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RESEARCH ARTICLE

Ghrelin induces clock gene expression in the liver of goldfish *in vitro* via protein kinase C and protein kinase A pathways

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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 phospholipase C/protein kinase C (PLC/PKC) and adenylate cyclase/protein kinase A (AC/PKA) intracellular signalling pathways was investigated. Ghrelin receptor transcripts, *ghs-r1a*, are present in the majority of goldfish hepatic cells. Ghrelin induced the mRNA expression of the positive (g*bmal1a*, *gclock1a*) and negative (*gper* genes) elements of the main loop of the molecular clock machinery, as well as *grev-erbα* (auxiliary loop) in cultured liver. These effects were blocked, at least in part, by a ghrelin antagonist. Incubation of liver with a PLC inhibitor (U73122), a PKC activator (phorbol 12-myristate 13-acetate) and a PKC inhibitor (chelerythrine chloride) demonstrated that the PLC/PKC pathway mediates such ghrelin actions. Experiments with an AC activator (forskolin) and a PKA inhibitor (H89) showed that *grev-erbα* regulation could be due to activation of PKA. Taken together, the 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.

KEY WORDS: Orexigenic peptides, Circadian system, Protein kinase C, Protein kinase A, Hepatic clock, Teleost

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 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 (food-entrainable oscillators, FEOs) in the circadian system remain unknown. In mammals, the oscillators of the circadian system respond to feeding inputs with different sensitivities, the liver being 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 that 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,b; 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 terms of expression and content in mouse stomach (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 to 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 to 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

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List of abbreviations

[D-Lys3]-GHRP-6	[D-Lys3]-growth hormone releasing peptide-6
AC	adenylate cyclase
Bmal1	brain and muscle ARNT-like 1
CHEL	chelerythrine chloride
Clock	circadian locomotor output cycles kaput
DIG	digoxigenin
FEO	food-entrainable oscillator
GHS-R	growth hormone secretagogue receptor
GRL	ghrelin
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

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 function.

Ghrelin actions are mediated by G-protein-coupled receptors known as growth hormone secretagogue receptors (GHS-Rs) or ghrelin receptors (Kaiya et al., 2013; Kojima et al., 1999). In Otophysi teleosts, two paralogue *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 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,c) 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 investigated the possible direct regulatory role of ghrelin on the hepatic molecular clock of goldfish. To achieve this aim, we first verified 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 investigated the possible involvement of the PLC/PKC and AC/PKA intracellular pathways in 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 to circadian clocks.

MATERIALS 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 dark photoperiod (lights on at 09:00 h). Fish were daily fed at 11:00 h (Zeitgeber time 2, ZT2) with food pellets (1% body mass, M_b; Novo GranoMix, JBL, GmbH and Co., Neuhofen, Germany). Goldfish (48 h fasted) were anaesthetized at ZT2 with 1 ml l^{-1} phenoxyethanol (ICN Biomedicals Inc., Irvine, CA, USA) and killed (overdose of anaesthetic). Then, the whole fish was immersed overnight in 4% paraformaldehyde diluted in 0.1 mol l^{-1} phosphate-buffered saline (PBS, pH 7.4). The following day, the liver was removed and post-fixed for 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, Cheshire, 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 dark photoperiod (lights on at 08:00 h). Fish were daily fed at ZT2 with food pellets (1% M_b; Bioflakes, Sera Pond, Heidelberg, Germany). On the day of the experiment, non-fed goldfish were anaesthetized in MS-222 (0.175 g l^{-1}, Sigma Aldrich, Carlsbad, CA, USA) at ZT2. Then, animals were killed (overdose of anaesthetic) and the liver was quickly sampled and distributed in the different wells (15 mg liver per well) of sterile 24-well culture 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 goldfish liver by *in situ* hybridization

The probes for *in situ* hybridization were synthesized from plasmids (pCR™4-TOPO® vector, Invitrogen, Carlsbad, CA, USA) containing 979 bp of goldfish *ghs-r1a* (Sánchez-Bretaño et al., 2015c). This probe targets a common fragment of goldfish *ghs-r1a1* and *ghs-r1a2* (GenBank accession numbers AB504275.1 and AB504276.1). Plasmids with the insert were linearized with *Spe*I and *Not*I, and antisense and sense mRNA probes were obtained with digoxigenin (DIG) RNA labelling mix (Roche Diagnostics, 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 described above was placed in TissueTek and sectioned at 8 µm using a cryostat. Sections were mounted on superfrost slides. *In situ* hybridization was performed as previously described (Escobar et al., 2013) with minor modifications. In brief, cryostat sections were washed twice in PBS over a period of 10 min before post-fixing in *Antigenfix* (DiaPath, Martinengo, Italy) for 20 min. Then, sections were treated for 5 min at 37°C with proteinase K (2 µg ml^{-1}, Sigma, Steinheim, Germany) diluted in PBS, and fixed in 4% paraformaldehyde for 15 min. Sections were rinsed twice in 2× standard saline citrate (SSC). Hybridization was performed at 65°C overnight in a

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Table 1. GenBank accession numbers of the genes of interest and primer sequences used in this study

Target gene	Accession no.	Primer sequence 5′→3′	Product (bp)
gper1a	EF690698	F: CAGTGGCTCGAATGAGCACCA R: TGAAGACCTGCTGTCCGTTGG	155
gper1b	KP663726	F: CTCGCAGCTCCACAAACCTA R: CACAACAGCTGCAGAGGAAT	159
gper2a	EF690697	F: TTTGTCAATCCCTGGAGCCGC R: AAGGATTTGCCCTCAGCCACG	116
gper3	EF690699	F: GGCTATGGCAGTCTGGCTAGTAA R: CAGCACAAAACCGCTGCAATGTC	130
gbmal1a	KF840401	F: AGATTCTGTTCGTCTCGGAG R: ATCGATGAGTCGTTCCCGTG	161
gclock1a	KJ574204	F: CGATGGCAGCATCTCTTGTGT R: TCCTGGATCTGCCGCAGTTCAT	189
grev-erbα	KU242427	F: CGTTCATCTCAGGCACCACT R: AACTGACCTGCAGACACCAG	166
gβ-actin	AB039726	F: CAGGGAGTGATGGTTGGCA R: AACACGCAGCTCGTTGTAGA	168

F, forward; R, reverse.

humidified chamber using 100 µl hybridization buffer (50% deionized formamide, 2× SSC, 5× Denhardt's solution, 50 µg ml^{-1} yeast tRNA, 4 mmol l^{-1} EDTA, 2.5% dextran sulfate) containing the DIG-labelled probe (3 µg ml^{-1}). After hybridization, slides were washed successively in 2× SSC at 65°C (2×30 min), 2× SSC/50% formamide at 65°C (2×30 min), 0.2× SSC (1×15 min) and 0.1× SSC (1×15 min) at room temperature. Slides were then washed in 100 mmol l^{-1} Tris-HCl (pH 7.5) containing 150 mmol l^{-1} NaCl for 10 min, washed in the same buffer containing 0.1% Triton X-100 and 0.5% skimmed milk powder (2×30 min), and incubated overnight at room temperature with anti-DIG alkaline phosphatase Fab fragments (1:2000; Roche Pharma, Mannheim, Germany). 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), 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 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 prepared 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 per group). Liver portions were pre-incubated for 2 h in 1 ml of control medium (15 mg liver ml^{-1} per well, quantified as 15 µl of tissue; Sánchez-Bretaño et al., 2016). The control medium consists of Dulbecco's modified Eagle's medium (DMEM; 17.3 g l^{-1} Sigma Aldrich) modified for fish tissues by adding $NaHCO_3$ (3.7 g l^{-1}) and antibiotics (10 ml l^{-1} penicillin–streptomycin and 500 mg l^{-1} gentamicin; Sigma Aldrich). After the 2 h pre-incubation 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 or 5 h depending on the experiment (see figures). The liver cultures were maintained under constant dim light and temperature (21±1°C) conditions. At the end of each culture time, liver samples were collected, quickly frozen in liquid nitrogen and maintained at −80°C until clock gene expression was quantified.

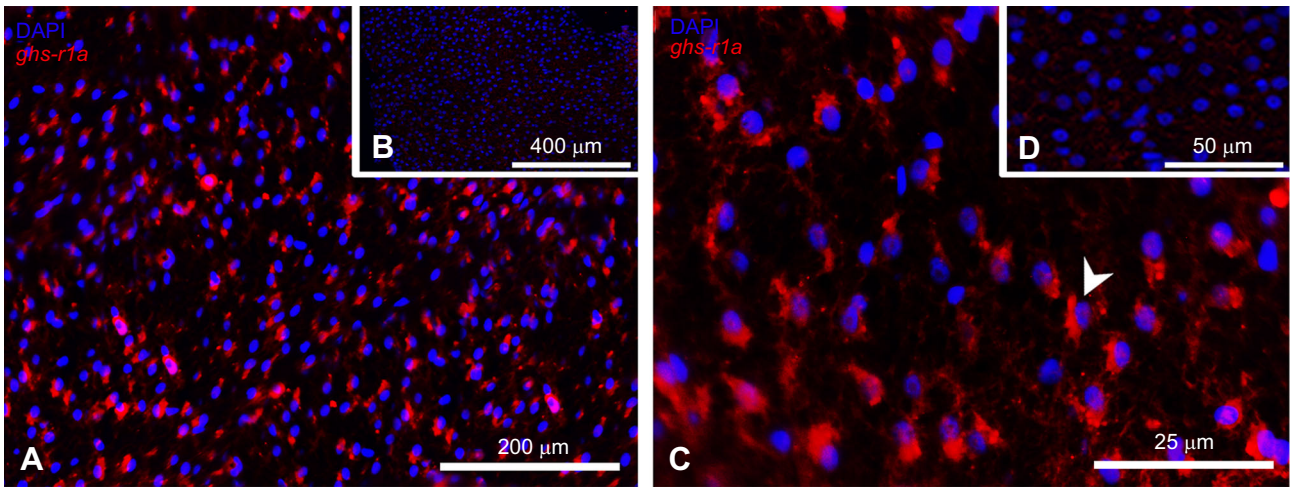

Fig. 1. Representative transverse sections of goldfish liver showing *ghs-r1a*-positive cells by *in situ* hybridization. (A) Liver section showing *ghs-r1a* antisense riboprobe staining (red) surrounding the nucleus (blue). (B) Liver section showing the absence of *ghs-r1a* sense riboprobe staining.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

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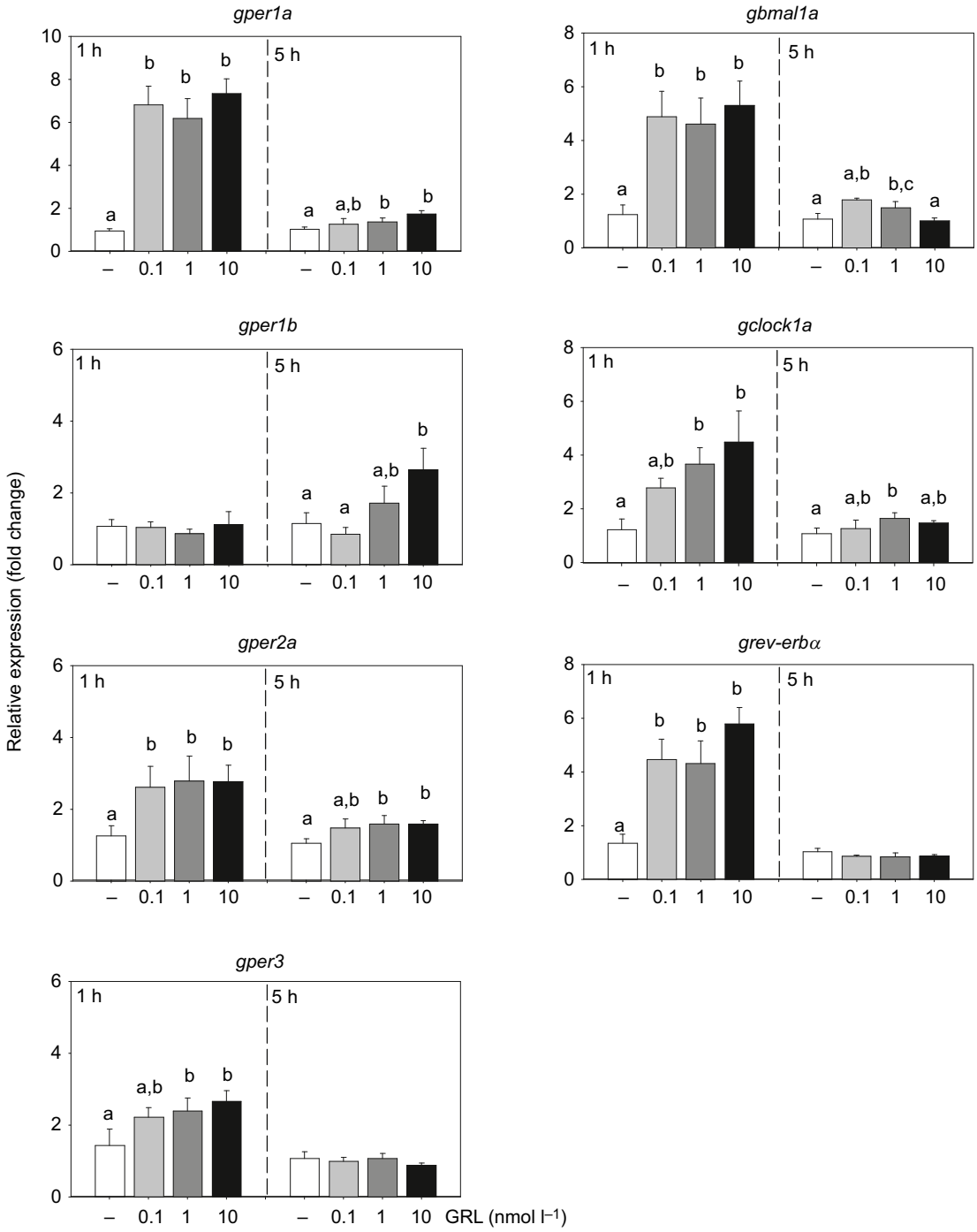

Fig. 2. Relative expression of clock genes in cultured goldfish liver treated with ghrelin for 1 or 5 h. Different concentrations of ghrelin (GRL; 0, 0.1, 1 and 10 nmol l^{-1}) were added to the culture medium. Data obtained by RT-qPCR are shown as means±s.e.m. (n=6, liver aliquots from 6 different fish) in relative units (ΔΔCt method). Differences among groups [Student–Newman–Keuls (SNK) test] are indicated by different letters when significant (one-way ANOVA, P<0.05).

Drugs

Stock solutions were prepared and stored at 4°C until used. The 17-amino acid isoform of goldfish ghrelin [GTS(octanoyl) FLSPAQKPQGRRPP; Bachem, Bubendorf, Switzerland] and the PKA inhibitor H89 (Sigma Aldrich) were prepared in distilled water at a concentration of 2 and 15 mmol l^{-1}, 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) were prepared in absolute ethanol at 1, 10 and 15 mmol l^{-1} concentrations, respectively. Stock solutions of the PKC inhibitor chelerythrine chloride (CHEL; Sigma Aldrich) and the PKC activator phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) were prepared in dimethyl sulfoxide (DMSO) at 5 and 20 mmol l^{-1} concentrations, respectively. All 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), the 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).

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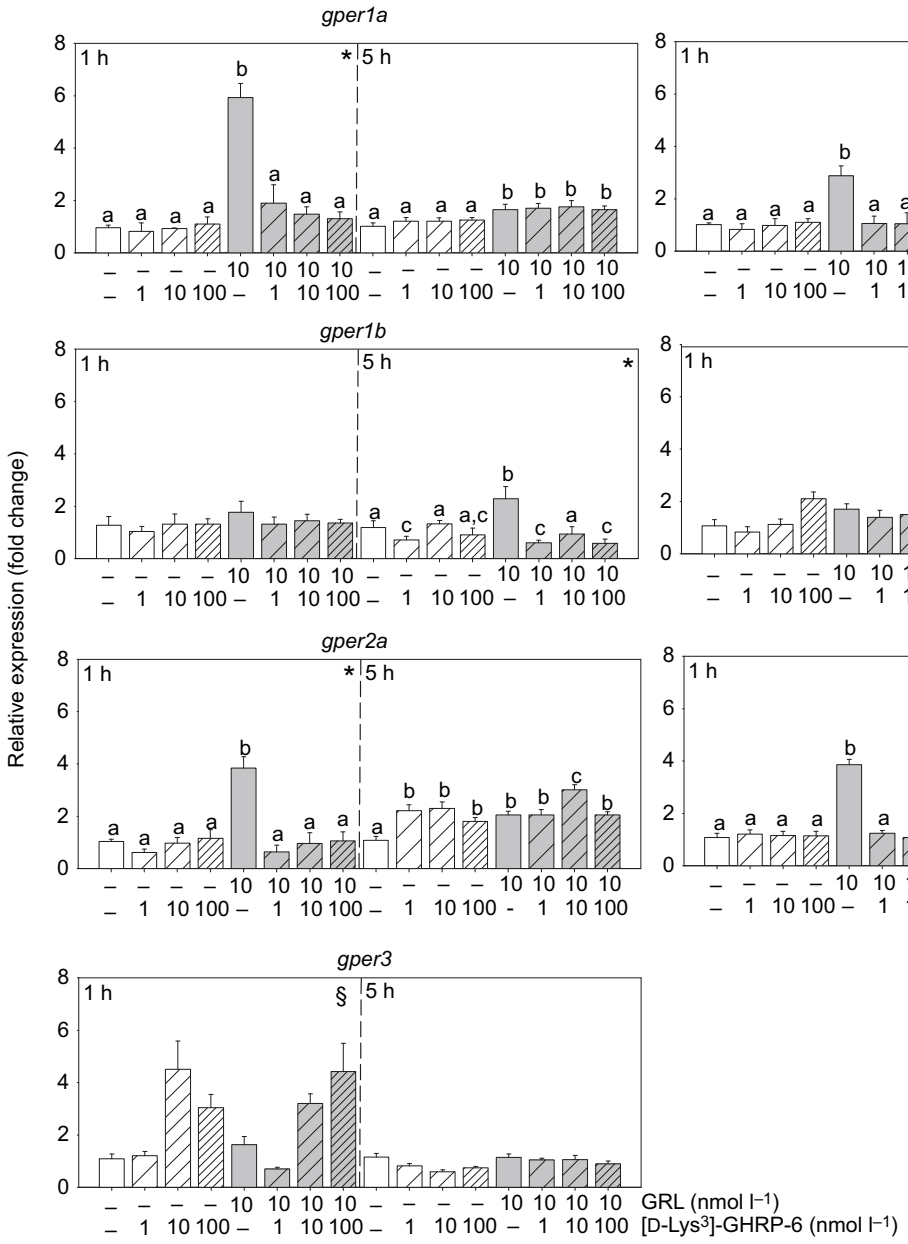

Fig. 3. Relative expression of clock genes in cultured goldfish liver treated with ghrelin and ghrelin receptor antagonist. The treatments were carried out for 1 or 5 h. The ghrelin receptor antagonist [D-Lys^3]-GHRP-6 (1, 10 and 100 nmol l^{-1}) was added 15 min prior to the addition of ghrelin (GRL; 10 nmol l^{-1}). Data obtained by RT-qPCR are shown as means±s.e.m. (n=6, liver aliquots from 6 different fish) in relative units (ΔΔCt method). Asterisks indicate significant antagonism of [D-Lys^3]-GHRP-6 on ghrelin stimulation (interaction P<0.05; two-way ANOVA). Lowercase letters 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 § indicates significant differences between 10 and 100 nmol l^{-1} [D-Lys^3]-GHRP-6 compared with 0 and 1 nmol l^{-1} [D-Lys^3]-GHRP-6 groups (*gper3*).

Quantification of clock gene expression by real-time PCR

Clock gene expression (*gper1a*, *gper1b*, *gper2a*, *gper3*, *gbmal1a*, *gclock1a* and *grev-erbα*) was quantified by real-time quantitative PCR (RT-qPCR) using *gβ-actin* as a reference gene, as previously described (Nisembaum et al., 2014). Specific primers and GenBank reference numbers are shown in Table 1. The RNA extraction (TRI® Reagent method, Sigma Chemical, Madrid, Spain), DNase treatment (Promega), cDNA synthesis (Invitrogen) and RT-qPCR reactions (iTaq™ SYBR® Green Supermix in a CFX96™ Real-Time System, BioRad Laboratories, Hercules, CA, USA) were carried out following the manufacturer's instructions with minor modifications (Nisembaum et al., 2014). Total RNA (1 µg) was reverse transcribed and PCR reactions were developed in a final volume of 10 µl (2 µl of cDNA per sample). PCR conditions were 30 s at 95°C, and 40 cycles consisting of 5 s at 95°C and 30 s at 60°C 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 the ΔΔCt method were logarithmically transformed in order to normalize the variance and to obtain homoscedasticity. A probability level of P<0.05 was considered

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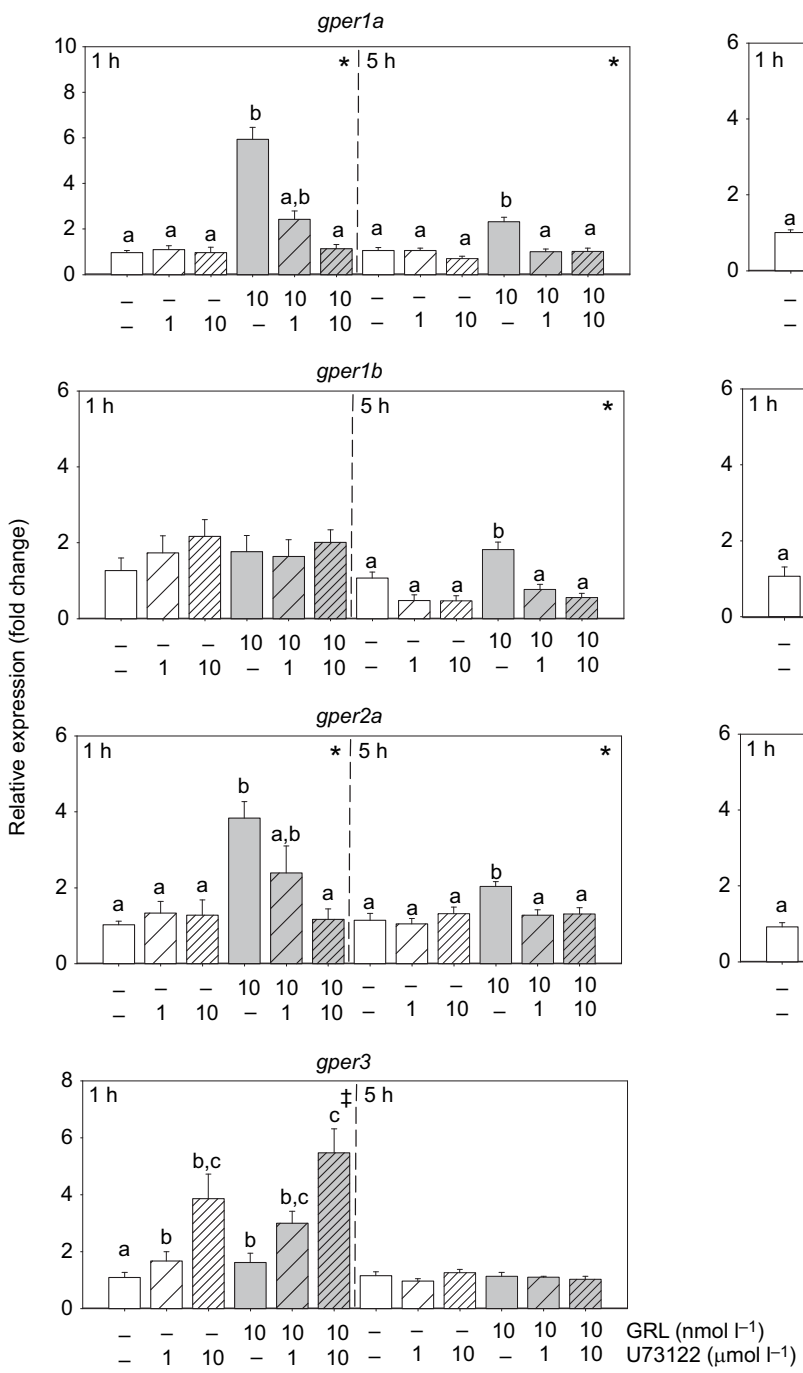

Fig. 4. Relative expression of clock genes in cultured goldfish liver treated with ghrelin and phospholipase C (PLC) inhibitor. The treatments were carried out for 1 or 5 h. The PLC inhibitor U73122 (1 or 10 μmol l^{-1}) was added 15 min prior to the addition of ghrelin (GRL; 10 nmol l^{-1}). Data obtained by RT-qPCR are shown as means±s.e.m. (*n*=6, liver aliquots from 6 different fish) in relative units (ΔΔCt method). Asterisks indicate significant inhibition by U73122 of ghrelin stimulation (interaction $P<0.05$; two-way ANOVA). ‡ indicates a significant stimulation by U73122. Lowercase letters indicate differences among ghrelin and U73122 groups (SNK test).

statistically significant. Analysis of the relative expression changes in the ghrelin concentration–response curves was conducted using one-way ANOVA followed by the *post hoc* Student–Newman–Keuls (SNK) test. A two-way ANOVA followed by a *post hoc* SNK was used when 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 goldfish liver

The ghrelin receptor *ghs-r1a* was found to be widely expressed in the goldfish liver (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.

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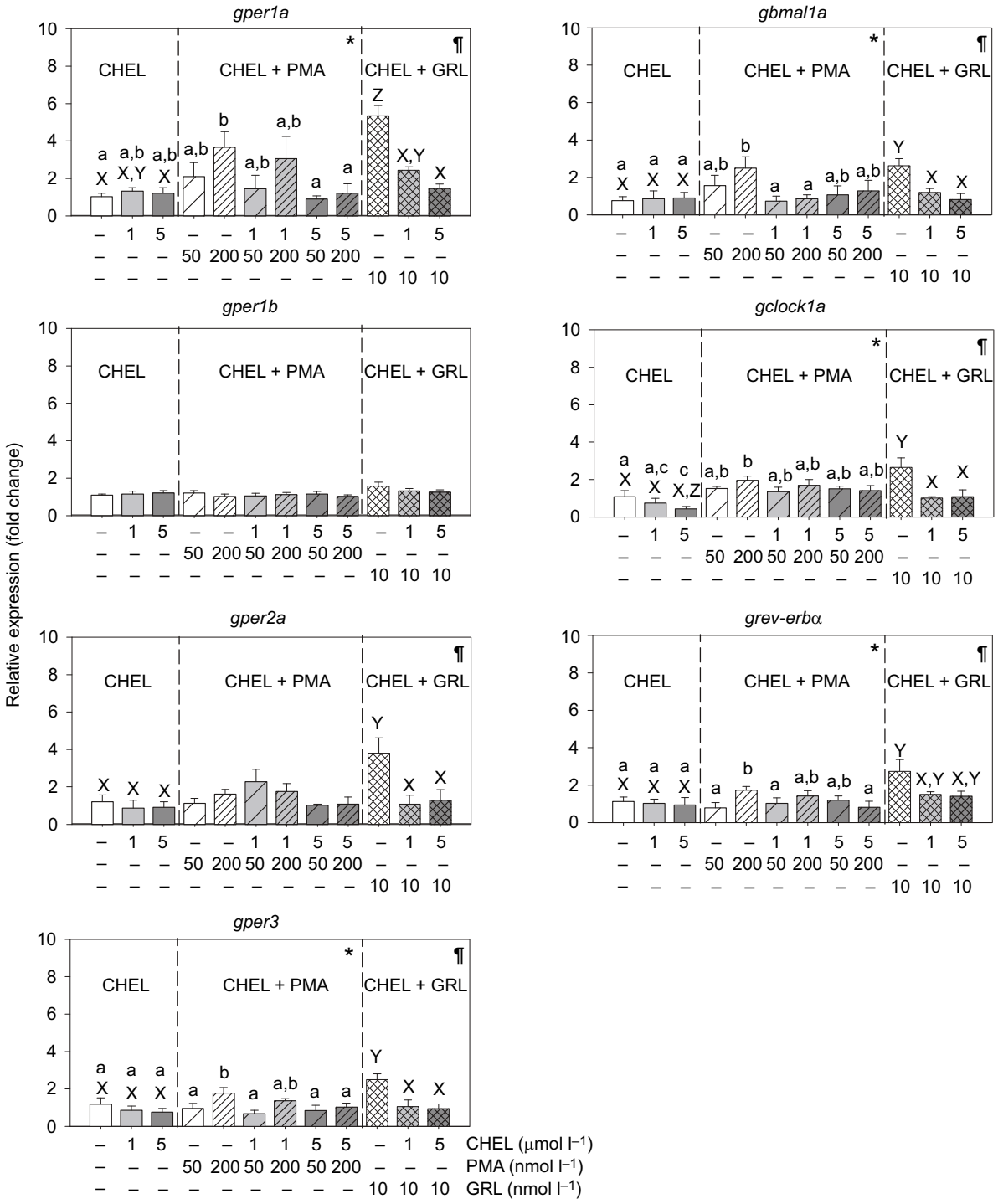

Fig. 5. Relative expression of clock genes in cultured goldfish liver treated with ghrelin, PKC activator and PKC inhibitor for 1 h. The PKC inhibitor chelerythrine chloride (CHEL; 1 or 5 μmol l^{-1}) was added 15 min prior to the addition of the PKC activator phorbol 12-myristate 13-acetate (PMA; 50 or 200 nmol l^{-1}) or ghrelin (GRL; 10 nmol l^{-1}). Data obtained by RT-qPCR are shown as means±s.e.m. (n=6, liver aliquots from 6 different fish) in relative units (ΔΔCt method). Asterisks indicate significant inhibition by CHEL of PMA stimulation (interaction P<0.05; two-way ANOVA). Lowercase letters 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 indicate differences among ghrelin and CHEL groups (SNK test).

Ghrelin as a regulator of clock gene expression in cultured liver

Ghrelin modified the expression of some clock genes in the goldfish liver *in vitro* (Fig. 2). With the exception of *gper1b*, all the clock genes studied were induced by 0.1–10 nmol l^{-1} ghrelin at 1 h post-treatment (for *gclock1a* and *gper3*, induction was only observed with ghrelin concentrations greater than 1 nmol l^{-1}). The highest induction was found for *gper1a* transcripts, which were increased around 7-fold, 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- to 5-fold). All the

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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 *gper1* was exposure of the liver to ghrelin (10 nmol l^{-1}) for 5 h a requirement for a 3-fold increase of transcripts to be observed.

The specificity of the ghrelin-evoked induction of clock gene expression was tested by using a ghrelin antagonist, [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*, levels of which were modified by the antagonist after a 5 h incubation (*gper2a*, *gclock1a*) or after a 1 h incubation (*gper3*). Pre-incubation of liver samples 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 induction was not observed at 5 h, probably because of 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

Pre-incubation (15 min) with the PLC inhibitor U73122 prior to the addition of ghrelin totally abolished the induction of hepatic clock gene expression (*gper1a*, *gper1b*, *gper2a*, *gbmal1a* and *grev-erbα*) evoked by the presence of ghrelin in the culture medium for 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 induction of gene expression. Ghrelin effects at longer exposures (5 h) were blocked by 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 remaining clock genes was not affected by U73122 at either of the tested concentrations (1 and 10 µmol l^{-1}).

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). Induction of expression by ghrelin in cultured liver was totally blocked by pre-treatment with CHEL for the majority of the studied clock genes (*gper1a*, *gper2a*, *gper3*, *gbmal1a* and *gclock1a*), with the exception of *grev-erbα*, where the induction of mRNA levels produced by ghrelin was only partially blocked. This PKC inhibitor had minor effects on basal clock gene expression at the tested concentrations (1 and 5 µmol l^{-1}), except for a slight reduction of *gclock1a* transcripts (Fig. 5). As a positive control for the involvement of PKC in clock gene expression, the liver was treated with a direct PKC activator, PMA, for 1 h (Fig. 5). This activator exerted slight effects on clock gene expression at a low concentration (50 nmol l^{-1}), but significantly induced *gper1a*, *gper3*, *gbmal1a*, *gclock1a* and *grev-erbα* expression at a higher concentration (200 nmol l^{-1}; Fig. 5). Pre-treatment of liver with CHEL (5 µmol l^{-1}) 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.

Involvement of the AC/PKA pathway in the ghrelin regulation of clock gene expression

Forskolin, an AC activator, induced the expression of *gper2a*, *gper3*, *gclock1a* and *grev-erbα* while it had no effect on the rest of the clock genes analysed after 1 h of treatment (Fig. 6). Pre-treatment with the PKA inhibitor H89 abolished forskolin effects in all cases. High concentrations of H89 (200 µmol l^{-1}) 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 pre-treatment 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 evidence for a direct effect of ghrelin on clock gene expression in the liver of a teleost. We found that ghrelin (acyl-ghrelin-17) induces clock gene expression via its receptor located in 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, the 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 mRNA expression of *grev-erbα* (auxiliary loop) over short time periods (1 h), and of *gper1b* over longer periods (5 h) in cultured goldfish liver. This induction of hepatic clock gene 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* gene expression (*gper1a*, *gper2* and *gper3*) in the liver at 1 h post-injection, an 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 goldfish liver.

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 obtained from this teleost (Nisembaum et al., 2014). We therefore suggest the involvement of a hepatic ghrelin receptor in the majority of the observed actions of ghrelin as a modulator of clock gene expression. The specificity of the ghrelin effect on *gclock1a* and *gper3* remains unsolved as the ghrelin receptor 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 hepatic cells. This is in agreement with the previous identification by PCR of the ghrelin receptor in the liver of some teleosts, including 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 previously been suggested as a determinant in the modulation of the circadian system in mammals. PKC is involved in the phase shift of the firing rate of suprachiasmatic nucleus 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 is involved in the effects of melatonin and neuropeptide Y on phase advance in rodents (Biello et al., 1997; McArthur et al., 1997). The *in vitro* induction of clock gene expression in goldfish liver by the activation of the PLC/PKC pathway (by PMA) indicates that this intracellular pathway is

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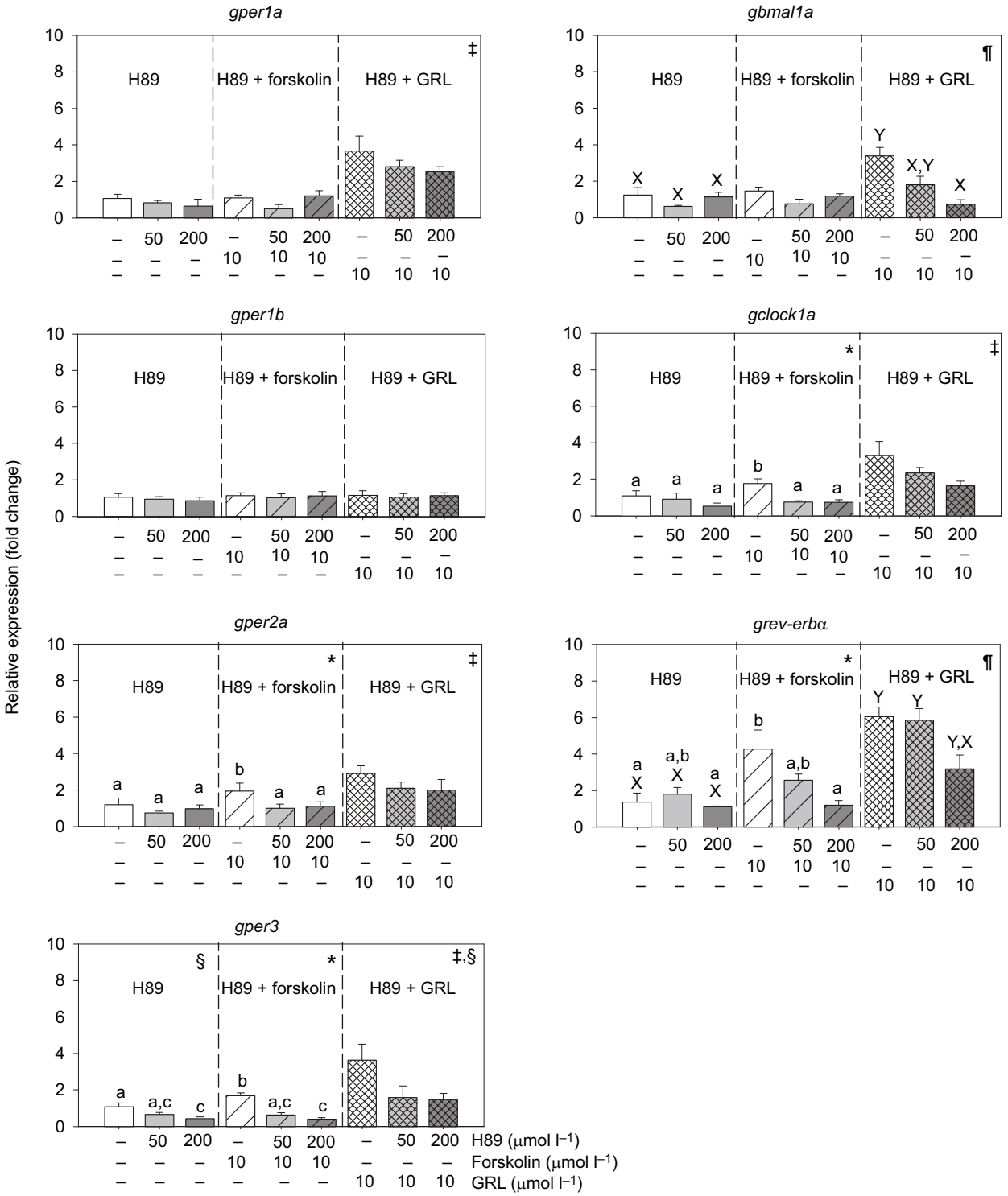

Fig. 6. Relative expression of clock genes in cultured goldfish liver treated with ghrelin, adenylate cyclase (AC) activator and protein kinase A (PKA) inhibitor for 1 h. The PKA inhibitor H89 (50 or 200 μmol l^{-1}) was added 15 min prior to the addition of the AC activator forskolin (10 μmol l^{-1}) or ghrelin (GRL; 10 nmol l^{-1}). Data obtained by RT-qPCR are shown as means±s.e.m. (*n*=6, liver aliquots from 6 different fish) in relative units (ΔΔCt method). Asterisks indicate significant inhibition by H89 of forskolin stimulation (interaction $P<0.05$; two-way ANOVA). Lowercase letters 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 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 a significant effect of ghrelin (*gper1a*, *gper2a*, *gper3*, *gclock1a*) and § indicates a significant effect of H89 (*gper3*) compared with controls.

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involved in the functionality of liver circadian oscillators in teleosts. Furthermore, the present results show that this intracellular pathway is involved in the induction of hepatic clock genes by ghrelin, as this effect is blocked by pre-treatment with PLC or PKC inhibitors (U73122 and CHEL, respectively). In the case of *gclock1a* and *gper3*, the PLC inhibitor (like the ghrelin antagonist) increased the basal expression of these genes, but the inhibitor of PKC (CHEL) totally blocked the ghrelin effects. Thus, we suggest that the PKC is at least one of the mechanisms underlying the ghrelin induction of these clock genes. It should be noted that our results demonstrate the involvement of the PLC/PKC system in 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 signal transduction via the PLC/PKC pathway in order to induce clock gene expression. Interestingly, we found that liver clock genes show similar responses to 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 the present results, it seems that the PKC pathway underlies the regulatory effect of ghrelin on clock genes in goldfish liver.

The intracellular Gs/AC/PKA pathway is also involved in the activation of the GHS-R1 ghrelin receptor in mammals (Kohno et al., 2003). In cultured goldfish 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 increase (4-fold) of *grev-erbα* transcript levels, suggesting a dependence on this intracellular signalling pathway. The specificity of forskolin is evidenced by the blocking of its effects with the PKA inhibitor H89, supporting the involvement of the AC/PKA pathway in the regulation of the liver circadian oscillator in this teleost. In agreement with our findings in goldfish liver, the AC/PKA intracellular pathway has been linked to the molecular functioning of endogenous clocks in some phylogenetically distant species. cAMP, which activates PKA, is a stabilizer and modulator of *per* gene transcripts in the fruit fly *Drosophila melanogaster* (Li et al., 2014) and mammals (Hastings et al., 2014; Motzkus et al., 2000; Zmrzljak et al., 2013). Moreover, PKA induces *Per1* expression in humans (Motzkus et al., 2007) and 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 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 suggest 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 increase in *grev-erbα* expression is supported by the induction of this gene by both forskolin and ghrelin, and the counteraction of this induction 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 analogues or AC inhibitors, given that forskolin did not induce expression of this gene. The relationship between the AC/PKA pathway and the circadian system has previously been suggested in the signal transduction of the light–dark cycle by cAMP response elements (CRE) (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 (such as the liver). This could justify the lower relevance of the AC/PKA intracellular pathway (compared with the PLC/PKC pathway) in the transduction of this hormonal signal to the hepatic clock.

In the present study, we used ghrelin-17, the biologically active isoform of ghrelin, which exerts orexigenic actions in goldfish (Kang et al., 2011; Miura et al., 2009). The range of ghrelin concentrations used (0.1–10 nmol l^{-1}) has previously been reported as physiologically significant in goldfish cultured pituitary, where different ghrelin isoforms (ghrelin-12 and ghrelin-19) induced luteinizing hormone and growth hormone 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 gene 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 gene expression in cultured liver shows key properties (acute and short time effects) of synchronizing agents. A similar fast and acute effect on *per2* expression has been established for light synchronization in zebrafish (Vatine et al., 2011).

Our results strongly suggest that ghrelin modulates the clock machinery in the liver, a key target for the interplay between the circadian system and metabolism. Considering the well-known role of this peptide in the signalling of energy status, it is plausible to suggest that ghrelin may be acting as a link in the regulation of both energy balance and the circadian system in teleosts. The high levels of circulating ghrelin (e.g. during starvation) might modify clock gene 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 cross-talk between signals of nutritional reserves (such as ghrelin) and the circadian system in order to maintain metabolic balance in the liver and even in the whole organism.

In conclusion, the 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* confirms 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 may be that this hormone leads to the disruption of the hepatic rhythmicity, which would make the synchronizing action of ghrelin on the hepatic clock less likely. 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.

Competing interests

The authors declare no competing or financial interests.

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 analysed the data. A.M.B., A.S.-B. and E.I. performed the *in vitro*

cultures and analysed 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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Data availability
The *rev-erbα* sequence is published in GenBank (KU242427).

References
Abizaid, A. and Horvath, T. L. (2012). Ghrelin and the central regulation of feeding and energy balance. *Indian J. Endocrinol. Metab.* **16**, 617-626.
Albrecht, U. (2012). Timing to perfection: the biology of central and peripheral circadian clocks. *Neuron* **74**, 246-260.
Bechtold, D. A. (2008). Energy-responsive timekeeping. *J. Genet.* **87**, 447-458.
Biello, S. M., Golombek, D. A., Schak, K. M. and Harrington, M. E. (1997). Circadian phase shifts to neuropeptide Y in vitro: cellular communication and signal transduction. *J. Neurosci. Off. J. Soc. Neurosci.* **17**, 8468-8475.
Bodosi, B., Gardi, J., Hajdu, I., Szentirmai, E., Obal, F., Jr and Krueger, J. M. (2004). Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **287**, R1071-R1079.
Bonsall, D. R. and Lall, G. S. (2013). Protein kinase C differentially regulates entrainment of the mammalian circadian clock. *Chronobiol. Int.* **30**, 460-469.
Cai, W.-J., Yuan, X.-C., Yuan, Y.-C., Xie, S.-Q., Gong, Y., Su, H. and Qiao, Y. (2015). Sequence, genomic organization and expression of ghrelin receptor in grass carp, *Ctenopharyngodon idellus*. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **179**, 54-61.
Chen, C.-Y., Asakawa, A., Fujimiya, M., Lee, S.-D. and Inui, A. (2009). Ghrelin gene products and the regulation of food intake and gut motility. *Pharmacol. Rev.* **61**, 430-481.
Costa, L. S., Serrano, I., Sánchez-Vázquez, F. J. and López-Olmeda, J. F. (2016). Circadian rhythms of clock gene expression in Nile tilapia (*Oreochromis niloticus*) central and peripheral tissues: influence of different lighting and feeding conditions. *J. Comp. Physiol. B.* **186**, 775-785.
Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F. and Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev.* **14**, 2950-2961.
del Pozo, A., Vera, L. M., Sánchez, J. A. and Sánchez-Vázquez, F. J. (2012). Molecular cloning, tissue distribution and daily expression of cry1 and cry2 clock genes in European seabass (*Dicentrarchus labrax*). *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **163**, 364-371.
Delporte, C. (2013). Structure and physiological actions of ghrelin. *Scientifica* **2013**, 518909-518934.
Dunlap, J. C. (1999). Molecular bases for circadian clocks. *Cell* **96**, 271-290.
Feliciano, A., Vivas, Y., de Pedro, N., Delgado, M. J., Velarde, E. and Isorna, E. (2011). Feeding time synchronizes clock gene rhythmic expression in brain and liver of goldfish (*Carassius auratus*). *J. Biol. Rhythms* **26**, 24-33.
Escobar, S., Servili, A., Espigares, F., Guegen, M. M., Brocal, I., Felip, A., Gomez, A., Carrillo, M., Zanuy, S. and Kah, O. (2013). Expression of kisspeptins and kiss receptors suggests a large range of functions for kisspeptin systems in the brain of the European sea bass. *PLoS One* **8**, e70177.
Ginty, D. D., Kornhauser, J. M., Thompson, M. A., Bading, H., Mayo, K. E., Takahashi, J. S. and Greenberg, M. E. (1993). Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science* **260**, 238-241.
Gnanapavan, S., Kola, B., Bustin, S. A., Morris, D. G., McGee, P., Fairclough, P., Bhattacharya, S., Carpenter, R., Grossman, A. B. and Korbonits, M. (2002). The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J. Clin. Endocrinol. Metab.* **87**, 2988-2991.
Grey, C. L. and Chang, J. P. (2011). Differential involvement of protein kinase C and protein kinase A in ghrelin-induced growth hormone and gonadotrophin release from goldfish (*Carassius auratus*) pituitary cells: PKC and PKA in GRLN-induced goldfish GH and LH release. *J. Neuroendocrinol.* **23**, 1273-1287.
Grey, C. L. and Chang, J. P. (2013). Differential modulation of ghrelin-induced GH and LH release by PACAP and dopamine in goldfish pituitary cells. *Gen. Comp. Endocrinol.* **191**, 215-224.
Hastings, M., O'Neill, J. S. and Maywood, E. S. (2007). Circadian clocks: regulators of endocrine and metabolic rhythms. *J. Endocrinol.* **195**, 187-198.
Hastings, M. H., Brancaccio, M. and Maywood, E. S. (2014). Circadian pacemaking in cells and circuits of the suprachiasmatic nucleus. *J. Neuroendocrinol.* **26**, 2-10.
Kaiya, H., Miura, T., Matsuda, K., Miyazato, M. and Kangawa, K. (2010). Two functional growth hormone secretagogue receptor (ghrelin receptor) type 1a and 2a in goldfish, Carassius auratus. *Mol. Cell. Endocrinol.* **327**, 25-39.
Kaiya, H., Kangawa, K. and Miyazato, M. (2013). Ghrelin receptors in non-mammalian vertebrates. *Front. Endocrinol.* **4**, 81.
Kang, K. S., Yahashi, S., Matsuda, K., Kang, K. S., Yahashi, S. and Matsuda, K. (2011). The effects of ghrelin on energy balance and psychomotor activity in a goldfish model: an overview. *Int. J. Pept. Int. J. Pept.* **2011**, e171034.
Kohno, D., Gao, H.-Z., Muroya, S., Kikuyama, S. and Yada, T. (2003). Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca2+ signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes* **52**, 948-956.
Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H. and Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**, 656-660.
Lee, J. M., Schak, K. M. and Harrington, M. E. (1999). Inhibition of protein kinase A phase delays the mammalian circadian clock. *Brain Res.* **835**, 350-353.
Lee, B., Almad, A., Butcher, G. Q. and Obrietan, K. (2007). Protein kinase C modulates the phase-delaying effects of light in the mammalian circadian clock. *Eur. J. Neurosci.* **26**, 451-462.
LeSauter, J., Hoque, N., Weintraub, M., Pfaff, D. W. and Silver, R. (2009). Stomach ghrelin-secreting cells as food-entrainable circadian clocks. *Proc. Natl. Acad. Sci. USA* **106**, 13582-13587.
Li, Y., Guo, F., Shen, J. and Rosbash, M. (2014). PDF and cAMP enhance PER stability in Drosophila clock neurons. *Proc. Natl. Acad. Sci. USA* **111**, E1284-E1290.
Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. *Methods* **25**, 402-408.
López-Olmeda, J. F., Tartaglione, E. V., de la Iglesia, H. O. and Sánchez-Vázquez, F. J. (2010). Feeding entrainment of food-anticipatory activity and *per1* expression in the brain and liver of zebrafish under different lighting and feeding conditions. *Chronobiol. Int.* **27**, 1380-1400.
Madrid, J. A., Boujard, T. and Sánchez-Vázquez, F. J. (2001). Feeding rhythms. In *Food Intake in Fish* (ed. D. Houlihan, T. Boujard and M. Jobling), pp. 189-215. Oxford: Blackwell Science Ltd.
Martín-Robles, A. J., Isorna, E., Whitmore, D., Muñoz-Cueto, J. A. and Pendón, C. (2011). The clock gene Period3 in the nocturnal flatfish *Solea senegalensis*: molecular cloning, tissue expression and daily rhythms in central areas. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **159**, 7-15.
McArthur, A. J., Hunt, A. E. and Gillette, M. U. (1997). Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: activation of protein kinase C at dusk and dawn. *Endocrinology* **138**, 627-634.
Mistlberger, R. E. (2011). Neurobiology of food anticipatory circadian rhythms. *Physiol. Behav.* **104**, 535-545.
Miura, T., Maruyama, K., Kaiya, H., Miyazato, M., Kangawa, K., Uchiyama, M., Shioda, S. and Matsuda, K. (2009). Purification and properties of ghrelin from the intestine of the goldfish, *Carassius auratus*. *Peptides* **30**, 758-765.
Motzkus, D., Maronde, E., Grunenberg, U., Lee, C. C., Forssmann, W.-G. and Albrecht, U. (2000). The human *PER1* gene is transcriptionally regulated by multiple signaling pathways. *FEBS Lett.* **486**, 315-319.
Motzkus, D., Loumi, S., Cadenas, C., Vinson, C., Forssmann, W.-G. and Maronde, E. (2007). Activation of human period-1 by PKA or CLOCK/BMAL1 is conferred by separate signal transduction pathways. *Chronobiol. Int.* **24**, 783-792.
Nader, N., Chrousos, G. P. and Kino, T. (2010). Interactions of the circadian CLOCK system and the HPA axis. *Trends Endocrinol. Metab.* **21**, 277-286.
Nisembaum, L. G., de Pedro, N., Delgado, M. J. and Isorna, E. (2014). Crosstalking between the "gut-brain" hormone ghrelin and the circadian system in the goldfish. Effects on clock gene expression and food anticipatory activity. *Gen. Comp. Endocrinol.* **205**, 287-295.
Oishi, K., Amagai, N., Shirai, H., Kadota, K., Ohkura, N. and Ishida, N. (2005). Genome-wide expression analysis reveals 100 adrenal gland-dependent circadian genes in the mouse liver. *DNA Res.* **12**, 191-202.
Panda, S., Hogenesch, J. B. and Kay, S. A. (2002). Circadian rhythms from flies to human. *Nature* **417**, 329-335.
Patton, D. F., Katsuyama, Â. M., Pavlovski, I., Michalik, M., Patterson, Z., Parfyonov, M., Smit, A. N., Marchant, E. G., Chung, J., Abizaid, A. et al. (2014). Circadian mechanisms of food anticipatory rhythms in rats fed once or twice daily: clock gene and endocrine correlates. *PLoS ONE* **9**, e112451.
Reddy, A. B., Maywood, E. S., Karp, N. A., King, V. M., Inoue, Y., Gonzalez, F. J., Lilley, K. S., Kyriacou, C. P. and Hastings, M. H. (2007). Glucocorticoid signaling synchronizes the liver circadian transcriptome. *Hepatology* **45**, 1478-1488.
Sánchez-Bretaño, A., Alonso-Gómez, Á. L., Delgado, M. J. and Isorna, E. (2015a). The liver of goldfish as a component of the circadian system: integrating a network of signals. *Gen. Comp. Endocrinol.* **221**, 213-216.
Sánchez-Bretaño, A., Blanco, A. M., Unniappan, S., Kah, O., Bertucci, J. I., Alonso-Gómez, A. L., Valenciano, A. I., Isorna, E. and Delgado, M. J. (2015b). 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*, e0141043.

Sánchez-Bretaño, A., Gueguen, M.-M., Cano-Nicolau, J., Kah, O., Alonso-Gómez, A. L., Delgado, M. J. and Isorna, E. (2015c). Anatomical distribution and daily profile of gper1b gene expression in brain and peripheral structures of goldfish (*Carassius auratus*). *Chronobiol. Int.* **32**, 889-902.

Sánchez-Bretaño, A., Callejo, M., Montero, M., Alonso-Gómez, A. L., Delgado, M. J. and Isorna, E. (2016). Performing a hepatic timing signal: glucocorticoids induce *gper1a* and *gper1b* and repress *gclock1a* and *gbmal1a* in the liver of goldfish. *J. Comp. Physiol. B.* **186**, 73-82.

Sánchez-Vázquez, F. J. and Madrid, J. A. (2001). Feeding anticipatory activity. In *Food Intake in Fish* (ed. D. Houlihan, T. Boujard and M. Jobling), pp. 216-232. Oxford: Blackwell Science Ltd.

Sato, T., Nakamura, Y., Shiimura, Y., Ohgusu, H., Kangawa, K. and Kojima, M. (2012). Structure, regulation and function of ghrelin. *J. Biochem.* **151**, 119-128.

Schak, K. M. and Harrington, M. E. (1999). Protein kinase C inhibition and activation phase advances the hamster circadian clock. *Brain Res.* **840**, 158-161.

Schibler, U., Gotic, I., Saini, C., Gos, P., Curie, T., Emmenegger, Y., Sinturel, F., Gosselin, P., Gerber, A., Fleury-Olela, F. et al. (2015). Clock-Talk: interactions between central and peripheral circadian oscillators in mammals. *Cold Spring Harb. Symp. Quant. Biol.* **80**, 223-232.

Schmutz, I., Albrecht, U. and Ripperger, J. A. (2012). The role of clock genes and rhythmicity in the liver. *Mol. Cell. Endocrinol.* **349**, 38-44.

Spieler, R. E. (1992). Feeding-entrained circadian rhythms in fishes. In *Rhythms in Fishes* (ed. M. A. Ali), pp. 137-147. New York: Springer US.

Stephan, F. K. (2002). The “other” circadian system: food as a Zeitgeber. *J. Biol. Rhythms* **17**, 284-292.

Sujino, M., Furukawa, K., Koinuma, S., Fujioka, A., Nagano, M., Iigo, M. and Shigeyoshi, Y. (2012). Differential entrainment of peripheral clocks in the rat by glucocorticoid and feeding. *Endocrinology* **153**, 2277-2286.

Tinoco, A. B., Nisembaum, L. G., de Pedro, N., Delgado, M. J. and Isorna, E. (2014). Leptin expression is rhythmic in brain and liver of goldfish (*Carassius auratus*). Role of feeding time. *Gen. Comp. Endocrinol.* **204**, 239-247.

Tischkau, S. A., Gallman, E. A., Buchanan, G. F. and Gillette, M. U. (2000). Differential cAMP gating of glutamatergic signaling regulates long-term state changes in the suprachiasmatic circadian clock. *J. Neurosci.* **20**, 7830-7837.

Travnickova-Bendova, Z., Cermakian, N., Reppert, S. M. and Sassone-Corsi, P. (2002). Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. *Proc. Natl. Acad. Sci. USA* **99**, 7728-7733.

Tsang, A. H., Barclay, J. L. and Oster, H. (2014). Interactions between endocrine and circadian systems. *J. Mol. Endocrinol.* **52**, R1-R16.

Unniappan, S. and Peter, R. E. (2004). *In vitro* and *in vivo* effects of ghrelin on luteinizing hormone and growth hormone release in goldfish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **286**, R1093-R1101.

Vatine, G., Vallone, D., Gothilf, Y. and Foulkes, N. S. (2011). It's time to swin! Zebrafish and the circadian clock. *FEBS Lett.* **585**, 1485-1494.

Vera, L. M., Negrini, P., Zagatti, C., Frigato, E., Sánchez-Vázquez, F. J. and Bertolucci, C. (2013). Light and feeding entrainment of the molecular circadian clock in a marine teleost (*Sparus aurata*). *Chronobiol. Int.* **30**, 649-661.

Yannielli, P. C., Molyneux, P. C., Harrington, M. E. and Golombek, D. A. (2007). Ghrelin effects on the circadian system of mice. *J. Neurosci.* **27**, 2890-2895.

Yin, Y., Li, Y. and Zhang, W. (2014). The growth hormone secretagogue receptor: its intracellular signaling and regulation. *Int. J. Mol. Sci.* **15**, 4837-4855.

Zhang, L., Abraham, D., Lin, S.-T., Oster, H., Eichele, G., Fu, Y.-H. and Ptáček, L. J. (2012). PKCγ participates in food entrainment by regulating BMAL1. *Proc. Natl. Acad. Sci. USA* **109**, 20679-20684.

Zhou, L., Gao, Q., Zhang, P., Guo, S., Gu, J., Hao, W. and Cao, J.-M. (2014). Activation of growth hormone secretagogue receptor induces time-dependent clock phase delay in mice. *Am. J. Physiol. Endocrinol. Metab.* **307**, E515-E526.

Zmrzljak, U. P., Korenčič, A., Košir, R., Goličnik, M., Sassone-Corsi, P. and Rozman, D. (2013). Inducible cAMP early repressor regulates the *Period 1* gene of the hepatic and adrenal clocks. *J. Biol. Chem.* **288**, 10318-10327.