; Arendt, J., Direct radioimmunoassay for melatonin in plasma. *Clinical chemistry* **1983,** 29, (2), 396-7.

 doi:10.20944/preprints201702.0101.v1

54. Misztal, T.; Romanowicz, K.; Barcikowski, B., Seasonal changes of melatonin secretion in relation to the reproductive cycle in sheep. *Journal of animal and feed sciences* **1996**, 56:35–48.

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Figure 1A

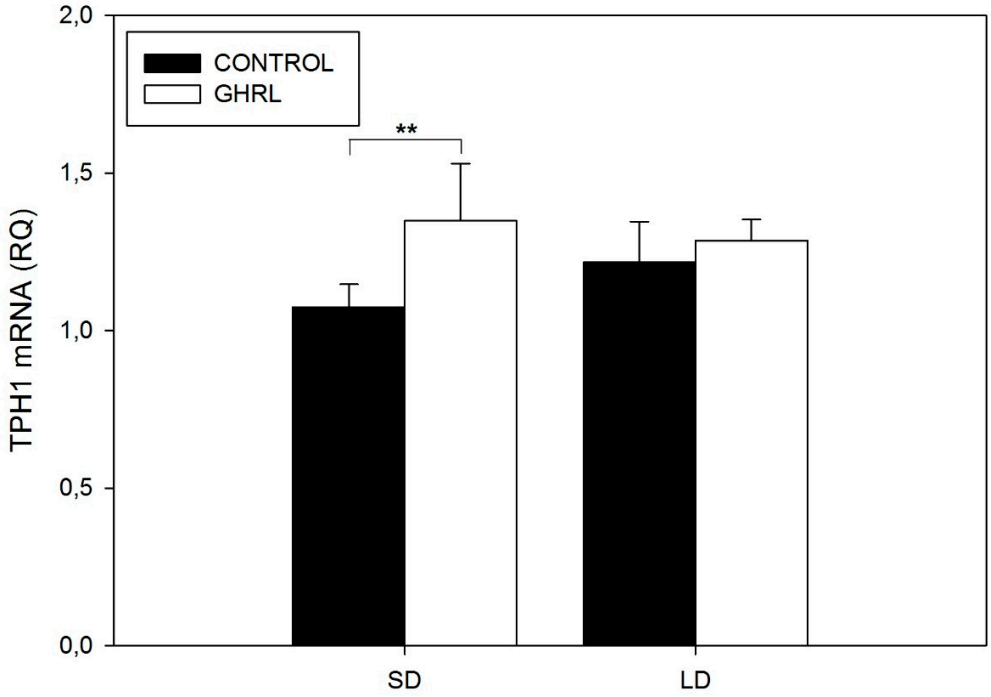

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Figure 1B

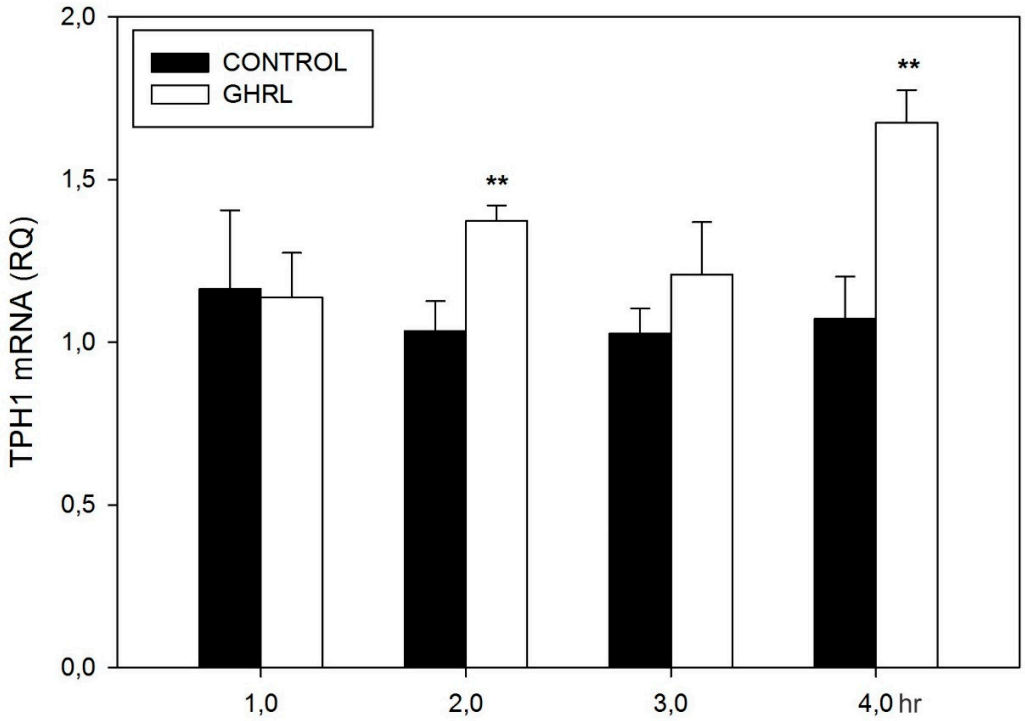

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Figure 1C

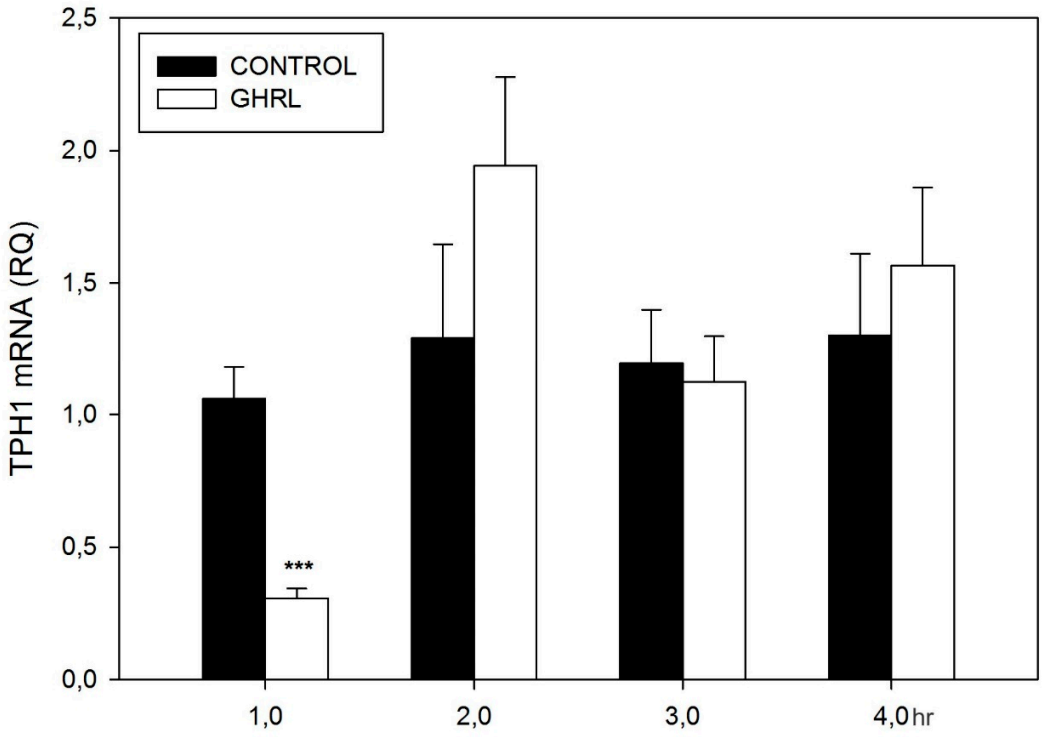

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Figure 1D

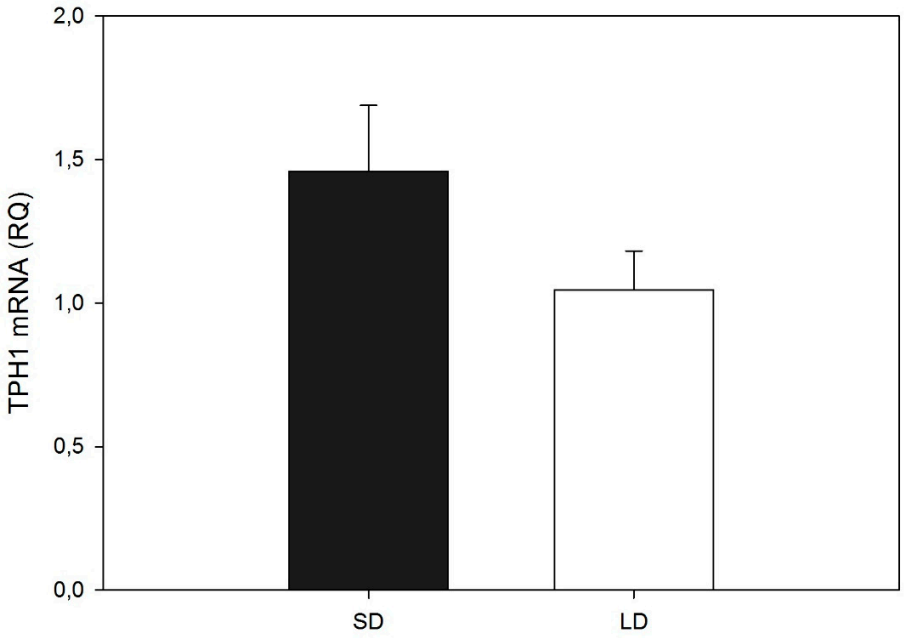

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Figure 2A

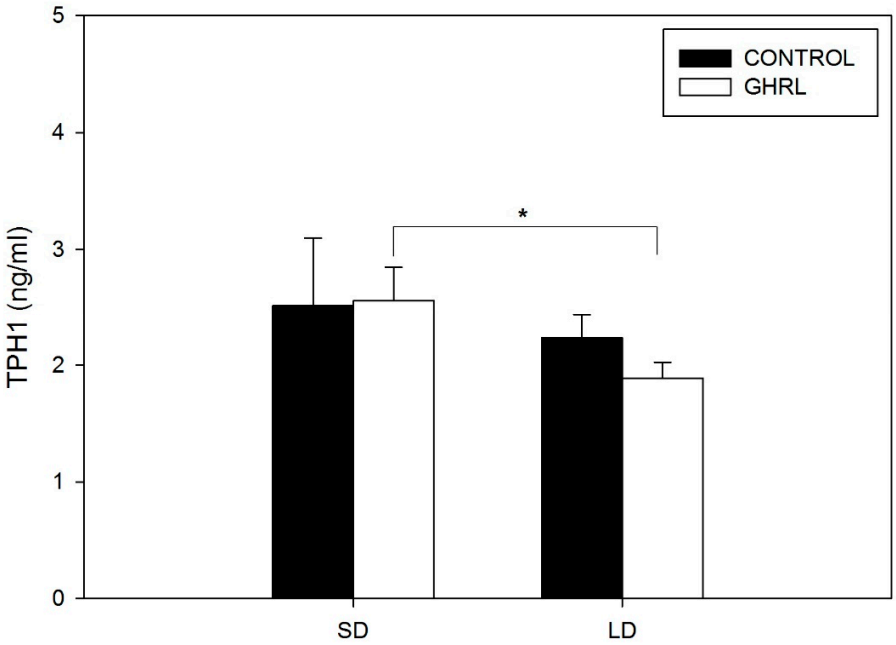

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Figure 2B

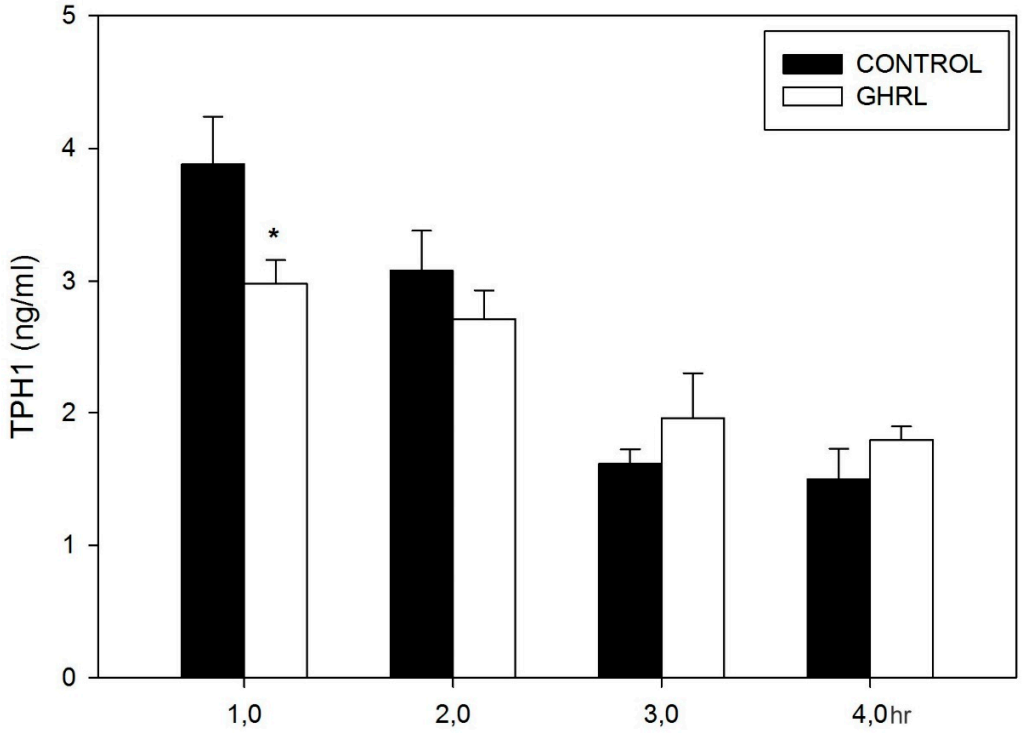

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Figure 2C

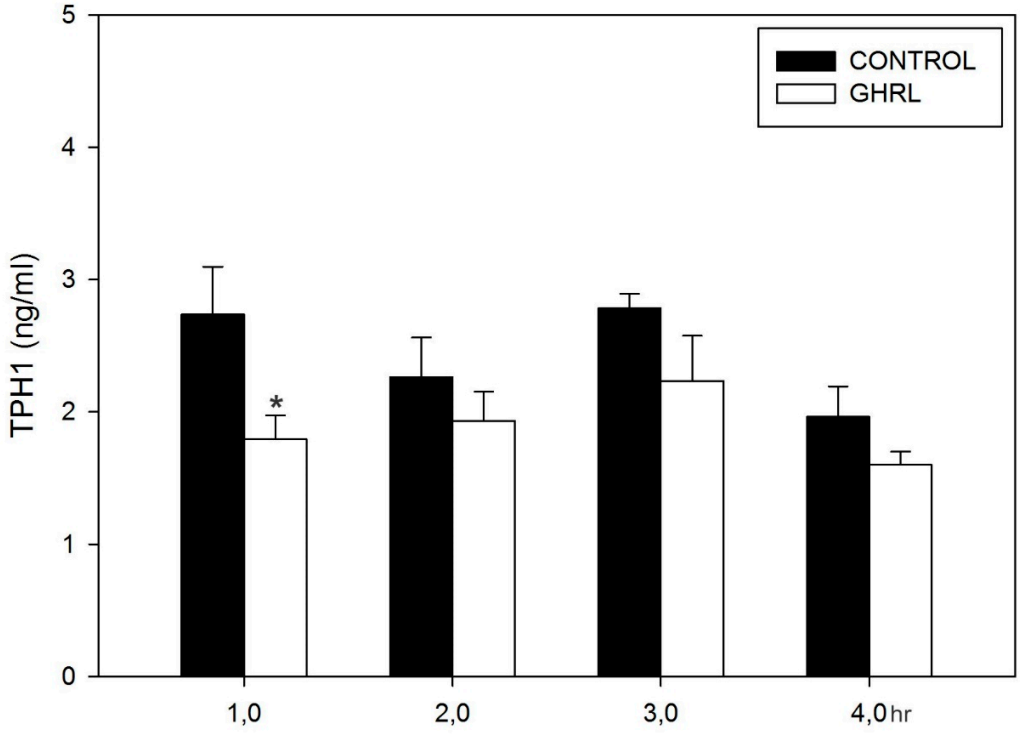

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Figure 3A

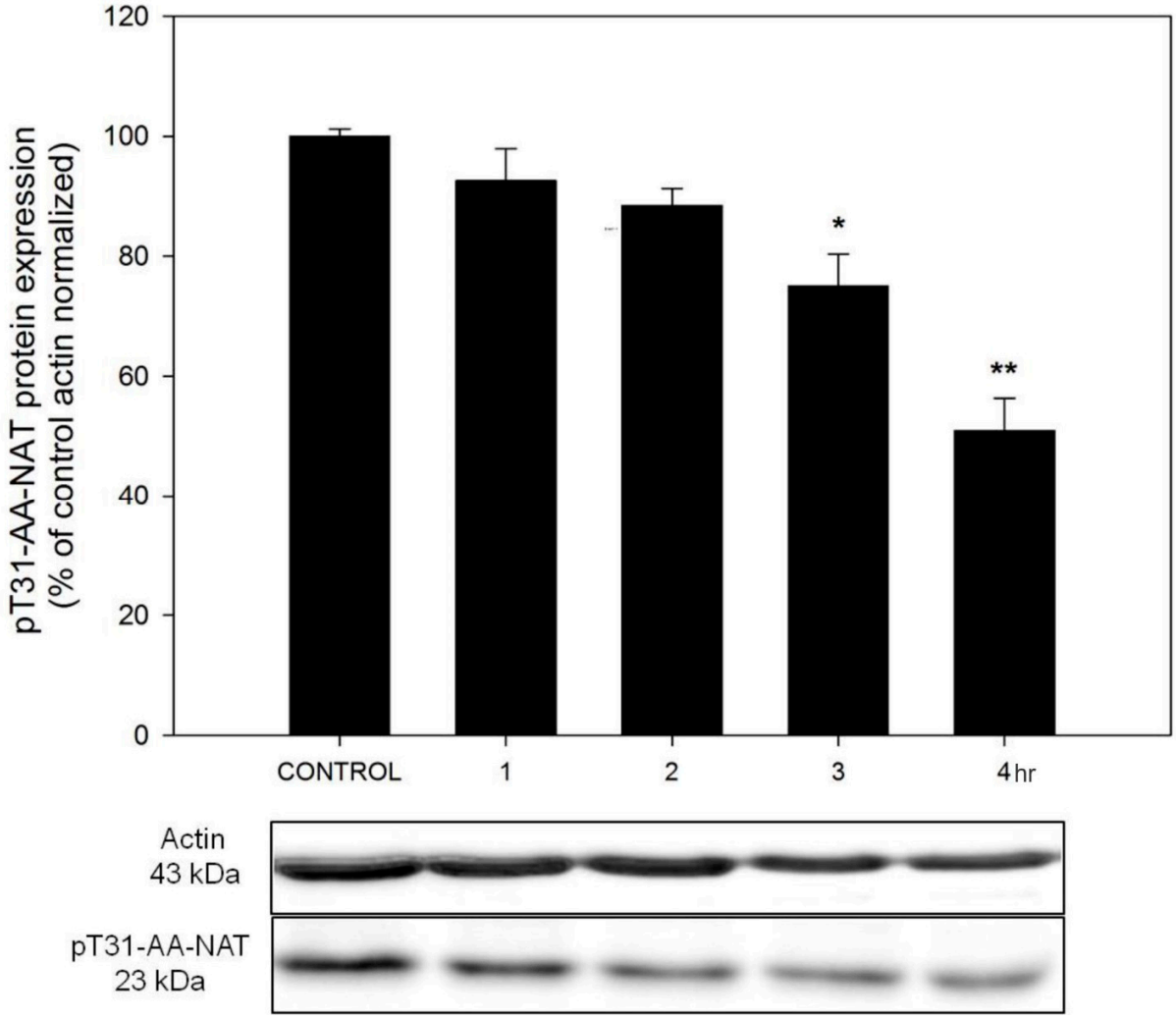

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Figure 3B

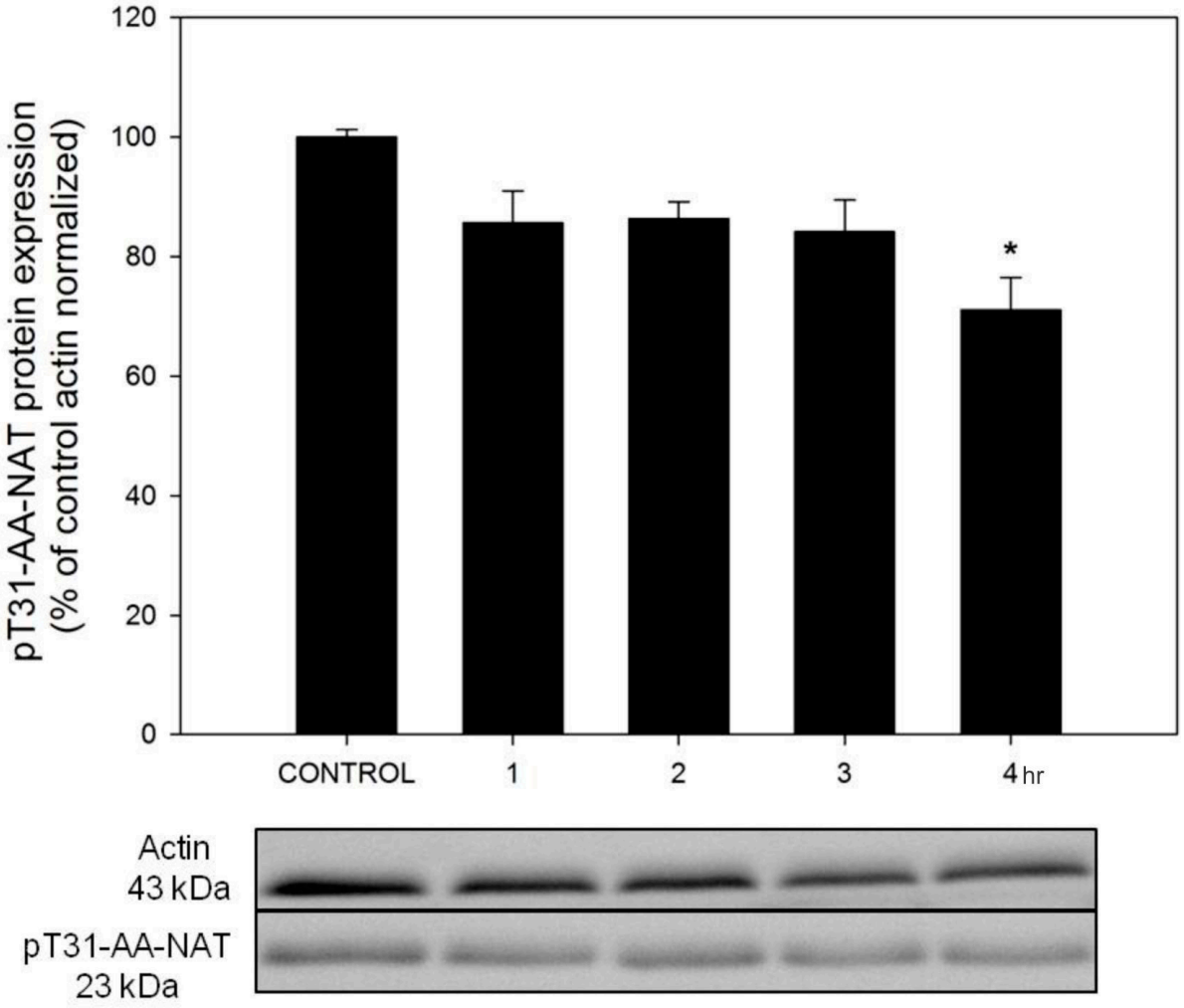

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Figure 4A

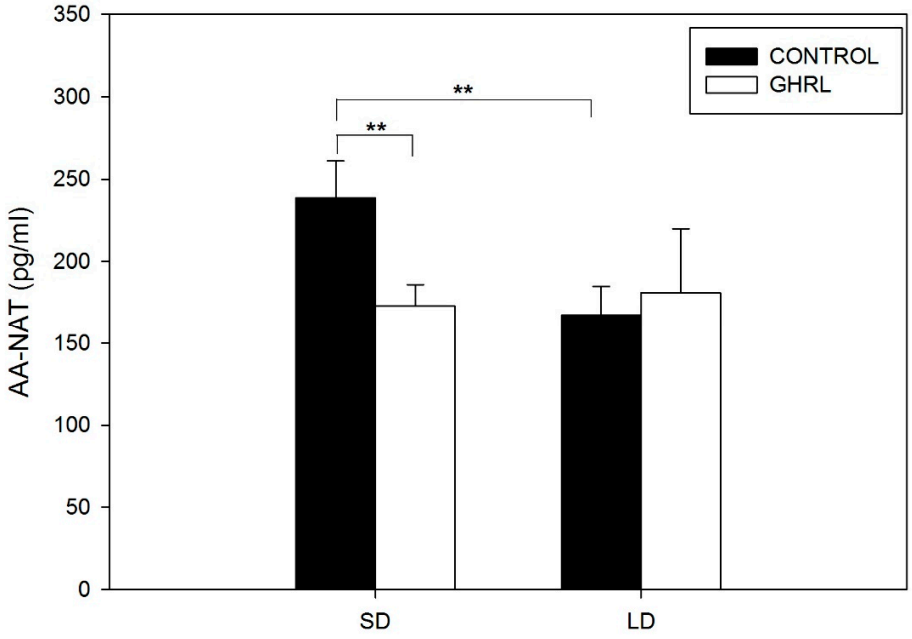

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Figure 4B

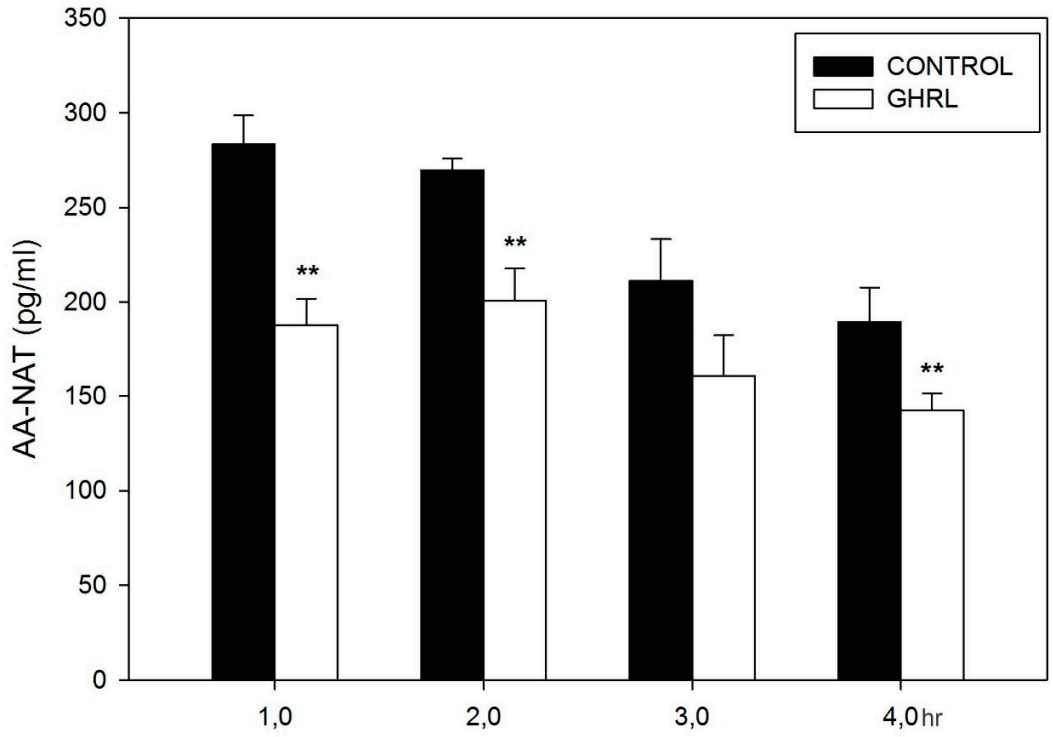

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Figure 4C

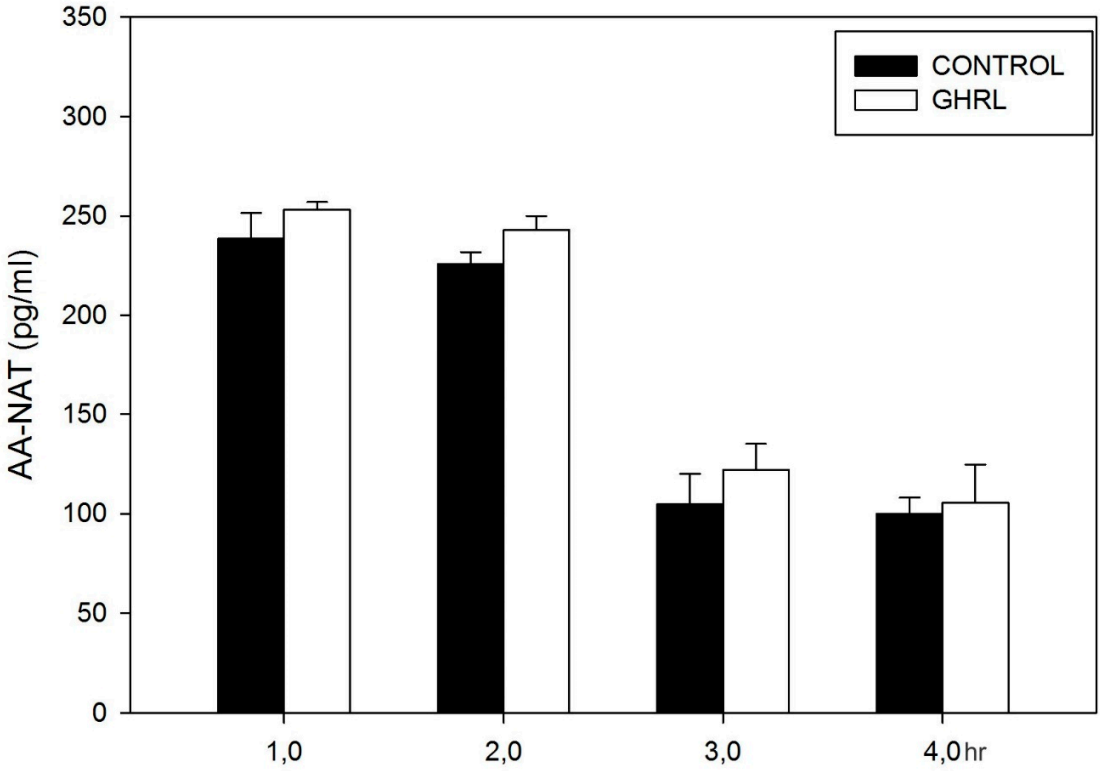

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Figure 5