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SUMMARY STATEMENT

The changes in clock gene expression induced by ghrelin in the goldfish liver suggest a novel role for this peptide in the entrainment of the circadian system.

ABSTRACT

The liver is the most important link between the circadian system and metabolism. As a food entrainable oscillator, the hepatic clock needs to be entrained by food-related signals. The objective of the present study was to investigate the possible role of ghrelin (an orexigenic peptide mainly synthesized in the gastrointestinal tract) as an endogenous synchronizer of the liver oscillator in teleosts. To achieve this aim, we first examined the presence of ghrelin receptors in the liver of goldfish. Then, the ghrelin regulation of clock gene expression in the goldfish liver was studied. Finally, the possible involvement of the PLC/PKC and AC/PKA intracellular signaling pathways was investigated. Ghrelin receptor transcripts, *ghs-r1a,* are present in the majority of the goldfish hepatic cells. Ghrelin induces the mRNA expression of the positive (g*bmal1a*, *gclock1a*) and negative (g*per genes*) elements of the main loop of the molecular clock machinery, as well as of *grev-erbα* (auxiliary loop) in cultured liver. These effects are blocked, at least in part, by a ghrelin antagonist. Incubation of liver with a phospholipase-C inhibitor (U73122), a protein-kinase-C activator (phorbol-12-myristate-13-acetate) and a protein-kinase-C inhibitor (chelerythrine-chloride) demonstrates that the PLC-PKC pathway mediates such ghrelin actions. Studies with an adenylate cyclase activator (forskolin) and a protein-kinase-A inhibitor (H89) show that *grev-erbα* regulation could be due to an activation of protein-kinase-A. Taken together, present results show for the first time in vertebrates a direct action of ghrelin on hepatic clock genes and support a role for this hormone as a temporal messenger in the entrainment of liver circadian functions.

INTRODUCTION

The circadian system controls physiological rhythms that let organisms anticipate cyclic environmental changes. In vertebrates, this endogenous timing system consists of multiple coupled central and peripheral oscillators that are entrained by environmental cues (Albrecht, 2012; Tsang et al., 2014). The molecular basis of these oscillators is well conserved among vertebrates (Dunlap, 1999; Panda et al., 2002) and it is based on interlocked auto-regulatory feedback loops of genes known as clock genes. The positive limb of the main loop includes *clock* and *bmal1* genes (Circadian Locomotor Output Cycles Kaput, and Brain and Muscle ARNT-Like 1, respectively). These genes form the heterodimer CLOCK-BMAL1 that activates the transcription of the negative elements, the *per* (*Period*) and *cry* (*Cryptochrome*) clock genes, whose protein products inhibit CLOCK-BMAL1 transactivation (Hastings et al., 2007; Nader et al., 2010; Schibler et al., 2015). This main auto-regulatory loop is stabilized by an auxiliary loop formed by *rev-erbα* (V-erbA-related protein EAR-1) and *ror* (Retinoic acid related orphan receptor) genes, which mainly modulates *bmal1* expression (Nader et al., 2010; Schibler et al., 2015).

It is well known that the light-dark and feeding cycles may act as potent synchronizers of locomotor activity daily rhythms in vertebrates, including teleosts (Bechtold, 2008; Madrid et al., 2001; Mistlberger, 2011; Sánchez-Vázquez and Madrid, 2001; Spieler, 1992; Stephan, 2002). However, the food-related signals that entrain the molecular clocks (or food entrained oscillators, FEOs) in the circadian system remain unknown. In mammals, the oscillators of the circadian system respond to feeding inputs with different sensitivities, being the liver one of the most sensitive peripheral oscillators in these vertebrates (Albrecht, 2012; Damiola et al., 2000; Reddy et al., 2007; Schibler et al., 2015; Schmutz et al., 2012; Sujino et al., 2012). In teleosts, the liver is highly sensitive to the feeding/fasting cycle and food related signals (Costa et al. 2016; del Pozo et al., 2012; Feliciano et al., 2011; López-Olmeda et al., 2010; Vera et al.,

2013; Sánchez-Bretaño et al. 2015b). Some studies suggest that the liver in fish may be acting as an oscillator which is synchronized by photoperiod and feeding schedule (del Pozo et al., 2012; Feliciano et al., 2011; López-Olmeda et al., 2010; Martín-Robles et al., 2011; Sánchez-Bretaño et al., 2015a and 2015b; Tinoco et al., 2014; Vera et al., 2013).

Ghrelin is a peripheral orexigenic peptide hormone mainly involved in energy balance by stimulating food intake, carbohydrate utilization and adiposity (Abizaid and Horvath, 2012; Delporte, 2013), although it also exerts a wide variety of physiological functions(Delporte, 2013; Sato et al., 2012). This hormone displays a daily rhythm in mice stomach expression and content (LeSauter et al., 2009), and in rat hypothalamus and plasma (Bodosi et al., 2004; Patton et al., 2014). Ghrelin is also rhythmically expressed in the hypothalamus, pituitary and anterior intestine of goldfish, *Carassius auratus* Linnaeus 1758 (Sánchez-Bretaño et al., 2015c). Such ghrelin rhythms have been mainly related with the feeding-fasting cycle, and it has been suggested that this hormone may drive food anticipatory activity acting as an output of the FEOs (LeSauter et al., 2009; Nisembaum et al., 2014; Patton et al., 2014). Additionally, some studies in mouse and goldfish point out ghrelin as an input of circadian clocks, by signalling feeding-fasting rhythms. Indeed, ghrelin induces a phase advance and a delay of the spontaneous firing rhythm and clock gene expression *in vivo* and in cultured mouse suprachiasmatic nuclei (Yannielli et al., 2007; Zhou et al., 2014). In goldfish, the peripheral administration of ghrelin stimulates *per* expression in hypothalamus and liver (Nisembaum et al., 2014). While this background is available, the direct action of ghrelin on the liver oscillator is unexplored to date, which would be a requirement to support the role of this hormone as a temporal messenger in the entrainment of circadian liver functions.

Ghrelin actions are mediated by G-protein coupled receptors known as growth hormone secretagogue receptors (GHS-R) or ghrelin receptors (Kaiya et al., 2013; Kojima et al., 1999). In otophysi teleosts, two paralog *ghs-r* genes have been identified (GHS-R1 and GHS-R2),

which has been tetraploidized in the members of the Cyprininae subfamily (e.g., goldfish) resulting in the presence of four receptor subtypes (GHS-R1a1, GHS-R1a2, GHS-R2a1 and GHS-R2a2, Kaiya et al., 2010). Among the different GHS-R subtypes, GHS-R1a seems to be involved in most of the ghrelin physiological actions (Gnanapavan et al., 2002; Kaiya et al., 2013; Yin et al., 2014). This receptor is mainly coupled to the phospholipase C (PLC)- protein kinase C (PKC) pathway (Kojima et al., 1999; Yin et al., 2014), but it can also trigger alternative intracellular pathways, including the adenylyl cyclase (AC)- protein kinase A (PKA) pathway (Kohno et al., 2003).

Considering the relevance of the liver in synchronizing feeding inputs in both mammals and fish (supporting its role as a food entrainable oscillator), and the role of ghrelin as a signal of the feeding-fasting cycle, it is plausible that this hormone might link the energy status and the circadian system by acting as an input of the hepatic oscillator. The similar anatomical distribution of *GHS-R1a* and *per1b* expression in the forebrain and gut of goldfish (Sánchez-Bretaño et al., 2015a; Sánchez-Bretaño et al., 2015c) supports the possible role of ghrelin as an input of circadian clocks in this teleost. To test this possible role of ghrelin, the present study investigates the possible direct regulatory role of ghrelin on the hepatic molecular clock of goldfish. To achieve this aim, we first reported the expression of the ghrelin receptor GHS-R1a in the liver of this teleost by *in situ* hybridization. Second, we demonstrated that ghrelin modulates the *in vitro* expression of hepatic clock genes (*gper1a*, *gper1b*, *gper2a*, *gper3*, *gbmal1a*, *gclock1a* and *grev-erbα*) in a concentration-dependent manner, and that these effects are counteracted by the ghrelin antagonist [D-Lys3]-Growth hormone releasing peptide-6 ([D-Lys3]-GHRP-6). Finally, we have investigated the possible involvement of the PLC/PKC and AC/PKA intracellular pathways on such ghrelin-induced modulation of clock genes in the goldfish hepatic clock, providing for the first time in vertebrates a putative mechanism by which ghrelin can act as an input of circadian clocks.

MATERIAL AND METHODS

Animals and sampling

For the anatomical experiments, goldfish (2.0 ± 0.5 g; n=7) obtained from a local supplier (Rennes, France) were maintained in 60 l aquaria with filtered and aerated fresh water (22 ± 1°C) under a 12 h light: 12 h darkness (12L:12D) photoperiod (lights on at 9 AM). Fish were daily fed at 11 AM (*zeitgeber* time 2, ZT2) with food pellets (1% body weight, bw; Novo GranoMix, JBL, GmbH & Co., Neuhofen, Germany). Goldfish (48 h fasted) were anesthetized at ZT2 with phenoxyethanol 1 ml/l (ICN Biomedicals Inc., Irvine, CA, USA) and sacrificed. Then, the whole fish was immersed overnight in 4% paraformaldehyde diluted in 0.1 M phosphate buffer saline (PBS, pH 7.4). The following day, the liver was removed and post-fixed 3 h in the same solution. Samples were cryoprotected overnight with 30% sucrose (MP Biomedical, LLC, Illkirch, France), included in the frozen section medium Richard-Allan Scientific™ Neg-50 (Thermo Shandon Scientific, Cheshir, UK) and stored at -80°C.

For *in vitro* studies, goldfish (7.2 ± 0.5 g) obtained from a local supplier (Madrid, Spain) were maintained in 60 l aquaria with filtered and aerated fresh water (22 ± 1°C) under a 12 h light: 12 h darkness (12L:12D) photoperiod (lights on at 8 AM). Fish were daily fed at ZT2 with food pellets (1% bw; Bioflakes, Sera Pond, Heidelberg, Germany). The day of the experiment, non-fed goldfish were anesthetized in MS-222 (0.175 g/l, Sigma Aldrich, CA, USA) at ZT2. Then, animals were sacrificed and the liver was quickly sampled and distributed in different wells (15 mg liver/well) of sterile culture 24-well multidish plates.

Fish handling procedures complied with International Standards for the Care and Use of Laboratory Animals and were in accordance with the Guidelines of the European Union Council (2010/63/EU) for the use of research animals.

Location of *ghs-r1a* in the liver of goldfish by *in situ* hybridization

The probes for *in situ* hybridization (ISH) were synthesized from plasmids (pCR™4-TOPO® vector; Invitrogen, Carlsbad, CA, USA) containing a 979 bp of goldfish *ghs-r1a* (Sánchez-Bretaño et al., 2015c). This probe targets a common fragment of goldfish *ghs-r1a1* and *ghs-r1a2* (accession numbers AB504275.1 and AB504276.1). Plasmids with the insert were linearized with *SpeI* and *NotI*, and antisense and sense mRNA probes were obtained with DIG RNA labeling MIX (Roche Diagnostic, Mannheim, Germany) by *in vitro* transcription with T7 and T3 RNA polymerases (Promega, Madison, WI, USA). The specificity of the probes was confirmed with parallel series of slides hybridized with the respective sense RNA probes.

The liver obtained and stored as above described was included in TissueTek and sectioned at 8 μm using a cryostat. Sections were mounted onto superfrost slides. The ISH was performed as previously described (Escobar et al, 2013) with minor modifications. In brief, cryostat sections were washed in PBS two times during 10 min before postfixation in *Antigenfix* (DiaPath, Martinengo, Italy) for 20 min. After that, sections were treated for 5 min at 37°C with proteinase K (2 μg/ml, Sigma, Steinheim, Germany) diluted in PBS, and fixed in 4% paraformaldehyde for 15 min. Sections were rinsed twice in 2x standard saline citrate (SSC). Hybridization was performed at 65°C overnight in a humidified chamber using 100 μl hybridization buffer (50% deionized formamide; 2x SSC; 5x Denhardt's solution; 50 μg/ml of yeast tRNA; 4 mM EDTA; 2.5% dextran sulfate) containing the DIG-labeled probe (3 μg/ml). After hybridization, slides were washed in 2x SSC at 65°C (2x30 min), 2x SSC/50% formamide at 65°C (2x30 min), 0.2x SSC (1x15 min) and 0.1x SSC (1x15 min) at room temperature. Slides were then washed in 100 mM Tris-HCl (pH 7.5) containing 150 mM NaCl for 10 min, washed in the same buffer containing 0.1% Triton and 0.5% of skimmed milk powder (2x30 min), and incubated overnight at room temperature with anti-digoxigenin

alkaline phosphatase Fab fragments (1:2,000; Roche Pharma, Mannheim, Germany). On the next day, slides were incubated for 4.5 h with an HNPP (2-hydroxy-3-naphtoic acid -2′-phenylanilide phosphate)/FastRED detection kit (Roche Pharma, Mannheim, Germany), according to the manufacturer's instructions. Finally, slides were cover slipped with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Slides were observed with an epifluorescence microscope (Olympus Provis, equipped with a DP71 digital camera). Images were processed with either the Olympus Analysis or Zeiss Cell software. Micrographs were generated in the "TIFF" format and adjusted linearly for light and contrast using Photoshop CS6 before being assembled on plates.

Culture conditions

Liver cultures were performed as previously described (Sánchez-Bretaño et al., 2016). A portion of liver from a different fish was used in each experimental group (n=6 fish/group). Liver portions were preincubated during 2 h in 1 ml of control medium (15 mg liver/ml/well, quantified as 15 µl of tissue; Sánchez-Bretaño et al., 2016). The control medium consists of Dulbecco's modified Eagle medium (DMEM;17.3 g/l Sigma Aldrich, CA, USA) modified for fish tissues by adding $NaHCO_3$ (3.7 g/l) and antibiotics (penicillin-streptomicin 10 ml/l and gentamicin 500 mg/l; Sigma Aldrich, CA, USA). After the 2 h-preincubation period, medium was replaced by 1 ml of fresh DMEM containing the respective vehicle (control groups) or the corresponding drug (treated groups). Incubation time was either 1 h or 5 h depending on the experiment (see figures). The liver cultures were performed under constant dim light and temperature (21 ± 1ºC) conditions. At the end of each culture time, liver samples were collected, quickly frozen in liquid nitrogen and maintained at -80°C until clock genes expression was quantified.

Drugs

Stock solutions were prepared and stored at 4ºC until used. The 17–amino acid isoform of goldfish ghrelin (GTS(octanoyl)FLSPAQKPQGRRPP; Bachem, Bubendorf, Switzerland) and the PKA inhibitor, H89 (Sigma Aldrich, CA, USA), were prepared in distilled water at a concentration of 2 and 15 mM, respectively. Stock solutions of the PLC inhibitor, U73122 (Tocris Bioscience, Bristol, UK), the ghrelin antagonist, [D-Lys^3]-GHRP-6 (Bachem, Bubendorf, Switzerland), and the AC activator, forskolin (Sigma Aldrich, CA, USA), were prepared in absolute ethanol at 1, 10 and 15 mM concentrations, respectively. The PKC inhibitor, chelerythrine chloride (CHEL; Sigma Aldrich, CA, USA) and the PKC activator, phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, CA, USA), stock solutions were prepared in dimethyl sulfoxide (DMSO) at 5 and 20 mM concentrations, respectively. All the stock solutions were diluted in DMEM to reach the required final concentrations just before use. Whenever the experimental design required the use of the antagonist or an inhibitor (i.e. [D-Lys^3]-GHRP-6, U73122, CHEL, H89), such drug was added to the culture medium 15 min prior to the addition of the respective activator of gene expression (i.e. ghrelin-17, PMA, forskolin).

Clock genes expression quantification by Real Time PCR

Clock genes expression (*gper1a*, *gper1b*, *gper2a*, *gper3*, *gbmal1a*, *gclock1a* and *grev-erbα*) was quantified by RT-qPCR using *gβ-actin* as a reference gene as previously described (Nisembaum et al., 2014). Specific primers and Gene Data Bank Reference Numbers are shown in Table 1. The RNA extraction (TRI® Reagent method, Sigma Chemical, Madrid, Spain), DNase treatment (Promega, Madison, USA), cDNA synthesis (Invitrogen, Carlsbad, USA) and real-time PCR reactions (iTaqTM SYBR® Green Supermix in a CFX96TM Real-Time

System, Biorad Laboratories, Hercules, USA) were carried out following manufacturer instructions with minor modifications (Nisembaum et al., 2014). Total RNA (1 µg) was retrotranscribed and PCR reactions were developed in a final volume of 10 µl (2 µl of cDNA per sample). PCR conditions were 30 sec at 95ºC, and 40 cycles consisting of 5 sec at 95ºC and 30 sec at 60ºC for all genes. Calibration curves were made of serial dilutions of cDNA, exhibiting efficiencies of around 100%. Specificity of amplifications was ensured by melting curves and tested by agarose gels. The relative mRNA expression was determined by the ΔΔCt method (Livak and Schmittgen, 2001).

Statistical analysis

Data obtained from ΔΔCt method were logarithmically transformed in order to normalize the variance and to obtain homocedasticity. A probability level of $p<0.05$ was considered as statistically significant. Analysis of the relative expression changes in the ghrelin concentration-response curves was conducted by using one-way ANOVA followed by the post-hoc Student Newman Keuls (SNK) test. A two-way ANOVA followed by the post-hoc SNK was used when both the interaction of activators (ghrelin-17, PMA, forskolin) and inhibitors ([D-Lys^3]-GHRP-6, U73122, CHEL, H89) was studied (see details in the figure legends).

RESULTS

Location of ghrelin receptor in the liver of goldfish

A wide expression of the ghrelin receptor *ghs-r1a* was found in the liver of goldfish (Fig. 1). Almost all the hepatic cells showed a strong *ghs-r1a* signal surrounding the nucleus (Fig. 1A, C), while the sense riboprobes yielded no signal (Fig. 1B, D), supporting the specificity of the obtained signal in the goldfish liver.

Ghrelin as regulator of clock genes expression in cultured liver

Ghrelin modified the expression of some clock genes in the goldfish liver *in vitro* (Fig. 2). All the clock genes studied (with the exception of *gper1b*) were induced by 0.1, 1 and 10 nM ghrelin at 1 h post-treatment (excepting *gclock1a and gper3* in which the induction was observed with ghrelin concentrations greater than 1 nM). The highest induction was found for *gper1a* transcripts which were around 7-fold increased, while the induction of *gper2a* and *gper3* expression was smaller (around 2-fold). Expression of genes from the positive limb, i.e. *gbmal1a* and *gclock1a,* and from the auxiliary loop, *grev-erbα,* was also induced by ghrelin (around 4-5-fold). All the ghrelin-evoked upregulations of clock gene expression observed at 1 h were diminished after 5 h of ghrelin exposure, and even disappeared in the case of *gper3* and *grev-erbα.* Only in the case of *gper1b*, an exposure of the liver to ghrelin (10 nM) during 5 h was a requirement for a 3-fold increase of transcripts to be observed.

The specificity of the ghrelin-evoked inductions of clock gene expression was tested by using a ghrelin antagonist, the [D-Lys^3]-GHRP-6 (Fig. 3). The presence of this antagonist in the culture medium did not modify the expression of clock genes by itself, with the exception of *gper2a*, *gclock1a* and gper3 whose levels were modified by the antagonist after a 5-h incubation (the first two) or after a 1-h incubation (the latter). The preincubation of liver portions with the ghrelin antagonist abolished the stimulatory effect of ghrelin on *gper1a*, *gper2a*, *gbmal1a* and *grev-erbα* expression. This blocking effect was observed after 1 h of exposure to the antagonist, coincident with the time when the inductions evoked by ghrelin were the highest. This blocking effect of the antagonist was also observed at 5 h in the case of *gper1b*. In the case of *gclock1a* and *gper2a*, the counteraction of the ghrelin-evoked inductions was not observed at 5 h, probably due to the significant increase of transcripts observed in the presence of the antagonist alone at this time.

Involvement of the PLC-PKC pathway in the ghrelin regulation of clock gene expression

The preincubation (15 min) with the PLC inhibitor (U73122) prior to the addition of ghrelin totally abolished the induction of hepatic clock genes expression (*gper1a, gper1b, gper2a, gbmal1a* and *grev-erbα*) evoked by the presence of this peptide in the culture medium during 1 h (Fig. 4). In the case of *gclock1a* and *gper3,* the direct stimulatory effect of U73122 on mRNA levels found at 1 h post-incubation probably hampered the blocking effects of the PLC inhibitor on ghrelin-inductions of gene expression. Ghrelin effects at longer exposures (5 h) were blocked by the U73122 only in the case of *gper1a, gper1b* and *gper2a,* while the inhibitor by itself induced *gbmal1a, gclock1a* and *grev-erbα* expression The basal expression of the rest of clock genes was not affected by U73122 at any of the tested concentrations (1 and 10 μM).

The possible role of PKC in the regulation of clock genes by ghrelin was assessed by the use of CHEL, a specific inhibitor of this kinase (Fig. 5). The ghrelin-induction of clock genes expression in cultured liver was totally blocked by the pretreatment with CHEL for the majority of the studied genes (*gper1a, gper2a, gper3, gbmal1a* and*gclock1a*), with the exception of *grev-erbα,* where the induction of mRNA levels produced by ghrelin was only blocked partially. This PKC inhibitor had minor effects on basal clock genes expression at the tested concentrations (1 and 5 μM), except for a slight reduction of *gclock1a* transcripts (Fig. 5). As a positive control for the involvement of PKC on clock genes expression, the liver was treated with a direct PKC activator, the PMA, for 1 h (Fig. 5). This activator exerted slight effects on clock gene expression at a low concentration (50 nM), but significantly induced *gper1a, gper3, gbmal1a, gclock1a* and *grev-erbα* expression at a higher concentration (200 nM) (Fig. 5). The pretreatment of liver with CHEL (5 μM) prior to the addition of PMA

diminished the induction of *gper1a, gper3, gbmal1a,* and *grev-erbα* produced by the activator. Neither ghrelin nor PMA modified *gper1b* expression after 1 h of treatment.

The AC-PKA pathway in the ghrelin regulation of clock gene expression

Forskolin, an AC activator, induced the expression of *per2a, gper3, gclock1a* and *grev-erbα* while it had no effect on the rest of the clock genes analyzed after 1 h of treatment (Fig. 6). The pretreatment with the PKA inhibitor, H89, abolished forskolin effects in all the cases. High concentrations of H89 (200 μM) decreased the expression of *gper3* in cultured liver by itself. The stimulatory effect of ghrelin on *gbmal1a* and *grev-erbα* expression was partially blocked by the pretreatment with H89 (significant interaction in two-way ANOVA), but this PKA inhibitor did not block ghrelin-induced upregulation of *gper1a* and *gper2a* expression. In the case of *gper3,* the down regulatory effect of H89 and the stimulatory effect of ghrelin on its expression seem to be independent actions.

DISCUSSION

In the present work, we report evidences for a direct effect of ghrelin on clock genes expression in the liver of a teleost. We found that ghrelin (acyl-ghrelin-17) induces clock genes expression via its own receptor located in the hepatic cells, and that the intracellular PLC-PKC and AC-PKA pathways are involved in this direct effect**.** This is the first report that links ghrelin with the molecular basis of functional metabolic oscillators in vertebrates.

First, present results show that ghrelin induces the mRNA expression of the positive (*gbmal1a, gclock1a*) and negative (*pger1a, gper2a, gper3*) elements of the main loop of the molecular clock machinery, as well as of *grev-erbα* (auxiliary loop) at short times (1 h), and of *gper1b* at longer times (5 h) in cultured liver of goldfish. This induction of hepatic clock genes

expression by ghrelin seems to be an acute and fast response that disappears after 5 h of exposure to the hormone (except for *gper1b*). These results are in accordance with previous studies in goldfish, where the acute intraperitoneal administration of ghrelin-19 induced *per* genes (*gper1a*, *gper2* and *gper3*) in the liver at 1 h post-injection, effect that disappeared 3 h later (Nisembaum et al., 2014). The delay observed in *gper1b* induction by ghrelin could be due to a different sensitivity of this clock gene to this hormone compared with the other *per* genes present in the liver of goldfish.

The ghrelin receptor antagonist, [D-Lys^3]-GHRP-6, partially blocked the ghrelin-evoked effects on hepatic clock genes in cultured liver, in agreement with previous *in vivo* results carried out in this teleost (Nisembaum et al., 2014). Then, we suggest the involvement of a hepatic ghrelin receptor in the majority of the observed actions of ghrelin as modulator of clock genes expression. The specificity of ghrelin effect on *gclock1a* and *gper3* remains unsolved as the antagonist increases basal levels of these two clock genes by itself. In support of such direct actions of ghrelin on liver cells, our results show for the first time in vertebrates a wide distribution of the ghrelin receptor *ghs-r1a* in the hepatic cells. This is in agreement with the previous identification by PCR of ghrelin receptor in the liver of some teleosts, including the goldfish (Cai et al., 2015; Kaiya et al., 2010).

The GHS-R1a ghrelin receptor subtype seems to be linked to the PLC-PKC intracellular transduction pathway (Chen et al., 2009; Grey and Chang, 2011; Yin et al., 2014). A circuitry that includes the activation of PLC and the regulation of different transcriptional factors has been previously suggested as determinant in the modulation of the circadian system in mammals. The PKC is involved in the phase shift of the firing rate of SCN cells *in vitro* (Schak and Harrington, 1999). The PLC/PKC system also mediates light (Bonsall and Lall, 2013; Lee et al., 2007) and food entrainment (Zhang et al., 2012), and it is involved in the effects of melatonin and NPY on phase advance in rodents (Biello et al., 1997; McArthur et al., 1997).

The *in vitro* induction of clock genes expression in goldfish liver by the activation of the PLC/PKC pathway (by PMA) indicates that this intracellular pathway is involved in the functionality of liver circadian oscillators in teleosts. Furthermore, present results show that this intracellular pathway is involved in the induction of hepatic clock genes by ghrelin, as this effect is blocked by the pretreatment with PLC or PKC inhibitors (U73122 and CHEL, respectively). In the case of *clock1a* and *per3*, the PLC inhibitor (like the ghrelin antagonist) increases the basal expression of these genes, but the inhibitor of PKC (CHEL) totally blocks the ghrelin effects. Thus, it may be suggested that the PKC is at least one mechanism underlying the ghrelin induction of these clock genes. It should be noted that our results demonstrate the involvement of the PLC-PKC system on ghrelin actions at 1 h, but other mechanisms may be involved at longer times. Overall, these experiments indicate a relationship among the presence of ghrelin, the activation of ghrelin receptors in the hepatocytes and the signaling transduction via PLC-PKC pathway in order to induce clock gene expression. Interestingly, we found that liver clock genes show similar responses to the effects of both ghrelin and the PKC activator (PMA), with a high induction of *gper1a*, intermediate sensitivity for *gper2a*, *gper3*, *gbmal1a* and *gclock1a*, and an evident insensitivity of *gper1b* at 1 h post-treatment. From present results, it seems that the PKC pathway is underlying the regulatory effect of ghrelin on clock genes in goldfish liver.

The intracellular Gs-AC-PKA pathway has been also involved in the activation of the GHS-R1 ghrelin receptor in mammals (Kohno et al., 2003). In goldfish cultured liver, the direct activation of the AC-PKA pathway by forskolin produced a slight induction of some clock genes (*gper2a*, *gper3* and *gclock1a*) and a pronounced effect on *grev-erbα* transcript amount (4-fold increase), suggesting a dependence on this intracellular signaling pathway. The specificity of these forskolin effects is evidenced by its blockade with the PKA inhibitor (H89), supporting that the AC-PKA pathway is involved in the regulation of the liver circadian

oscillator in this teleost. In agreement with our result in goldfish liver, the AC-PKA intracellular pathway has been linked to the molecular functioning of endogenous clocks in some highly phylogenetically distant species. The cAMP levels are stabilizers and modulators of *per* gene transcripts in the fruit fly (*Drosophila melanogaster* Meigen 1830; Li et al., 2014) and mammals (Hastings et al., 2014; Motzkus et al., 2000; Zmrzljak et al., 2013). Moreover, the PKA induces *Per1* in humans (Motzkus et al., 2007), adjusts endogenous clocks in the presence of light pulses (Tischkau et al., 2000) and its inhibition delays the mammalian clock (Lee et al., 1999). Our results in fish, in agreement with these previous reports in mammals, suggest that the possible functional role of the AC-PKA pathway (as the PLC-PKC) in the regulation of clock genes is conserved throughout phylogeny.

Despite the fact that the AC-PKA pathway seems to be involved in the regulation of hepatic clock gene expression, current results point out that the effects of ghrelin on clock genes are independent of this intracellular pathway, except for *grev-erbα* and probably *gbmal1a.* The involvement of the AC-PKA pathway in the *grev-erbα*-increment is supported by the induction of this gene by both forskolin and ghrelin, and the counteraction of such inductions by the PKA inhibitor. The blockade of the ghrelin-evoked increase in *gbmal1a* levels with the PKA inhibitor needs to be further explored with cAMP analogs or AC inhibitors, given that forskolin did not induce the expression of this gene. The relationship between the AC-PKA pathway and the circadian system has been previously suggested in the signaling transduction of light-dark cycle by CRE elements (Ginty et al., 1993; Motzkus et al., 2007; Travnickova-Bendova et al., 2002), which play a key role in the light entrainable oscillators. Ghrelin, as a food intake regulator and energy balance signal, is expected to be mainly related to the food entrainable oscillators (as the liver). This could justify the lower relevance of the AC-PKA intracellular pathway (compared to the PLC/PKC pathway) in the transduction of this hormonal signal to the hepatic clockwork.

In the present study we used ghrelin-17, the biologically active isoform of ghrelin, that exerts orexigenic actions in goldfish (Kang et al., 2011; Miura et al., 2009). The range of ghrelin concentrations used (0.1-10 nM) has been previously reported as physiologically significant in goldfish cultured pituitary, where different ghrelin isoforms (ghrelin-12 and ghrelin-19) induced LH and GH release (Grey and Chang, 2013; Unniappan and Peter, 2004). The fact that two isoforms, ghrelin-19 (the isoform used in *in vivo* studies in goldfish; Nisembaum et al., 2014) and ghrelin-17 (current experiments), modulate clock genes expression in liver suggests that both forms of ghrelin might play physiological roles in fish, and emphasizes the relevance of this hormone as an input of the hepatic oscillator. In addition, the observed effect of ghrelin on clock genes expression in cultured liver shows key properties (acute and short time effects) to be synchronizing agents. A similar fast and acute effect on *per2* expression has been established for light synchronization in zebrafish (Vatine et al., 2011).

Current results strongly suggest that ghrelin modulates clockworks in the liver, a key target for the interplay between circadian system and metabolism. Considering the well-known role of this peptide in the signaling of energy status, it is plausible to suggest that ghrelin may be acting as a link in the regulation of both energy balance and circadian system in teleosts. The high levels of circulating ghrelin (e.g. during starvation) might modify clock genes expression in the hepatic oscillator in an acute but strong manner. Then, this hormone may be acting as an input to reset the hepatic metabolism via modulation of the hepatic oscillator entrainable by food. The fact that most of the genes that show circadian oscillations in the liver are related to metabolic processes (Oishi et al., 2005; Reddy et al., 2007) supports the crosstalking between signals of nutritional reserves (as ghrelin) and the circadian system in order to maintain metabolic balance in the liver and even in the whole organism.

In conclusion, present results demonstrate for the first time in vertebrates the direct effect of ghrelin on the modulation of the molecular machinery of the hepatic oscillator by

inducing the expression of some clock genes, via the intracellular PKC-PLC and to a lesser extent the AC-PKA pathways. Whether the acute response of clock genes to ghrelin observed *in vitro* leads to a physiological role of this orexigenic hormone as an endogenous input of the circadian system in fish remains to be elucidated. As ghrelin induced both negative and positive clock genes, it could be possible that this hormone leads to the disruption of the hepatic rhythmicity, which would make the synchronizing action of ghrelin on the hepatic clock less probable. However, it is also possible that ghrelin sensitizes the liver to other signals (i.e. the liver could respond differentially to other signals in the presence or absence of ghrelin). This interesting but unexplored physiological role of ghrelin deserves to be further studied.

LIST OF SYMBOLS AND ABREVIATIONS

[D-Lys3]-GHRP-6 [D-Lys3]-Growth hormone releasing peptide-6
AC Adenylatecyclase
Bmal1 Brain and muscle ARNT-Like 1
cAMP Cyclic adenosine monophosphate
CHEL Chelerythrine chloride
Clock Circadian locomotor output cycles kaput
Cry Cryptochrome
DAPI 4′,6-diamidino-2-phenylindole
DIG Digoxigenin
DMEM Dulbecco's modified Eagle medium
FEOs FoodEntrainable Oscillators
GHS-R Growth hormone secretagogue receptors
GRL Ghrelin
HNPP 2-hydroxy-3-naphtoic acid -2′-phenylanilide phosphate
LEOs Light Entrainable Oscillators
NPY Neuropeptide Y
PBS Phosphate buffered saline
Per Period
PKA Protein kinase A

PKC Protein kinase C

PLC Phospholipase C

PMA Phorbol 12-myristate 13-acetate

SNK Student Newman Keuls

SSC Standard saline citrate

ZT *Zeitgeber* time

COMPETING INTERESTS

No competing interests declared.

AUTHOR CONTRIBUTIONS

A.L.A.G., A.S.B. and E.I. conceived and designed the experiments. A.L.A.G., A.S.B., E.I. and M.J.D. interpreted the findings. A.S.B. and O.K. performed the HIS experiments and analyzed the data. A.M.B., A.S.B. and E.I. performed the *in vitro* cultures and analyzed the data. A.L.A.G., A.M.B., A.S.B., E.I. and M.J.D. drafted and revised the manuscript.

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Figures

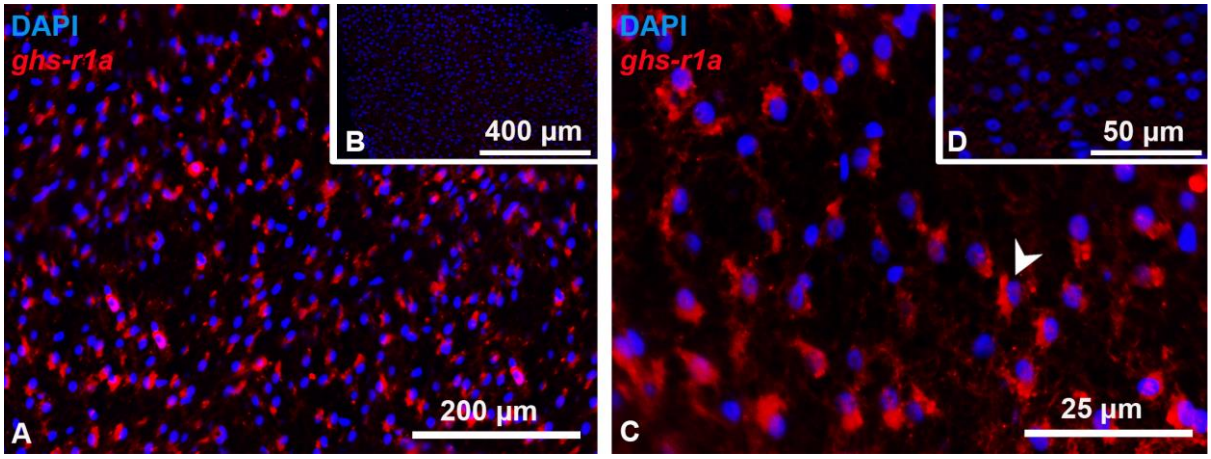

Figure 1. Transversal representative sections of goldfish liver showing *ghs-r1a* positive cells found by *in situ* hybridization. A. Liver section showing *ghs-r1a* antisense riboprobe staining surrounding the nucleus in red. **B**. Liver section showing the absence of *ghs-r1a* sense riboprobe staining. **C**. Detail of nucleus surrounded by red *ghs-r1a* mRNA riboprobe staining (arrowhead). **D**. Detail of hepatocytes showing the absence of *ghs-r1a* sense riboprobe staining.

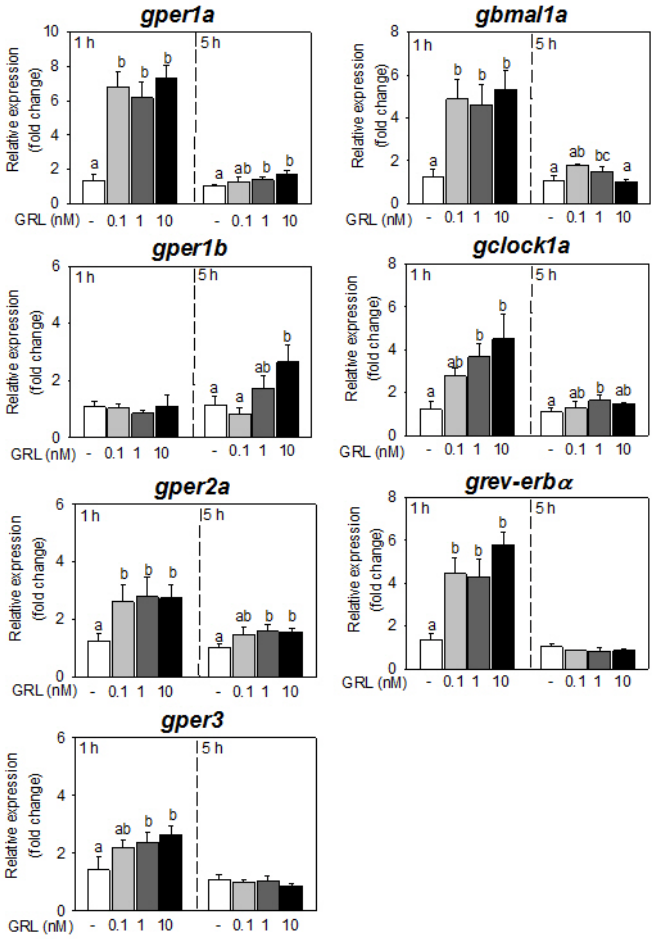

Figure 2. Relative expression of clock genes in cultured liver treated with ghrelin (GRL) during 1 or 5 h. Different concentrations of ghrelin (0, 0.1, 1, and 10 nM) were added to the culture medium. Data obtained by RT-qPCR are shown as the mean ± s.e.m. (n=6, liver aliquots from 6 different fish) in relative units (ΔΔCt method). Differences among groups (SNK test) are indicated by different letters when significant (one-way ANOVA $p<0.05$).

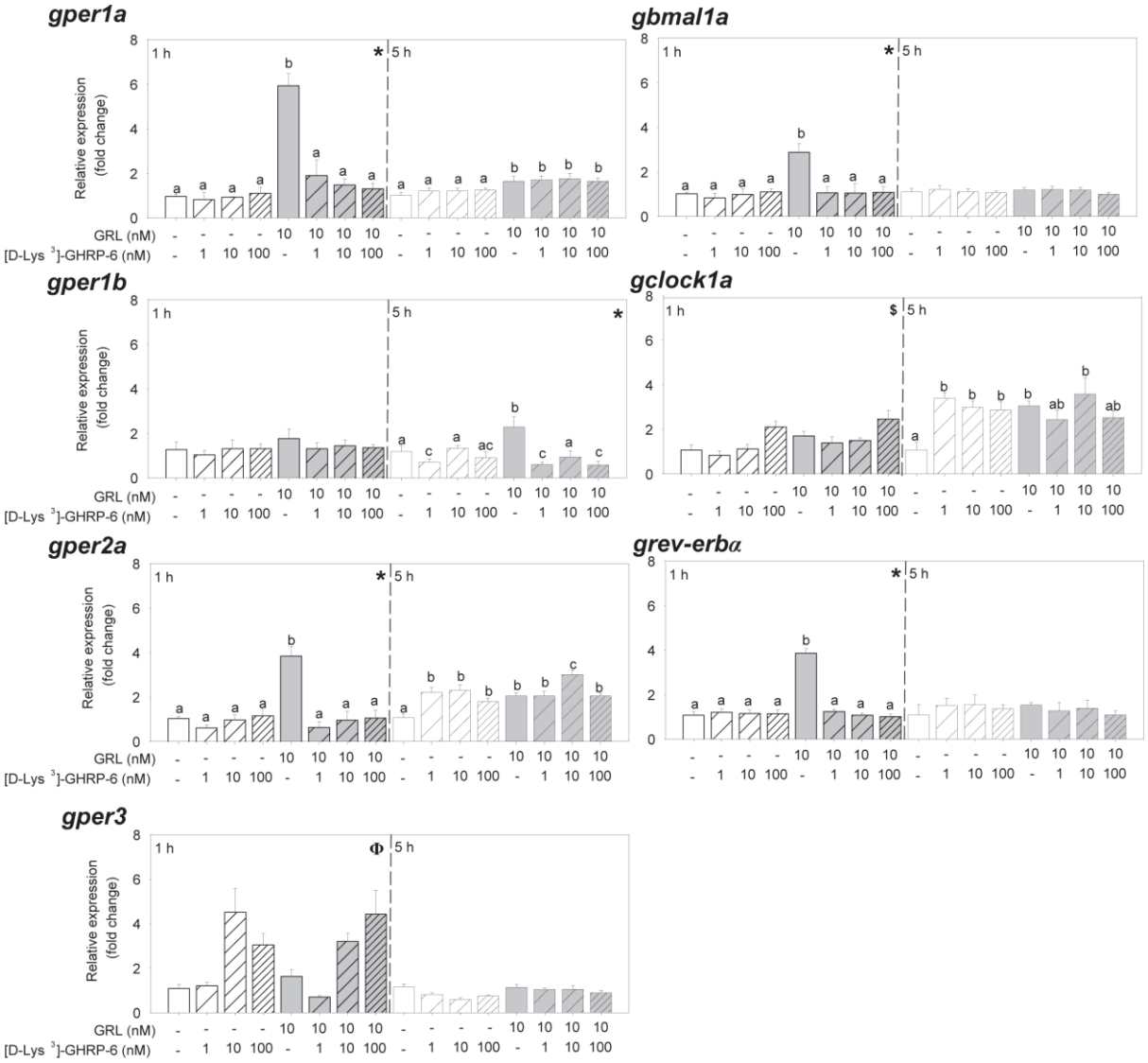

Figure 3. Relative expression of clock genes in goldfish cultured liver treated with ghrelin (GRL) and the antagonist of ghrelin receptor ([D-Lys^3]-GHRP-6). The treatments were carried out for 1 h or 5 h. The antagonist (1, 10 and 100 nM) was added 15 min prior to the addition of ghrelin (10 nM). Data obtained by RTq-PCR are shown as the mean ± s.e.m. (n=6, liver aliquots from 6 different fish) in relative units (ΔΔCt method). Asterisk indicates significant antagonism of [D-Lys^3]-GHRP-6 on ghrelin stimulation (interaction $p<0.05$; two-way ANOVA). Small letters (a, b, c) indicate differences among ghrelin and [D-Lys^3]-GHRP-6 groups (SNK test). When the two-way ANOVA was significant ($p<0.05$), but there was no

significant interaction between factors, $ indicates significant effects of ghrelin (*gclock1a*), and ϕ indicates significant differences between 10 and 100 nM [D-Lys^3]-GHRP-6 compared to 0 and 1 nM [D-Lys^3]-GHRP-6 groups (g*per3*).

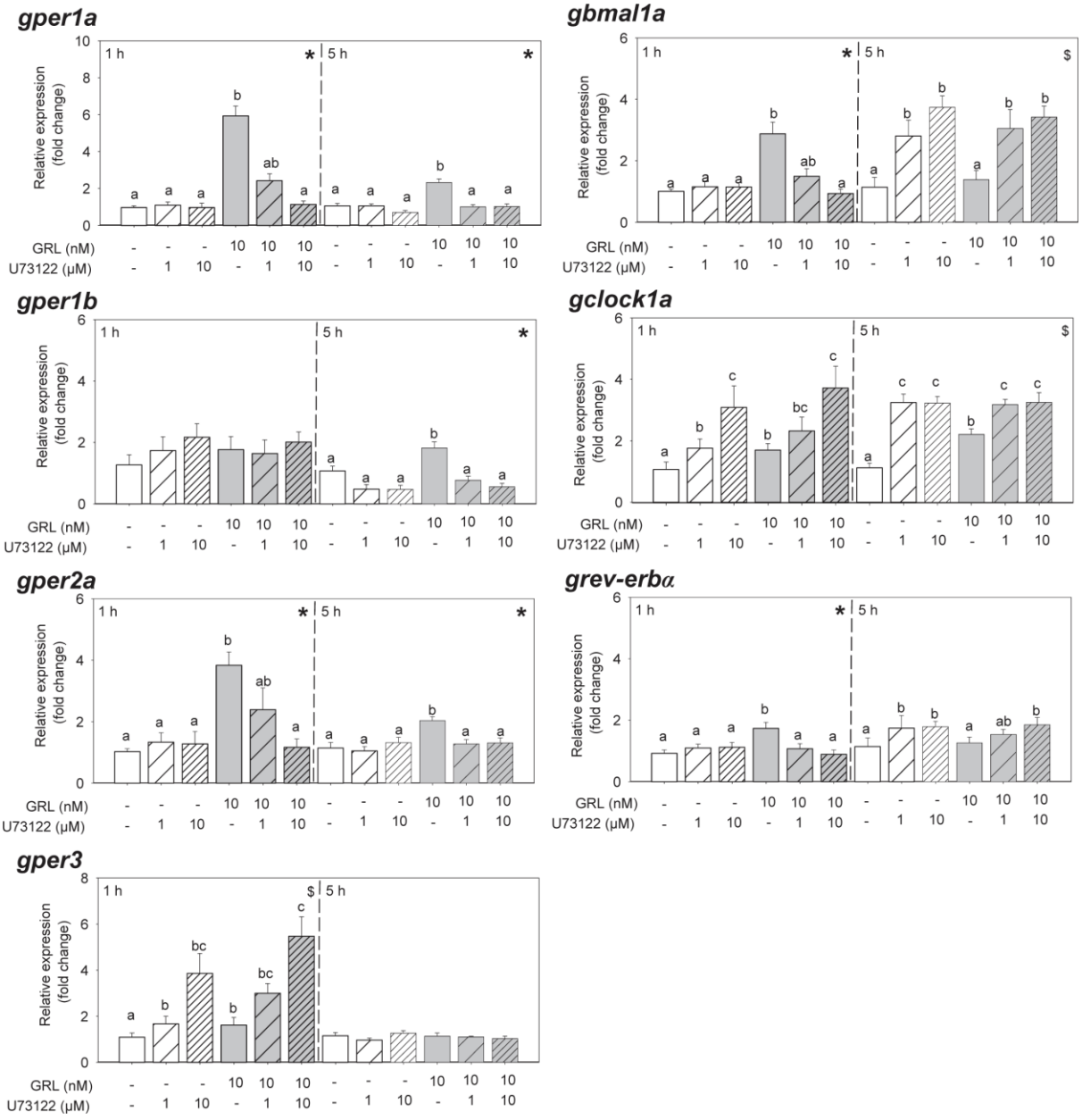

Figure 4. Relative expression of clock genes in cultured liver treated with ghrelin (GRL) and the PLC inhibitor (U73122). The treatments were carried out for 1 h or 5 h. The U73122 (1 or 10 μM) groups were preincubated during 15 min prior to the addition of ghrelin (10 nM). Data obtained by RT-qPCR are shown as the mean ± s.e.m. (n=6, liver aliquots from 6 different fish) in relative units (ΔΔCt method). Asterisk indicates significant inhibition by U73122 of ghrelin stimulation (interaction $p<0.05$; two-way ANOVA). $ indicates a significant stimulation by U73122. Small letters (a, b, c) indicate differences among ghrelin and U73122 groups (SNK test).

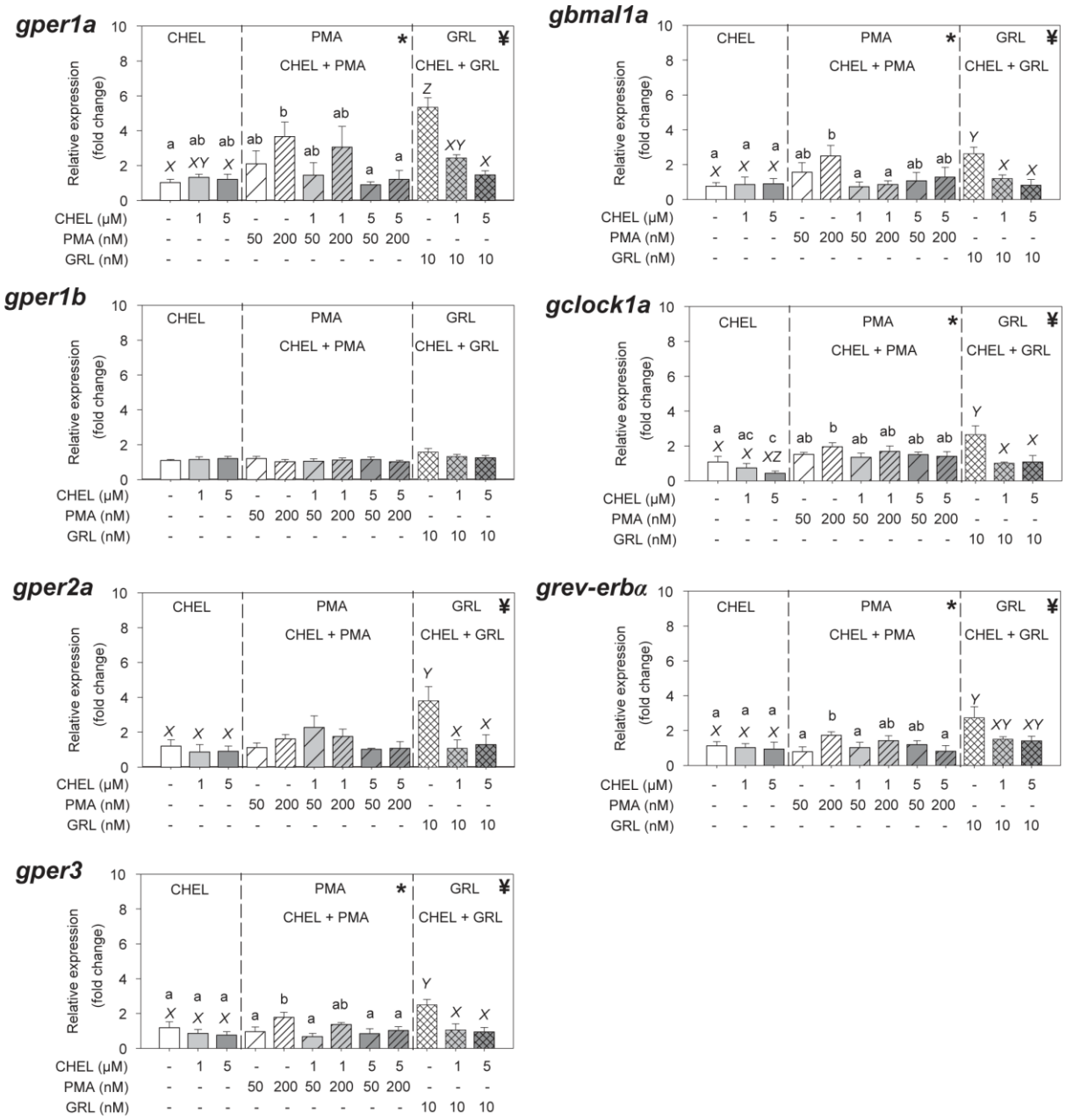

Figure 5. Relative expression of clock genes of cultured liver treated with ghrelin (GRL), phorbol 12-myristate 13-acetate (PMA, PKC activator), and chelerythrine chloride (CHEL, PKC inhibitor) during 1 h. The CHEL (1 or 5 μM) groups were preincubated during 15 min prior to the addition of PMA (50 or 200 nM) or ghrelin (10 nM). Data obtained by RT-qPCR are shown as the mean ± s.e.m. (n=6, liver aliquots from 6 different fish) in relative units (ΔΔCt method). Asterisk indicates significant inhibition by CHEL of PMA stimulation (interaction $p<0.05$; two-way ANOVA). Small letters (a, b, c) indicate differences among PMA and CHEL groups (SNK test). ¥ indicates significant inhibition by CHEL of ghrelin stimulation

(interaction $p<0.05$; two-way ANOVA). Capital letters (X, Y, Z) indicate differences among ghrelin and CHEL groups (SNK test).

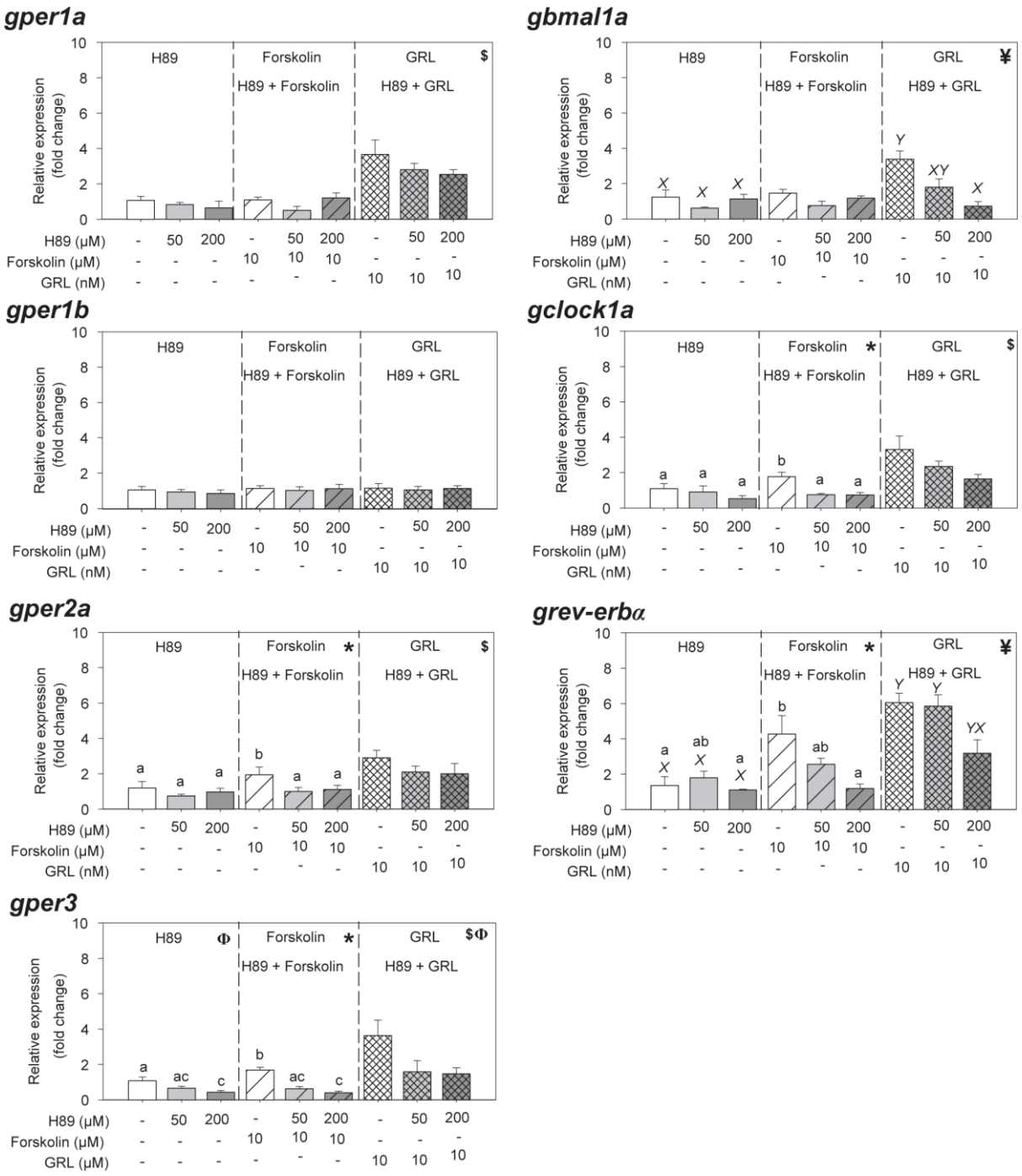

Figure 6. Relative expression of clock genes in cultured liver treated with ghrelin (GRL), forskolin (AC activator) and H89 (PKA inhibitor) during 1 h. Groups with H89 (50 or 200 μM) were preincubated during 15 min prior to the addition of forskolin (10 μM) or ghrelin (10 nM). Data obtained by RT-qPCR are shown as the mean ± s.e.m. (n=6, liver aliquots from 6 different fish) in relative units (ΔΔCt method). Asterisk indicates significant inhibition by H89 of forskolin stimulation (interaction $p<0.05$; two-way ANOVA). Small letters (a, b, c) indicate

differences among forskolin and H89 groups (SNK test). ¥ indicates significant inhibition by H89 of ghrelin stimulation (interaction $p<0.05$; two-way ANOVA). Capital letters (X, Y, Z) indicate differences among ghrelin and H89 groups (SNK test). When the two-way ANOVA was significant ($p<0.05$), but there was no significant interaction between factors, $ indicates significant effect of ghrelin (*gper1a, gper2a, gclock1a*) and ϕ indicates significant effect of H89 (*gper3*) compared to its controls.

Table 1. Accession numbers of the genes of interest and primers sequences used in this study.

Target gene	*Accession number*	*Primer sequences 5' → 3'*		**product (pb)**
gper1a	EF690698	F R	CAGTGGCTCGAATGAGCACCA TGAAGACCTGCTGTCCGTTGG	155
gper1b	KP663726	F R	CTCGCAGCTCCACAAACCTA CACAACAGCTGCAGAGGAAT	159
gper2a	EF690697	F R	TTTGTCAATCCCTGGAGCCGC AAGGATTTGCCCTCAGCCACG	116
gper3	EF690699	F R	GGCTATGGCAGTCTGGCTAGTAA CAGCACAAAACCGCTGCAATGTC	130
gbmal1a	KF840401	F R	AGATTCTGTTCGTCTCGGAG ATCGATGAGTCGTTCCCGTG	161
gclock1a	KJ574204	F R	CGATGGCAGCATCTCTTGTGT TCCTGGATCTGCCGCAGTTCAT	189
grev-erba	KU242427	F R	CGTTCATCTCAGGCACCACT AACTGACCTGCAGACACCAG	166
gβ-actin	AB039726	F R	CAGGGAGTGATGGTTGGCA AACACGCAGCTCGTTGTAGA	168