Comparative Biochemistry and Physiology, Part A 283 (2023) 111458

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Daily rhythms of REV-ERBα and its role as transcriptional repressor of clock genes in fish hepatic oscillator

Nuria Saiz, Lisbeth Herrera-Castillo, Miguel Gómez-Boronat, María Jesús Delgado, Esther Isorna, Nuria de Pedro [*]

Department of Genetics, Physiology and Microbiology, Faculty of Biological Sciences, Complutense University of Madrid, Spain

A R T I C L E I N F O

Edited by Michael Hedrick

Keywords:
Goldfish
Circadian system
nr1d1
Nuclear receptors
FEO

A B S T R A C T

The REV-ERBα nuclear receptor is a key component of the molecular machinery of circadian oscillators in mammals. While the rhythmic expression of this receptor has been described in teleosts, several critical aspects of its regulation remain unknown, such as which synchronizers entrain its rhythm, and whether it can modulate the expression of other clock genes. The objective of this study was to gain deeper understanding of the role of REV-ERBα in the fish circadian system. To this end, we first investigated the cues that entrain the rhythm of *rev-erbα* expression in the goldfish (*Carassius auratus*) liver and hypothalamus. A 12-h shift in feeding time induced a parallel shift in the hepatic rhythm of *rev-erbα* expression, confirming that this gene is food-entrainable in the goldfish liver. In contrast, light seems the main driver of *rev-erbα* rhythmic expression in the hypothalamus. Next, we examined the effects of REV-ERBα activation on locomotor activity and hepatic expression of clock genes. Subchronic treatment with the REV-ERBα agonist SR9009 slightly decreased locomotor activity anticipating light onset and food arrival, and downregulated hepatic *bmal1a*, *clock1a*, *cry1a*, *per1a* and *pparα* expression. This generalized repressing action of REV-ERBα on the expression of hepatic clock genes was confirmed *in vitro* by using agonists (SR9009 and GSK4112) and antagonist (SR8278) of this receptor. Overall, the present work reveals that REV-ERBα modulates the daily expression of the main genes of the teleostean liver clock, reinforcing its role in the liver temporal homeostasis, which seems highly conserved in both fish and mammals.

1. Introduction

Most physiological processes in animals exhibit rhythms of approximately to 24 h, driven by a network of oscillators that form the circadian system (Buijs et al., 2016). The circadian system anticipates cyclical environmental changes caused by the Earth rotation and adjusts physiology and behavior to them, provided that oscillators are regularly tuned by external cues such as cyclical light/dark and food availability (Mendoza, 2007; Roenneberg and Merrow, 2003).

Cellular circadian oscillators have a highly conserved molecular mechanism, centered on a self-regulating transcriptional and translational feedback loop, referred to as the core clock (Patke et al., 2020; Takahashi, 2015; Vatine et al., 2011). In vertebrates, the positive limb of the core clock includes the products of the genes *bmal1* (*brain and muscle arnt-like protein 1*) and *clock* (*circadian locomotor output cycle kaput*), which possess helix-loop-helix domains that recognize *E*-box regulatory sequences. The BMAL1:CLOCK heterodimer binds to these regions and induces the expression of genes from the negative limb, *i.e. per* (*period*) and *cry* (*cryptochrome*), whose products form the heterodimer PER:CRY, and return to the nucleus. Here, they inhibit BMAL1:CLOCK activity, and thus their own transcription, reinitiating the cycle every 24 h. The core clock is interlocked with secondary feedback loops that stabilize its period, one of them involving REV-ERB (repressor, *reverse strand of protein ERB*) and ROR (activator, *retinoic acid related orphan receptor*), which compete for binding to RORE/REvRE (ROR/REV-ERB response element)/sequences in *bmal* and *clock* promoters. In turn, the BMAL1:CLOCK heterodimer induces *rev-erb* and *ror via E*-boxes (Guillaumond et al., 2005). Ultimately, REV-ERB, ROR, and BMAL1:CLOCK regulate the transcription of the clock-controlled genes (CCGs), generating observable daily rhythms (Stratmann and Schibler, 2012). The core clock feedback loops work in a similar way in mammals and teleosts, but it is noteworthy that fish possess a different number of gene paralogs (Sánchez-Bretaño et al., 2015; Vatine et al., 2011). Nonetheless, research on the function of auxiliary loops genes in fish is limited

* Corresponding author at: Department of Genetics, Physiology and Microbiology, Faculty of Biological Sciences, Complutense University of Madrid, Spain.
E-mail address: ndepedro@ucm.es (N. de Pedro).

https://doi.org/10.1016/j.cbpa.2023.111458
Received 3 May 2023; Received in revised form 29 May 2023; Accepted 1 June 2023
Available online 7 June 2023
1095-6433/© 2023 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

compared to mammals and other model organisms.

One of the most overt outputs of the circadian system in vertebrates, including fish, is locomotor activity. Most species become more active during a specific phase of the light-dark cycle, which is the main synchronizer for locomotor activity (Krylov et al., 2021; López-Olmeda et al., 2006; Refinetti, 2015). Moreover, food can also synchronize daily locomotor activity rhythms. For instance, when food availability is restricted to a single time of day, animals anticipate this feeding time, displaying a gradual increase in activity that occurs a few hours before a scheduled meal (Davidson, 2006). This food anticipatory activity (FAA) has been well characterized in fish (Guerra-Santos et al., 2017; Isorna et al., 2017; Sánchez-Vázquez et al., 2001).

The present study focuses on the role of the above mentioned nuclear receptor REV-ERBα (also known as NR1D1, nuclear receptor subfamily 1, group D, member 1) in the circadian system of teleosts. Lacking activating capacity, REV-ERBα represses transcription as a monomer or as a dimer by binding to RORE/RevRE sequences in the promoter of its target genes (competing with the binding of nuclear ROR, which are activators of transcription) and recruiting the co-repressors nuclear co-repressor 1 (NCOR1) and HDAC3 (Mosure et al., 2021; Raghuram et al., 2007).

As a clock gene, *rev-erbα* is expressed with a circadian pattern in both mammals (Bookout et al., 2006; Guillaumond et al., 2005; Yang et al., 2006), and fish (Bolton et al., 2021; Delaunay et al., 2000; Gómez-Boronat et al., 2022). Furthermore, as aforementioned, REV-ERB plays a key role in the functioning of mammalian biological timekeeping (Bugge et al., 2012; Cho et al., 2012; Ikeda et al., 2019; Stratmann and Schibler, 2012). Notably, REV-ERBα is especially involved in the rhythmicity of peripheral tissues of mammals (Pett et al., 2018). This is evidenced by the strong disruption that deletion or overexpression of *rev-erbα/β* causes in the circadian expression of core clock genes and in locomotor activity rhythms, with a particularly severe effect in the liver, impairing the oscillations of a large part of its transcriptome (Cho et al., 2012; Ikeda et al., 2019; Kornmann et al., 2007). These results show that the cycling of *rev-erbα* underpins the circadian rhythms of many of its target genes, highlighting the essential role of REV-ERBα in optimal circadian function. However, little is known about the cues that drive *rev-erbα* rhythms in teleosts. A recent report in goldfish (*Carassius auratus*) shows that *rev-erbα* daily rhythms can be sustained by scheduled feeding under constant darkness in the liver, but not in the hypothalamus, suggesting that fasting/feeding and light/dark cycles may affect *rev-erbα* expression rhythms differently depending on the tissue (Gómez-Boronat et al., 2022). This suggests a potential role of REV-ERB*α* in food entrainment, as supported by several reports in mammals indicating its involvement in integrating of intake signals into the circadian clock (Delezie et al., 2016; Tahara et al., 2011). In fish, REV-ERBα may also play a fundamental role in the rhythmicity of the liver transcriptome, including genes associated with metabolic enzymes and feeding regulation, since it has demonstrated an important role on the energy balance of goldfish (Saiz et al., 2022).

The transcriptional repressing activity of REV-ERBα is ligand-dependent, meaning that binding of the heme group (its physiological ligand) seems to be required for REV-ERBα to recruit co-repressors and actively inhibit transcription of its target genes (Raghuram et al., 2007; Yin et al., 2007). Following this discovery, different synthetic ligands have been developed (Kojetin and Burris, 2014; Murray et al., 2022; Wang et al., 2020). Some of these ligands act as agonists, mimicking the action of heme, such as GSK4112 and SR9009. The use of GSK4112 to *in vitro* assays is limited by it poor pharmacokinetic properties, but SR9009 is more potent and effective, with qualities that make it suitable for *in vivo* studies. SR8272 blocks enhancement of REV-ERBα-dependent repression induced by both GSK4112 and heme (Kojetin and Burris, 2011). These drugs that directly target REV-ERBα have been shown to modulate the molecular clock, circadian behavior, and metabolism in mammals (Grant et al., 2010; Kojetin et al., 2011; Solt et al., 2012), making them very useful as pharmacological tools for studying REV-ERBα function.

As detailed above, REV-ERBα is being increasingly recognized as a key component of the mammalian clocks, making it imperative to understand its involvement in the teleost circadian system. The fact that *rev-erbα* rhythms are present in fish suggests a similar role as a clock gene, but its entraining cues are still under investigation. It is also unknown whether REV-ERBα activation modulates the expression of clock genes or modifies circadian behavior in fish. Considering this, the aims of this work are, first, to identify the impact of uncoupling mealtime and photocycle on the rhythm of *rev-erbα* expression in neural and peripheral clocks. Secondly, to define the effect of REV-ERBα activation on daily locomotor activity rhythms, as one of the most apparent outputs of the circadian system. Finally, we seek to examine the role of REV-ERBα activation and inhibition over the expression of hepatic clock genes both *in vivo* and *in vitro*.

2. Methodology

2.1. Animals and ethics statement

Goldfish ($n = 112$) were procured from a local commercial supplier (ICA, Madrid, Spain) and housed in 60-l aquariums (6–8 fish per aquarium). The aquaria were filled with filtered freshwater at a temperature of 21 ± 1 °C, and continuous aeration was provided. The fish were kept under a photoperiod of 12 h of light and 12 h of darkness (12L:12D), with the lights being turned on at 08:00 a.m. (Zeitgeber time 0, ZT 0). The light source was fluorescent lamps (~400 lx at the bottom of aquaria). They were fed dry pellets (Sera Pond, Heisenberg, Germany) every day at 10:00 a.m. (ZT 2), amounting to 1.5% of their body weight (bw) and dispensed by programmed feeders. Animals were acclimated to these conditions at least two weeks previous to any assay. The housing conditions of the fish used for all the procedures are the ones described in this section, unless otherwise specified in the specific experimental design. The experimental protocols conform to the regulations laid out by the European Union Council (EU 63/2010) and the Spanish Government (RD 53/2013) for protecting animals employed in scientific purposes. These protocols were endorsed by the Community of Madrid (PROEX 107/20).

2.2. Experimental procedures

2.2.1. Daily rhythms of rev-erbα in liver and hypothalamus

Goldfish weighing 22.5 ± 1.5 g were divided into two experimental groups, with two different feeding schedules: The first group ($n = 36$, 6 fish/aquarium) was fed daily at mid-photophase (ZT 6, referred to as Scheduled Feeding 6, SF6), while the second group (n = 36, 6 fish/aquarium) was fed daily at mid-scotophase (ZT 18, referred to as SF18). After three weeks, fish were sampled every 4 h over a 24-h period (6 fish per sampling point at ZT 5, ZT 9, ZT 13, ZT 17, ZT 21, and ZT 1). The fish were euthanized using tricaine methanesulfonate (MS-222, 0.3 g/l, Sigma-Aldrich, Madrid, Spain), and their liver and hypothalamus were collected, flash-frozen in liquid nitrogen, and stored at −80 °C for *rev-erbα* expression analysis with qPCR. The collection of tissues during the scotophase was conducted under dim red light.

2.2.2. Effects of a sub-chronic treatment with SR9009 on clock gene expression in liver

Goldfish (15.5 ± 0.5 g bw, $n = 12$ fish/group) were divided into four aquaria (6 fish/aquarium, two for each experimental group) (control or SR9009, n = 12 fish/group). During 7 days, fish were anesthetized (MS-222, 0.14 g/l) and injected (intraperitoneal-IP, 10 μl/g bw) at 13:00 h (ZT 5, 3 h post-feeding) with either vehicle alone (85% teleost saline solution and 15% Kolliphor®) or containing the REV-ERBα agonist SR9009 (100 μg/g bw). All products were purchased to Sigma-Aldrich. After IP injections fish were released back into the aquaria, where they recovered their balance and locomotion within 1–2 min. Locomotor

activity was monitored throughout the experiment. On the eighth day, after 27 h of fasting (ZT 5), the fish were euthanized, and their liver was collected, frozen rapidly in liquid nitrogen, and stored at −80 °C until analysis.

2.2.3. Effects of REV-ERBα agonists and antagonist on clock gene expression in liver explants

Two pharmacological approaches were carried out by using organotypic cultures of liver explants (Sánchez-Bretaño et al., 2016). Briefly, liver explants (20 mg) of eight 24 h-fasted fish (29.3 ± 1.3 g bw, $n = 8$) were placed in plates at 23:00 h (ZT 15) and pre-incubated for 1 h in 1 ml Dulbecco's Modified Eagle Medium (DMEM, 9 g/l) supplemented with $NaHCO_3$ (990 mg/l), HEPES (2.43 g/l) and antibiotics (penicillin–streptomycin, 10 ml/l and gentamicin 500 mg/l). After pre-incubation, the medium was replaced with 1 ml of fresh DMEM containing the vehicle (dimethyl sulfoxide, DMSO, 0.04%) alone (control group) or including the agonists SR9009 (10 and 30 μM), GSK4112 (10 and 30 μM), and the antagonist SR8278 (10 and 30 μM). In addition, to investigate the specificity of the agonistic effects of GSK4112 we blocked the receptor by incubating liver explants in the presence of SR8278, including the following experimental groups: control (DMSO, 0.04%), GSK4112 (10 μM) alone, SR8278 (10 μM) alone, and agonist and antagonist together (10 μM SR8278 + 10 μM GSK4112 added 30 min later). All products were purchased to Sigma-Aldrich. The liver explants were incubated at constant temperature (21 ± 1 °C), 12L:12D photoperiod (lights on at 8:00 h, 400 lx) and a 5% CO_2 and 95% O_2 atmosphere. After 16 (ZT 8) and 24 h (ZT 16), the content of the wells was centrifuged, liver explants were retrieved, frozen in liquid nitrogen and maintained at −80 °C until clock gene expression analysis. Sampling during the scotophase was conducted under dim red light The sampling points ZT 8 and ZT 16 were chosen because they correspond to the expected time of increase in *bmal1a/clock1* transcripts and *rev-erbα* transcripts, respectively (Gómez-Boronat et al., 2018; present results).

2.3. Analysis of gene expression

The relative abundance of *rev-erbα*, *pparα*, *bmal1a*, *clock1a*, *cry1a* and *per1a* mRNA was determined by RT-qPCR as described in Saiz et al. (2022). Briefly, total RNA was extracted using the phenol/chloroform method (TRI® Reagent, Sigma-Aldrich) following the manufacturer's instructions in mechanically homogenized liver samples. Then, RNA samples were treated with RQ1 RNAse-Free DNAse (Promega, Madison, WI, USA) to prevent genomic contamination, and RNA was purified by precipitation to remove contaminants. After that, reverse transcription was carried out using 0.5 μg of total RNA, random primers (Invitrogen, Waltham, MA, USA), RNAse inhibitor (Promega, Madison, WI, USA), and SuperScript IV Reverse Transcriptase (Invitrogen, Waltham, MA, USA) following the manufacturer protocol.

Duplicate real-time quantitative PCR (RT-qPCR) assays were performed for each sample using iTaq TM Universal SYBR®Green Supermix (Bio-Rad Laboratories) in a 96-well plate. Each well contained 1 μl of sample cDNA, specific primers for each gene at a final concentration of 0.5 μM, and SYBR®Green Supermix to a final volume of 10 μl. Each PCR run included a standard curve of cDNA, and a negative control (water). The protocol involved an initial denaturation at 95 °C for 30 s followed by 40 cycles of a two-step amplification program (95 °C for 5 s and 60 °C for 30 s). To verify the reaction specificity, melting curves were systematically monitored (temperature gradient from 70 to 90 °C at a 0.5 °C/5 s rate). The primer sequences (Sigma-Aldrich) and GenBank reference numbers utilized for housekeeping and target genes are listed in Supplementary Table 1. Relative gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001)

2.4. Locomotor activity recordings

The method for recording daily locomotor activity of goldfish was detailed in previous studies (Saiz et al., 2021). To summarize, infrared photocells (E3S-AD12, Omron Corporation, Kyoto, Japan) were attached to the walls of aquaria and connected to an actimeter that transmits the number of light beam crossings (pulses) every 10 min to a data acquiring software Adq16 (Micronec, Madrid, Spain). 6 photocells were distributed along the walls of the aquaria, at a height of 3–15 cm above the bottom. To minimize external interferences, the walls of the aquaria were covered with opaque paper.

2.5. Statistics

Sigmaplot® 12 software was used for statistical analysis of relative expression. Shapiro–Wilk and Levene tests were conducted to ensure data normality and homoscedasticity, and data sets were transformed to a logarithmic or square root scale if necessary. To compare relative gene expression among groups, a one-way ANOVA was performed, followed by the *post-hoc* Student-Newman-Keuls (SNK) test, and considering $p < 0.05$ as the significance threshold. In the experiment that combined two treatments, two-way ANOVA was performed to search for individual effects of each drug or interactions between them. If an interaction was found, the groups were compared with one-way ANOVA. Locomotor activity data were analyzed with El Temps® software, which was used to obtain profiles of average daily activity rhythms, actograms, and periodograms. Chi-square periodograms were used to examine the existence of significant periods ($p < 0.05$) in activity rhythms. Differences between groups in activity in the 4 h pre-feeding, ZT 22-2 (percentage, considering total daily pulses 100%), as well as in the second half of the photophase (ZT 6-12), were determined by Student's *t*-test. Cosinor analysis was conducted to evaluate the existence of 24-h rhythms of *rev-erbα* expression, fitting the data to sinusoidal functions by the least square's method (Duggleby, 1981): $f(t) = M + A \bullet cos(\frac{t\pi}{12} - \Phi)$, $f(t)$ (mRNA abundance at a given time), M (rhythm-adjusted mean value), A (oscillation amplitude), t (time, h), and Φ (acrophase, time of highest expression). The significance of Cosinor analysis was determined by the ratio SE(A)/A < 0.3 (Nisembaum et al., 2012).

3. Results

The daily variations of *rev-erbα* expression in hypothalamus and liver of goldfish fed at different times of day are shown in Fig. 1. Both groups (SF6 and SF18) showed significant 24 h rhythms of *rev-erbα* mRNA in the hypothalamus. The SF6 group showed a higher amplitude and an acrophase at mid-scotophase (ZT 18), while in SF18 fish, the amplitude was 3-fold lower and the acrophase was 3-h advanced (ZT 15, Fig. 1a). Hepatic expression of *rev-erbα* in the goldfish displayed significant 24 h rhythms in both SF6 and SF18 fish (Fig. 1b), with a higher amplitude than in the hypothalamus. When the feeding schedule was shifted from midday (ZT 6) to midnight (ZT 18), the acrophase of the hepatic *rev-erbα* rhythm was as well 12 h displaced (from ZT 19 to ZT 7).

Locomotor activity of control and SR9009-treated fish is shown in Fig. 2. The actograms (Fig. 2a–b) and waveforms (Fig. 2c–d), show a daily (24 h) significant locomotor activity rhythm in both groups with higher activity during the light phase (ZT 0-12) than in the dark phase (ZT 12-24). However, although the rise in activity prior to feeding already began during the last hours of the dark phase in the control group (22.43 ± 1.6% of the activity happened from ZT 22 to ZT 2, Fig. 2c), this pattern was less pronounced ($p = 0.05$, Student's *t*-test) in the group treated with the agonist SR9009 (19.33 ± 0.9%, Fig. 2d). Also, SR9009-treated fish showed slightly reduced levels of activity in the second half of the photophase (23.6 ± 1.8% *vs.* 26.0 ± 1.5%), although this trend was not statistically significant (Fig. 2c–d).

The sub-chronic treatment with the REV-ERB agonist SR9009 produced a significant decrease ($p < 0.05$) in the hepatic expression of *bmal1a*, *clock1a*, *per1a*, and *pparα* compared to the control group (Fig. 3a, b, e, d), without significant modifications in *cry1a* expression

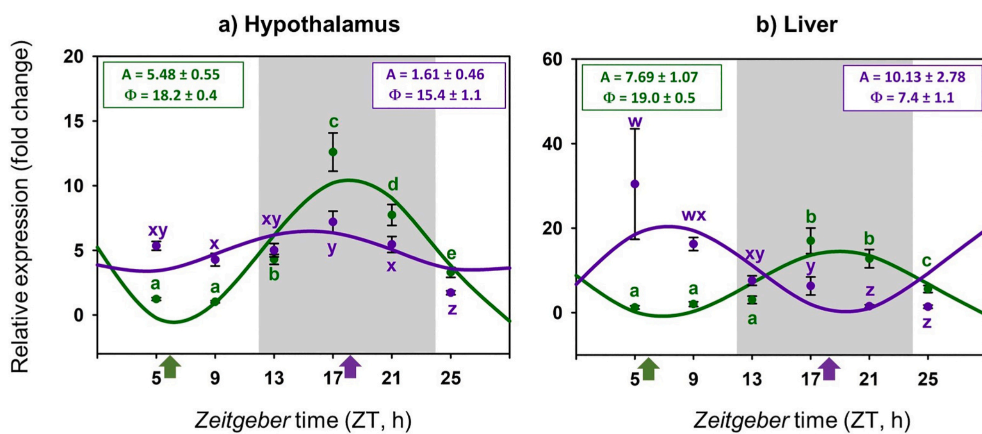

Fig. 1. Daily expression profile of *rev-erbα* in a) hypothalamus and b) liver of goldfish fed during mid-photophase and mid-scotophase. The grey area signals the dark phase of 12L:12D photoperiod, while the arrow indicates feeding time: at ZT 6, group SF6 (green); at ZT 18, group SF18 (purple). Data are expressed as mean ± SEM (n = 6) in relative units (data were normalized to the lowest expression group). The periodic sinusoidal functions, represented as purple (SF6 fish) or green wave (SF18 fish), represent significant rhythms by Cosinor analysis [SE(A)/A < 0.3]. The parameters defining the rhythms, such as amplitude (A), acrophase (Φ), and their standard errors, are displayed. Different letters express significant differences by one-way ANOVA (a–e in SF6 group; w-z in SF18 group). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

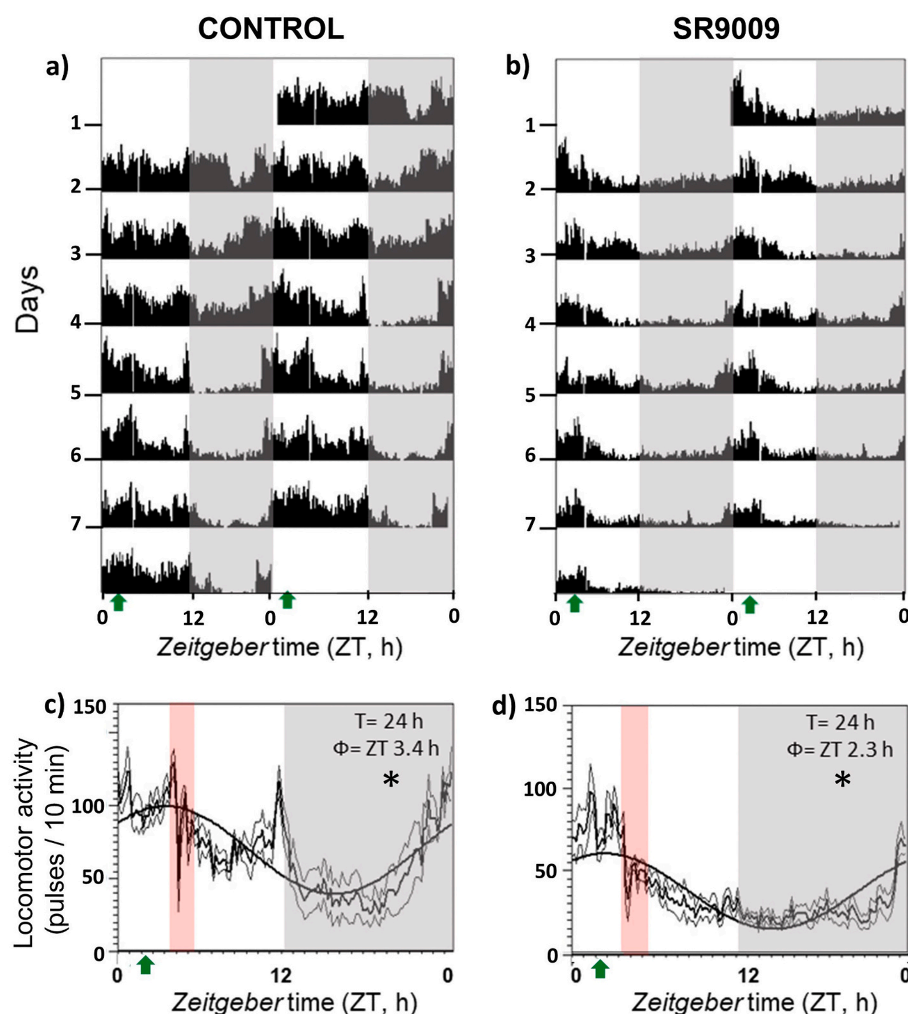

Fig. 2. Representative locomotor activity diagrams from goldfish intraperitoneally injected with vehicle (control) or SR9009 (100 μg/g bw) for 7 days. a, b: Actograms are presented in a double plot format (48 h time scale) to improve visualization. c, d: Average waveform of locomotor activity (values are the mean ± SEM). Light and dark phases are indicated by white and grey areas, respectively. The red zones indicate the injection time (ZT 5) and the green arrows point to feeding time (ZT 2). Period (T) and significancy of the rhythms according to Sokolove–Bushell periodograms, as well as acrophase (Φ) according to Cosinor analysis are shown. * indicates a significant rhythm $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3c).

The Fig. 4 shows the expression levels of the clock genes of the positive loop, *bmal1a* and *clock1a*, in the goldfish liver after 16 and 24 h of culture time in the presence of the agonists SR9009 and GSK4112 and the antagonist SR8278. Both agonists SR9009 (10 and 30 μM) and GSK4112 (30 μM) significantly reduced the expression of *bmal1a* at 24 h culture time (Fig. 4a, c). The hepatic expression of *clock1a* was also decreased by the two agonists, with SR9009 (10 μM) at 16 h culture time (Fig. 4b) and with the GSK4112 (30 μM) at 24 h (Fig. 4d). In agreement, the antagonist SR8278 increased the hepatic expression of *clock1a* (10 μM) at both incubation times (Fig. 4f), without statistically significant modifications on the expression of *bmal1a* (Fig. 4e).

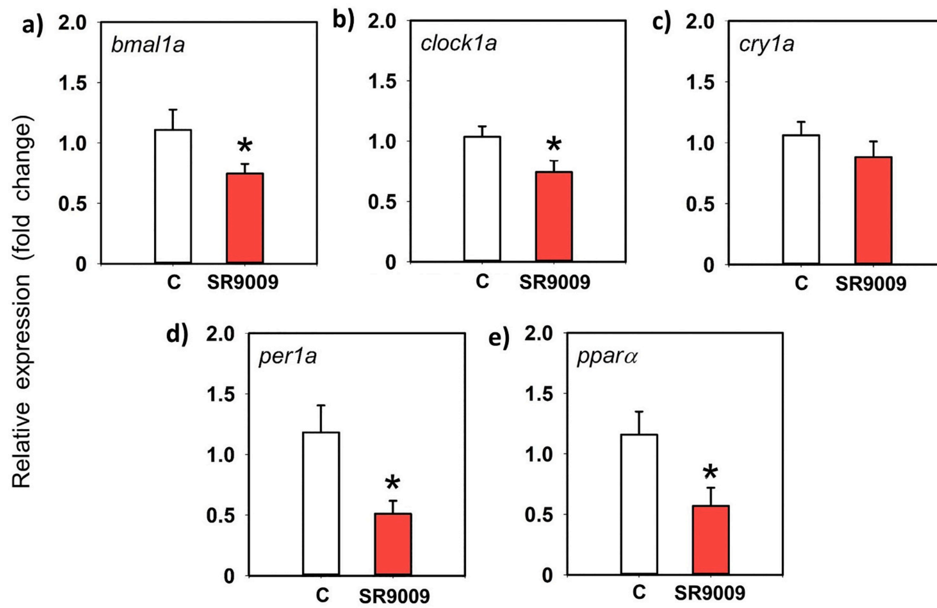

Fig. 3. Effect of sub-chronic intraperitoneal injection of the REV-ERBα agonist (SR9009) or vehicle (C) on hepatic expression of clock genes in *Carassius auratus*. Data are expressed as the mean + SEM (n = 12/group) in relative mRNA units (the SR9009 group is relativized to the control fish, C). *p < 0.05 (Student's *t*-test).

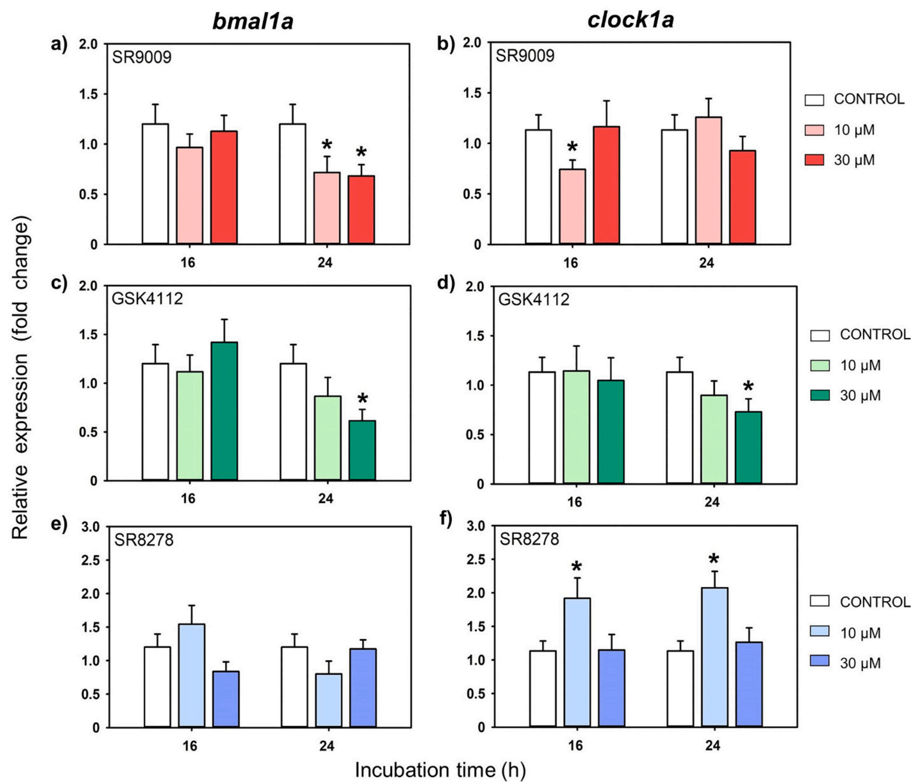

Fig. 4. *In vitro* effect of two REV-ERBα agonists, SR9009 (a, b) and GSK4112 (c, d), and the antagonist SR8278 (e, f) on hepatic expression of clock genes *bmal1a* and *clock1a* in *Carassius auratus*. Data are expressed as the mean + SEM (n = 8/group) in relative units of mRNA (relativizing the experimental groups to the control fish). *p < 0.05 compared to the control group at the same incubation time (one-way ANOVA; post-hoc test SNK).

The hepatic expression of the negative loop genes, *per1a* and *cry1a*, is shown in the Fig. 5. SR9009 (10 μM at 16 h and 30 μM at 24 h) down-regulated the expression of *per1a* (Fig. 5a). Comparable results were observed with GSK4112 at 16 (30 μM) and 24 h (10 μM, Fig. 5c). Both agonists (SR9009 and GSK4112) at 10 μM concentration of decreased the hepatic expression of *cry1a* at 24 h culture time (Fig. 5b, d). Furthermore, the antagonist SR8278 upregulated the expression of both *per1a* and *cry1a* at 16 h incubation time (Fig. 5e, f).

Hepatic expression of *pparα*, the member of the auxiliary loop in the molecular clock, was significantly reduced at both incubation times in presence of both agonists employed in the culture (Fig. 6a, b). However, it was increased by the presence of the highest concentration of the

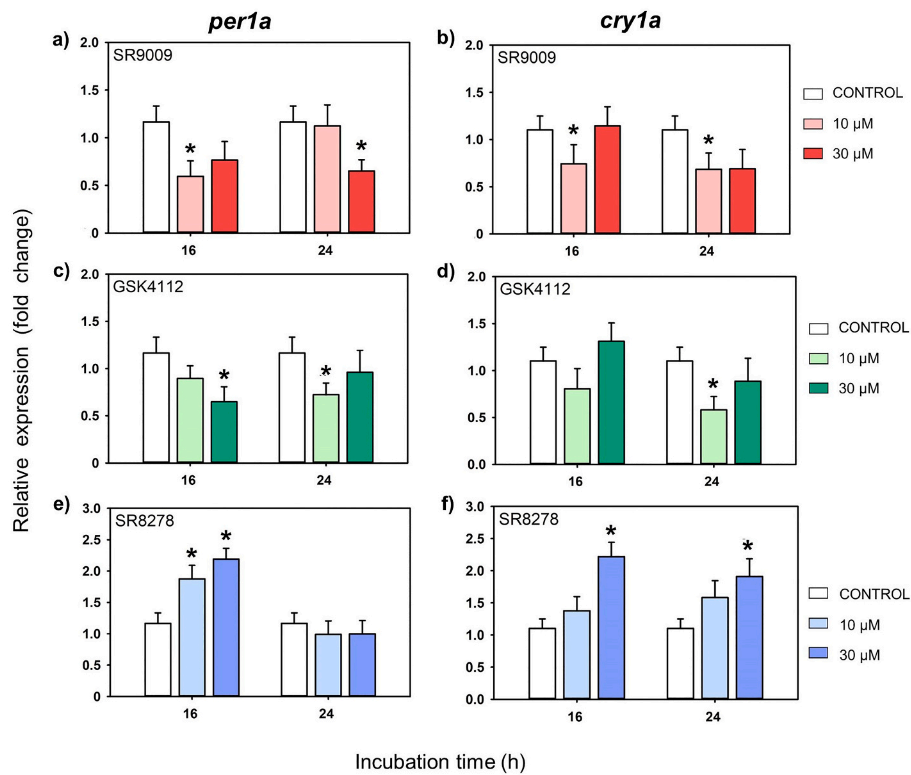

Fig. 5. *In vitro* effect of two REV-ERBα agonists SR9009 (a, b) and GSK4112 (c, d), and antagonist SR8278 (e, f) on hepatic expression of clock genes *per1a* and *cry1a* in *Carassius auratus*. Data are expressed as the mean + SEM ($n = 8$/group) in relative units of mRNA (relativizing the experimental groups to the control fish). *$p < 0.05$ compared to the control group at the same incubation time (one-way ANOVA; post-hoc test SNK).

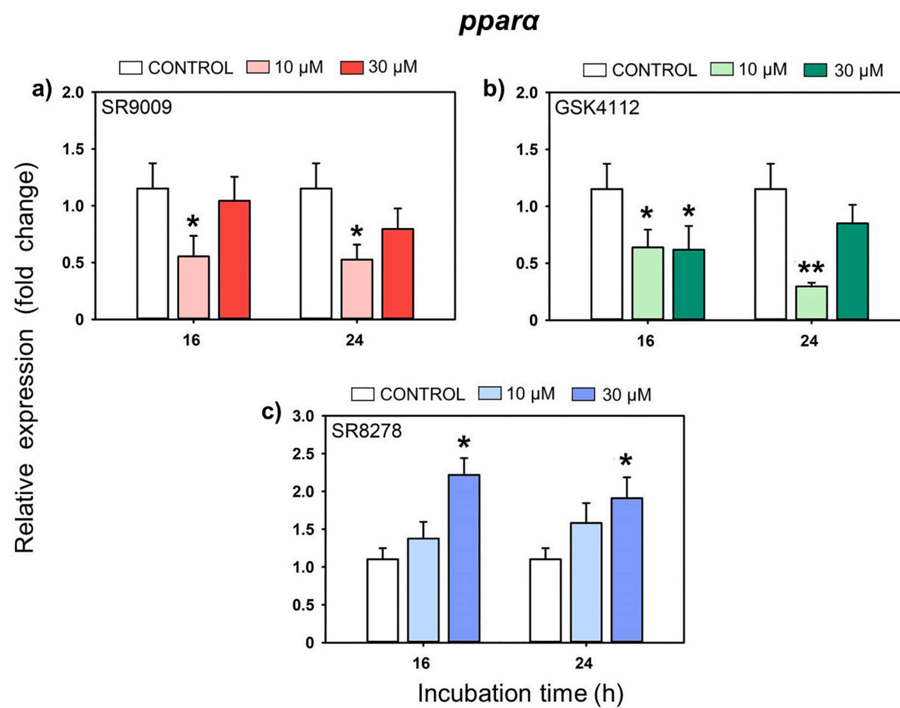

Fig. 6. *In vitro* effect of two REV-ERBα agonists, SR9009 (a) and GSK4112 (b), and antagonist SR8278 (c) on hepatic expression of clock gene *pparα* in *Carassius auratus*. Data are expressed as the mean + SEM ($n = 8$/group) in relative units of mRNA (relativizing the experimental groups to the control fish). *$p < 0.05$ and **$p < 0.01$ compared to the control group at the same incubation time (one-way ANOVA; post-hoc test SNK).

antagonist SR8278 at the two culture times (16 and 24 h, Fig. 6c), and was increased by the antagonist SR8278 at 30 μM (Fig. 6c).

The results from liver explants incubated in the presence of the agonist GSK4112 and the antagonist SR8278 alone or combined are shown in Figs. 7–9. After 16 h of culture, the antagonist SR8278 produced a significant increase of *bmal1a* transcripts, which was dampened when combined with the agonist GSK4112 (Fig. 7a). After 24 h of incubation, an interaction was found between the effects of both drugs on *bmal1a* expression, which was significantly decreased by GSK4112, an effect which was not observed when incubated together with the antagonist (Fig. 7c). The other element of the positive loop, *clock1a*, was independently downregulated by the agonist (GSK4112) and upregulated by the antagonist (SR8278) at 24 h of incubation, the combined group displaying intermediate expression levels (Fig. 7d). No modifications after 16 h of incubation were found (Fig. 7b).

Regarding the clock genes of the negative loop, hepatic *per1a* mRNA increased after 16 and 24 h of culture with the antagonist SR8278, as well as decreased after 24 h of treatment with the agonist GSK4112. In all cases, the antagonist partially counteracted the effects of the agonist (Fig. 8a, c). Furthermore, *cry1a* expression was reduced by the agonist after 24 but not 16 h of culture, an effect that was slightly reverted by the antagonist in the combined treatment (Fig. 8b, d).

The hepatic expression of *pparα* was significantly modified at both incubation times, as expected the agonist reduced its expression and the antagonist raised it, an effect that was more accused at 16 h. The downregulation induced by the agonist GSK4112 was not observed when liver explants were previously incubated with the antagonist (Fig. 9a, b).

4. Discussion

The present results contribute to a better understanding of the role of REV-ERBα in the circadian system of fish. On one hand, they confirm that the phase of *rev-erbα* expression rhythm is determined by feeding time in the liver but not in a central nervous system oscillator, suggesting a differential regulation of this gene among brain and peripheral tissues. On the other hand, they provide insight into the effects of this receptor activation on the hepatic circadian clock machinery itself, showing repressions on several core clock genes *in vivo* and *in vitro*. Results on locomotor activity are not conclusive, but suggest that REV-ERBα activation could reduce the activity increase observed in anticipation to the light onset and feeding time.

4.1. Feeding synchronization of rev-erbα expression in liver and hypothalamus

Quantification of *rev-erbα* mRNA in fish with a 12-h shift in mealtime confirms that its levels follow a daily rhythm in both goldfish liver and hypothalamus. The amount of transcripts peaks at the mid of the dark (~ZT 18) in both tissues under daytime feeding (SF6) conditions, matching recent reports (Gómez-Boronat et al., 2022). This pattern of expression in goldfish under 12L:12D and fed at midday is consistent with that observed in other diurnal teleosts such as zebrafish (Amaral and Johnston, 2012; Delaunay et al., 2000) or Atlantic salmon (Bolton et al., 2021). However, *rev-erbα* rhythms were absent in the liver of the latter species, under *ad libitum* feeding conditions (Betancor et al., 2014). Looking at the present data, a 12-h shift in feeding time caused an equivalent shift in *rev-erbα* acrophase in the liver of fish fed at mid-scotophase (SF18), suggesting that these cycles are preferentially entrained by the feeding schedule, as expected for this organ that is considered a FEO in both mammals and fish (Gómez-Boronat et al., 2018; Isorna et al., 2017; Mistlberger, 2006). In contrast, in the hypothalamus the phase of *rev-erbα* rhythm is not shifted by the 12-h feeding lag, confirming that food is not the main *Zeitgeber* for hypothalamic *rev-erbα*, aligning with recent studies that consider this region as a LEO (Gómez-Boronat et al., 2022). Nevertheless, the decrease in amplitude observed in goldfish fed at midnight reveals that daily oscillations of this nuclear receptor in the hypothalamus are altered by the uncoupling of photic and nutritional cues, as occurs for other clock genes (Gómez-Boronat et al., 2018). However, it is important to keep in mind that the ability of REV-ERBα to repress transcription depends not only on the amount of protein but also on its activation by its specific ligand, the heme group, which also shows daily oscillations in mammals (Rogers et al., 2008).

4.2. Effects of a 7-day sustained REV-ERBα activation on daily rhythms of locomotor activity

Being a predominantly diurnal species (Iigo and Tabata, 1996), all the goldfish in this study show higher activity during the photophase and exhibit a 24-h rhythmic pattern of locomotor activity. This 24-h rhythm was maintained under SR9009 treatment, consistent with

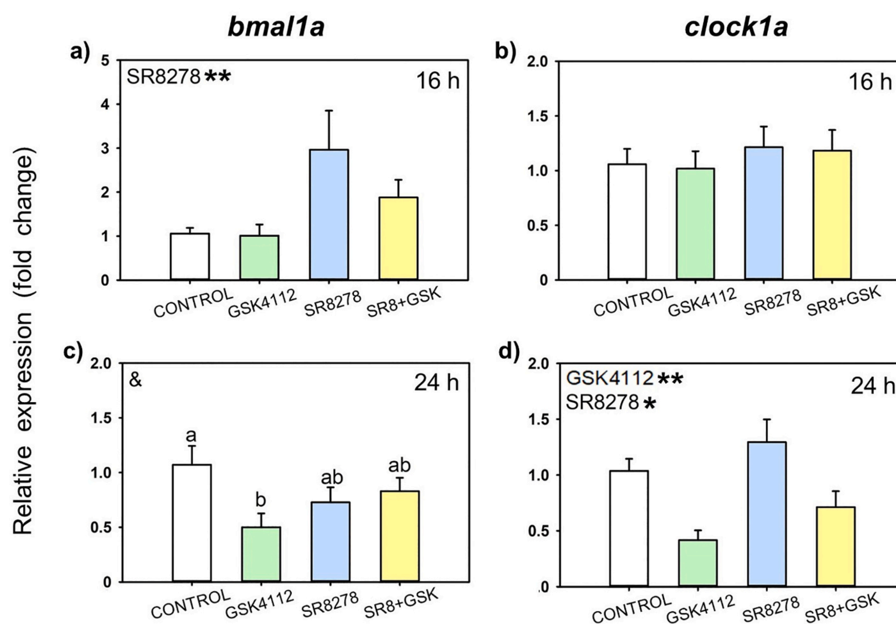

Fig. 7. *In vitro* effect of REV-ERBα agonist GSK4112 (10 μM) and antagonist SR8278 (10 μM) alone or combined (SR8278 + GSK4112) on hepatic expression of *bmal1a* and *clock1a* in *Carassius auratus*. Data expressed as the mean + SEM ($n = 8$/group) in relative units of mRNA (relativizing the experimental groups to the control fish). $*p < 0.05$, $**p < 0.01$ shows significant effect of SR8278 or GSK4112, and & indicates the interaction of both drugs (two-way ANOVA). Whenever there was a significant interaction, a one-way ANOVA followed by a SNK test was performed, different letters showing significant differences.

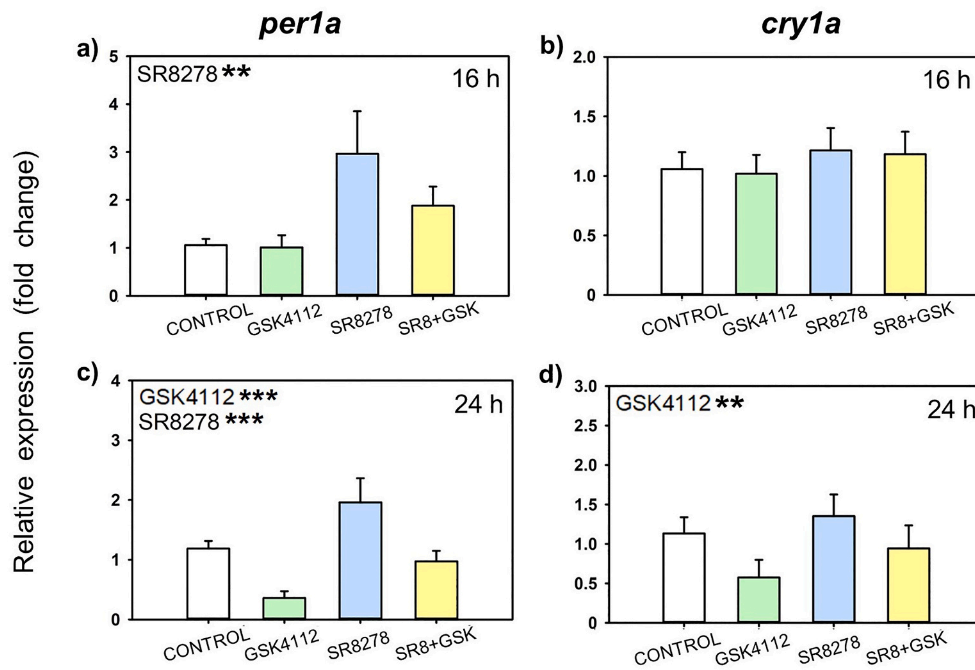

Fig. 8. *In vitro* effect of REV-ERBα agonist GSK4112 (10 μM) and antagonist SR8278 (10 μM) alone or combined (SR8278 + GSK4112) on hepatic expression of *per1a* and *cry1a* in *Carassius auratus*. Data expressed as the mean + SEM (n = 8/group) in relative units of mRNA (relativizing the experimental groups to the control fish). $^{**}p < 0.01$, $^{***}p < 0.001$ shows significant effect of SR8278 or GSK4112 (two-way ANOVA).

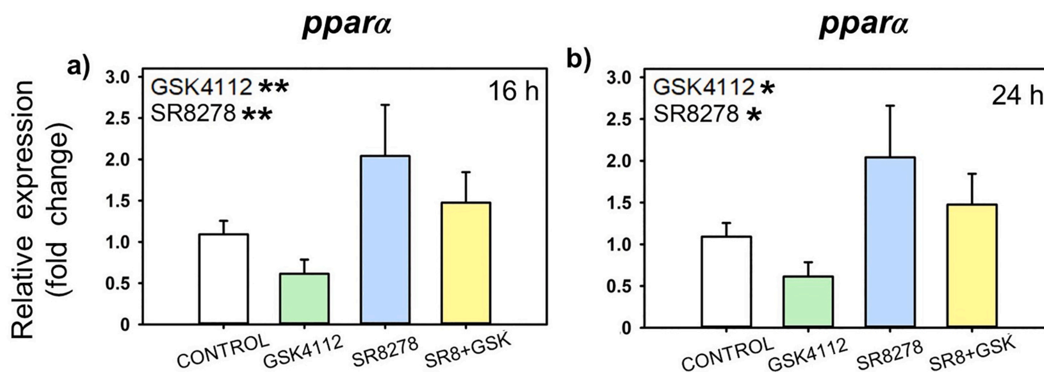

Fig. 9. *In vitro* effect of REV-ERBα agonist GSK4112 (10 μM) and antagonist SR8278 (10 μM) alone or combined (SR8278 + GSK4112) on hepatic expression of *pparα* in *Carassius auratus*. Data expressed as the mean + SEM (n = 8/group) in relative units of mRNA (relativizing the experimental groups to the control fish). $^{*}p < 0.05$, $^{**}p < 0.01$ shows significant effect of SR8278 or GSK4112 (two-way ANOVA).

previous findings in mice treated with the agonists SR9009, SR9011 and SR10067 (Solt et al., 2012) Although the daily rhythm was not lost, the administration of these agonists in mice has caused some alterations in circadian behavior, reducing wheel-running activity during the subjective active phase under free-running conditions, and more subtly in the scotophase under a light-dark cycle (Solt et al., 2012). In the present study, locomotor activity in the second half of the active phase also seemed lower in SR9009-treated fish. The role of REV-ERBα modulating the temporal organization of locomotor activity is also supported by modifications on daily rhythms of locomotor activity and sleep-wake cycles in *rev-erbα* knockout mice (Cho et al., 2012; Delezie et al., 2016; Mang et al., 2016; Sen et al., 2018). Although our results in goldfish seem to support the involvement of REV-ERBα in locomotor activity, the use of *rev-erb* knockout models in fish would be useful to assess this hypothesis.

Another aspect of circadian locomotor activity that appears to be influenced by REV-ERBα in mammals is the FAA, since this nuclear receptor affects the daily rhythm of food intake in mice (Delezie et al., 2012, 2016; Sen et al., 2018). In the present work, the FAA is noticeable in both groups of goldfish, with an apparently lower amplitude and later start in the SR9009-treated group. A more pronounced effect of SR9009 over locomotor activity and FAA may arise in free-running conditions, since deletion of REV-ERBα in mice overrides the circadian rhythm of food intake and locomotor activity under continuous darkness (Sen et al., 2018), whereas this does not happen under a daily photocycle (Delezie et al., 2012; Sen et al., 2018). The attenuated FAA observed in goldfish injected with SR9009 is also coherent with the anorexigenic effect of this agonist reported in this teleost (Saiz et al., 2022) and with the negative influence on feeding motivation that REV-ERBα produces in mice (Amador et al., 2016b; Feillet et al., 2017).

The efficacy and magnitude of REV-ERB agonists seems to depend on the time of day, as expected for drugs targeting a receptor with circadian oscillations. This could also explain the moderate effects we found on locomotor activity. In the present study, SR9009 was administered at ZT 5 in mid-photophase, while the acrophase of the daily rhythm of *rev-erbα* is at ZT 18 (present results; Gómez-Boronat et al., 2018). Yet, the most

pronounced effects of SR9009 on locomotor activity or sleep/wake behavior in rodents were observed after administration at ZT 6 (Amador et al., 2016a; Banerjee et al., 2014), concurring with the maximum amount of *rev-erbα* transcripts in nocturnal rodents (Guillaumond et al., 2005; Panda et al., 2002; Preitner et al., 2002; Yang et al., 2006).

4.3. Downregulatory effects of REV-ERB on clock gene expression in liver

The expression of *bmal1a* was reduced by REV-ERB agonists, both *in vivo* and *in vitro*, while the antagonist had the opposite effect. These results agree with those obtained in mice, with reductions of *bmal1* mRNA in osteoblast, microglial, and hepatic cells treated with GSK4112 (Grant et al., 2010; Guo et al., 2019; Hirai et al., 2015), an effect that can be overridden by the antagonist SR8278 (Hirai et al., 2015). REV-ERBα enhancement by SR9009 injections reduces *bmal1* transcripts in mice (Amador et al., 2016b), and the same is the case in cells overexpressing *rev-erbα* (Guillaumond et al., 2005). In the opposite way, the REV-ERBα antagonist SR8278 induces *bmal1* in microglial cells (Lee et al., 2020), and deficiency of REV-ERBα results in a constant upregulation of *bmal1* in mice fibroblasts (Liu et al., 2008). Combined deficiency of both REV-ERBs completely abolishes *bmal1* oscillations in mammals (Amador et al., 2016b; Bugge et al., 2012; Cho et al., 2012; Ikeda et al., 2019; Liu et al., 2008; Preitner et al., 2002), while addition of the agonist GSK4112 can phase-advance *bmal1* rhythms (Meng et al., 2008). The finding that *bmal1a* is repressed by REV-ERBα activation in fish suggests a regulatory mechanism analogous to that of mammals, in which the molecular basis, REV-ERBα binding to repeated RORE sequences in the *bmal* promoter, is well described (Guillaumond et al., 2005; Preitner et al., 2002; Yin and Lazar, 2005).

On the other hand, *clock1a* (whose protein dimerizes with BMAL1 to induce expression of clock-controlled genes), is also transcriptionally repressed in the liver of SR9009-treated fish, a downregulation that is reversed by the antagonist SR8278. These observations agree with studies in mammals showing the presence of a RORE in the promoter of this gene (Crumbley and Burris, 2011; Ueda et al., 2005), and impaired *clock* rhythms in both *rev-erbα*-deficient and SR9011-treated mice (Cho et al., 2012; Preitner et al., 2002; Solt et al., 2012). The fact that the transcriptional repression by REV-ERBα was evidenced in both *bmal* and *clock1a* demonstrates a dual role of REV-ERBα in regulating the activity of the positive limb of the core clock in fish.

Current results from *in vivo* and *in vitro* experiments also point to a generalized repressing action of REV-ERBα on the expression of the main members of the negative limb of the core clock (*per1* and *cry1*), as well as those from the positive limb, in the liver of goldfish. Similarly, inhibition of *cry1* expression by REV-ERBα in mammals has been suggested, since mammalian *cry1* contains two putative ROREs (Cho et al., 2012; Takeda et al., 2012; Ueda et al., 2005), and it is repressed by REV-ERBs independently of *bmal* (Wang et al., 2014). Regarding the *per* family, the *per1a* repression induced by REV-ERBα agonists in goldfish liver is in line with the action of agonist GSK4112 in embryonic stem cells P19, decreasing the expression of *per2* and *per3* (Mashhour et al., 2018). However, we have not found any other study, either in fish or in mammals, reporting a similar downregulatory effect on *per1*, and SR8278 does not modify *per1* expression in mouse microglia (Lee et al., 2020). However, REV-ERBα shares cistromic sites with BMAL in the promoters of the *per1* and *cry1* clock genes, which supports a possible regulation of these genes (Cho et al., 2012; Lee et al., 2016). It cannot be discarded that REV-ERBα exerts repression of genes from the negative limb indirectly, by decreasing the expression of *bmal* and *clock*, which are the main activators of *per* and *cry* genes.

PPARα is a nuclear receptor/transcription factor that directly regulates core clock components (Chen and Yang, 2014), and it is a known activator of *rev-erbα* and *bmal*, binding to PPRE (PPAR response element) sites in their promoters (Canaple et al., 2006; Gervois et al., 1999). The regulation between PPARα and REV-ERB appears to be reciprocal, as double *rev-erbα/β* knockouts have an impaired hepatic *pparα* rhythm (Cho et al., 2012). Present results show lower *pparα* mRNA in the liver of fish treated with agonists, both *in vivo* and *in vitro*. However, the mechanisms behind this response have yet to be elucidated, since RORE sequences have been identified in the promoter of mammalian *pparγ* (another member of the PPAR family), but not in pparα (Sundvold and Lien, 2001). Another factor to consider is that mir-122 hepatic expression is regulated by REV-ERBα, and this microRNA in turn inhibits the expression and activity of PPARs (Gatfield et al., 2009). Finally, as in the case of *per* and *cry*, the expression of *ppar* is controlled by BMAL:CLOCK, and the downregulation of this gene could be a consequence of *bmal* and *clock* repression by REV-ERBα. SR9009 and GSK4112-induced downregulation of *pparα* in goldfish liver expands the repressing capacity of REV-ERBα to additional genes that, belonging to auxiliary loops, take part in the induction of *bmal1* by activating its promoter.

The fact that all effects of the REV-ERBα agonists SR9009 and GSK4112 on clock gene expression were downregulatory is likely due to the absence of the C-terminal helix in the ligand-binding domain of REV-ERBα, which prevents it from activating gene transcription, unlike other nuclear receptors (Everett and Lazar, 2014; Kojetin and Burris, 2014). Along those lines, the REV-ERBα antagonist SR8278 blocks the effect of the agonist GSK4112, confirming the specificity of inhibition, as previously demonstrated in mammals (Kojetin et al., 2011). Also, the antagonist had effects on its own, indicating that there is some baseline activity of REV-ERBα in the absence of synthetic agonists, which is probably due to the constitutive presence of heme in the cells, as has been recently suggested (Murray et al., 2022). Nevertheless, the magnitude of the effect of agonists and antagonists was dependent on the time and dose, being more pronounced for a given dose or time depending on the gene analyzed, as expected since the expression of all target genes varies in a circadian fashion. In general, the effects of the agonists and antagonists on goldfish liver explants confirm the effects of SR9009 observed *in vivo*. These resembling results indicate that the transcriptional regulation of clock genes is mediated by the drugs directly at the hepatic level and not through any other intermediate process that might occur in the whole animal, such as neural or humoral communication with circadian oscillators located in different organs.

The present work contributes to the understanding of the role of REV-ERBα in the circadian system of teleosts. A shift on feeding time produces an uncoupling between central (hypothalamus) and peripheral (liver) oscillators, that could affect the temporal homeostasis and welfare of animals, as previously suggested (Buijs et al., 2016; Saiz et al., 2021). Here, activation and inhibition of REV-ERB activity has shown effects on an array of key genes of the teleost hepatic clock, both *in vivo* and *in vitro*, suggesting that REV-ERBα is an important element in maintaining the temporal homeostasis of this organ. Furthermore, some reports even suggest that the ROR/BMAL/REV-ERB loop may be an independent oscillator rather than simply having a stabilizing role, being one of the main forces generating circadian oscillations (Relógio et al., 2011). In this regard, the present results suggest a high functional conservation of this regulatory system in mammals and fish.

Funding

This research was funded by Spanish Ministerio de Ciencia e Innovación, PID2019-103969RBC32 to N.P. and M.J.D. N.S. and L.H.C. are predoctoral fellows from Universidad Complutense de Madrid (CT42/18-CT43-18; CT58/21).

Declaration of competing interest

The authors declare not conflicts of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2023.111458.

References

Amador, A., Huitron-Resendiz, S., Roberts, A.J., Kamenecka, T.M., Solt, L.A., Burris, T.P., 2016a. Pharmacological targeting the REV-ERBs in sleep/wake regulation. PLoS One 11 (9), e0162452. https://doi.org/10.1371/journal.pone.0162452.

Amador, A., Wang, Y., Banerjee, S., Kameneka, T.M., Solt, L.A., Burris, T.P., 2016b. Pharmacological and genetic modulation of REV-ERB activity and expression affects orexigenic gene expression. PLoS One 11 (3), e0151014. https://doi.org/10.1371/journal.pone.0151014.

Amaral, I.P.G.G., Johnston, I.A., 2012. Circadian expression of clock and putative clock-controlled genes in skeletal muscle of the zebrafish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 302, 193–206. https://doi.org/10.1152/ajpregu.00367.2011.

Banerjee, S., Wang, Y., Solt, L.A., Griffett, K., Kazantzis, M., Amador, A., El-Gendy, B.M., Huitron-Resendiz, S., Roberts, A.J., Shin, Y., Kamenecka, T.M., Burris, T.P., 2014. Pharmacological targeting of the mammalian clock regulates sleep architecture and emotional behaviour. Nat. Commun. 5, 5759. https://doi.org/10.1038/ncomms6759.

Betancor, M.B., McStay, E., Minghetti, M., Migaud, H., Tocher, D.R., Davie, A., 2014. Daily rhythms in expression of genes of hepatic lipid metabolism in Atlantic salmon (*Salmo salar* L.). PLoS One 9 (9), e10673. https://doi.org/10.1371/journal.pone.0106739.

Bolton, C.M., Bekaert, M., Eilertsen, M., Helvik, J.V., Migaud, H., 2021. Rhythmic clock gene expression in Atlantic salmon parr brain. Front. Physiol. 12, 2173. https://doi.org/10.3389/FPHYS.2021.761109/BIBTEX.

Bookout, A.L., Jeong, Y., Downes, M., Yu, R.T., Evans, R.M., Mangelsdorf, D.J., 2006. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell 126, 789–799. https://doi.org/10.1016/J.CELL.2006.06.049.

Bugge, A., Feng, D., Everett, L.J., Briggs, E.R., Mullican, S.E., Wang, F., Jager, J., Lazar, M.A., 2012. Rev-erbα and Rev-erbβ coordinately protect the circadian clock and normal metabolic function. Genes Dev. 26, 657–667. https://doi.org/10.1101/gad.186858.112.

Buijs, F.N., León-Mercado, L., Guzmán-Ruiz, M., Guerrero-Vargas, N.N., Romo-Nava, F., Buijs, R.M., 2016. The circadian system: a regulatory feedback network of periphery and brain. Physiology 31, 170–181. https://doi.org/10.1152/physiol.00037.2015.

Canaple, L., Rambaud, J., Dkhissi-Benyahya, O., Atrice Rayet, B., Tan, N.S., Michalik, L., Delaunay, F., Wahli, W., Laudet, V., 2006. Reciprocal regulation of brain and muscle arnt-like protein 1 and peroxisome proliferator-activated receptor defines a novel positive feedback loop in the rodent liver circadian clock. Mol. Endocrinol. 20 (8), 1715–1727. https://doi.org/10.1210/me.2006-0052.

Chen, L., Yang, G., 2014. PPARs integrate the mammalian clock and energy metabolism. PPAR Res. 2014, 653017 https://doi.org/10.1155/2014/653017.

Cho, H., Zhao, X., Hatori, M., Yu, R.T., Barish, G.D., Lam, M.T., Chong, L.W., Ditacchio, L., Atkins, A.R., Glass, C.K., Liddle, C., Auwerx, J., Downes, M., Panda, S., Evans, R.M., 2012. Regulation of circadian behaviour and metabolism by REV-ERB-α and REV-ERB-β. Nature 485, 123–127. https://doi.org/10.1038/nature11048.

Crumbley, C., Burris, T.P., 2011. Direct regulation of CLOCK expression by REV-ERB. PLoS One 6, e17290. https://doi.org/10.1371/journal.pone.0017290.

Davidson, A.J., 2006. Search for the feeding-entrainable circadian oscillator: a complex proposition. Am. J. Physiol. Regul. Integr. Comp. Physiol. 290 (6), R1524–R1526. https://doi.org/10.1152/ajpregu.00073.2006.

Delaunay, F., Thisse, C., Marchand, O., Laudet, V., Thisse, B., 2000. An inherited functional circadian clock in zebrafish embryos. Science 289 (5477), 297–300. https://doi.org/10.1126/SCIENCE.289.5477.297.

Delezie, J., Dumont, S., Dardente, H., Oudart, H., Gréchez-Cassiau, A., Klosen, P., Teboul, M., Delaunay, F., Pévet, P., Challet, E., 2012. The nuclear receptor REV-ERBα is required for the daily balance of carbohydrate and lipid metabolism. FASEB J. 26, 3321–3335. https://doi.org/10.1096/fj.12-208751.

Delezie, J., Dumont, S., Sandu, C., Reibel, S., Pevet, P., Challet, E., 2016. Rev-erbα in the brain is essential for circadian food entrainment. Sci. Rep. 6, 1–15. https://doi.org/10.1038/srep29386.

Duggleby, R.G., 1981. A nonlinear regression program for small computers. Anal. Biochem. 110 (1), 9–18. https://doi.org/10.1016/0003-2697(81)90104-4.

Everett, L.J., Lazar, M.A., 2014. Nuclear receptor Rev-erbα: up, down, and all around. Trends Endocrinol. Metab. 25, 586–592. https://doi.org/10.1016/j.tem.2014.06.011.

Feillet, C.A., Bainier, C., Mateo, M., Blancas-Velázquez, A., Salaberry, N.L., Ripperger, J.A., Albrecht, U., Mendoza, J., 2017. Rev-erbα modulates the hypothalamic orexinergic system to influence pleasurable feeding behaviour in mice. Addic. Biol. 22, 411–422. https://doi.org/10.1111/ADB.12339.

Gatfield, D., le Martelot, G., Vejnar, C.E., Gerlach, D., Schaad, O., Fleury-Olela, F., Ruskeepää, A.L., Oresic, M., Esau, C.C., Zdobnov, E.M., Schibler, U., 2009. Integration of microRNA miR-122 in hepatic circadian gene expression. Genes Dev. 23, 1313–1326. https://doi.org/10.1101/gad.1781009.

Gervois, P., Chopin-Delannoy, S., Fadel, A., Dubois, G., Kosykh, V., Fruchart, J.C., Najib, J., Laudet, V., Staels, B., 1999. Fibrates increase human REV-ERBα expression in liver via a novel peroxisome proliferator-activated receptor response element. Mol. Endocrinol. 13, 400–409. https://doi.org/10.1210/mend.13.3.0248.

Gómez-Boronat, M., Sáiz, N., Delgado, M.J., de Pedro, N., Isorna, E., 2018. Time-lag in feeding schedule acts as a stressor that alters circadian oscillators in goldfish. Front. Physiol. 9, 1749. https://doi.org/10.3389/fphys.2018.01749.

Gómez-Boronat, M., De Pedro, N., Alonso-Gómez, Á.L., Delgado, M.J., Isorna, E., Fernando Lopez-Olmeda, J., Oliveira, C., Bertucci, J.I., 2022. Nuclear receptors (PPARs, REV-ERBs, RORs) and clock gene rhythms in goldfish (*Carassius auratus*) are differently regulated in hypothalamus and liver. Front. Physiol. 13, 1–11. https://doi.org/10.3389/fphys.2022.903799.

Grant, D., Yin, L., Collins, J.L., Parks, D.J., Orband-Miller, L.A., Wisely, G.B., Joshi, S., Lazar, M.A., Willson, T.M., Zuercher, W.J., 2010. GSK4112, a small molecule chemical probe for the cell biology of the nuclear heme receptor rev-erbα. ACS Chem. Biol. 5, 925–932. https://doi.org/10.1021/cb100141y.

Guerra-Santos, B., López-Olmeda, J.F., de Mattos, B.O., Baião, A.B., Pereira, D.S.P., Sánchez-Vázquez, F.J., Cerqueira, R.B., Albinati, R.C.B., Fortes-Silva, R., 2017. Synchronization to light and mealtime of daily rhythms of locomotor activity, plasma glucose and digestive enzymes in the Nile tilapia (*Oreochromis niloticus*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 204, 40–47. https://doi.org/10.1016/J.CBPA.2016.11.006.

Guillaumond, F., Dardente, H., Giguère, V., Cermakian, N., 2005. Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. J. Biol. Rhythm. 20, 391–403. https://doi.org/10.1177/0748730405277232.

Guo, D.K., Zhu, Y., Sun, H.-Y., Xu, X.-Y., Zhang, S., Hao, Z.-B., Wang, G.-H., Mu, C.-C., Ren, H.-G., 2019. Pharmacological activation of REV-ERBα represses LPS-induced microglial activation through the NF-κB pathway. Acta Pharmacol. Sin. 40, 26–34. https://doi.org/10.1038/s41401-018-0064-0.

Hirai, T., Tanaka, K., Togari, A., 2015. α_{1B}-Adrenergic receptor signaling controls circadian expression of Tnfrsf11b by regulating clock genes in osteoblasts. Biol. Open 4, 1400–1409. https://doi.org/10.1242/bio.012617.

Iigo, M., Tabata, M., 1996. Circadian rhythms of locomotor activity in the goldfish *Carassius auratus*. Physiol. Behav. 60, 775–781. https://doi.org/10.1016/0031-9384(96)00131-X.

Ikeda, R., Tsuchiya, Y., Koike, N., Umemura, Y., Inokawa, H., Ono, R., Inoue, M., Sasawaki, Y., Grieten, T., Okubo, N., Ikoma, K., Fujiwara, H., Kubo, T., Yagita, K., 2019. REV-ERBα and REV-ERBβ function as key factors regulating mammalian circadian output. Sci. Rep. 9, 1–9. https://doi.org/10.1038/s41598-019-46656-0.

Isorna, E., de Pedro, N., Valenciano, A.I., Alonso-Gómez, Á.L., Delgado, M.J., 2017. Interplay between the endocrine and circadian systems in fishes. J. Endocrinol. 232, R141–R159. https://doi.org/10.1530/JOE-16-0330.

Kojetin, D.J., Burris, T.P., 2011. A role for Rev-erbα ligands in the regulation of adipogenesis. Curr. Pharm. Des. 17, 320–324. https://doi.org/10.2174/138161211795164211.

Kojetin, D.J., Burris, T.P., 2014. REV-ERB and ROR nuclear receptors as drug targets. Nat. Rev. Drug Discov. 13 (3), 197–216. https://doi.org/10.1038/nrd4100.

Kojetin, D., Wang, Y., Kamenecka, T.M., Burris, T.P., 2011. Identification of SR8278, a synthetic antagonist of the nuclear heme receptor REV-ERB. ACS Chem. Biol. 6, 131–134. https://doi.org/10.1021/cb1002575.

Kornmann, B.B., Schaad, O., Bujard, H., Takahashi, J.S., Schibler, U., 2007. System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. PLoS Biol. 5 (2), e34 https://doi.org/10.1371/journal.pbio.0050034.

Krylov, V.V., Izvekov, E.I., Pavlova, V.V., Pankova, N.A., Osipova, E.A., 2021. Circadian rhythms in zebrafish (*Danio rerio*) behaviour and the sources of their variability. Biol. Rev. 96, 785–797. https://doi.org/10.1111/BRV.12678.

Lee, J., Lee, S., Chung, S., Park, N., Son, G.H., An, H., Jang, J., Chang, D.J., Suh, Y.G., Kim, K., 2016. Identification of a novel circadian clock modulator controlling BMAL1 expression through a ROR/REV-ERB-response element-dependent mechanism. Biochem. Biophys. Res. Commun. 469, 580–586. https://doi.org/10.1016/j.bbrc.2015.12.030.

Lee, J., Kim, D.E., Griffin, P., Sheehan, P.W., Kim, D.H., Musiek, E.S., Yoon, S.Y., 2020. Inhibition of REV-ERBs stimulates microglial amyloid-beta clearance and reduces amyloid plaque deposition in the 5XFAD mouse model of Alzheimer's disease. Aging Cell 19 (2), e13078. https://doi.org/10.1111/acel.13078.

Liu, A.C., Tran, H.G., Zhang, E.E., Priest, A.A., Welsh, D.K., Kay, S.A., 2008. Redundant function of REV-ERBα and β and non-essential role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms. PLoS Genet. 4 (2), e1000023 https://doi.org/10.1371/journal.pgen.1000023.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

López-Olmeda, J.F., Madrid, J.A., Sánchez-Vázquez, F.J., 2006. Light and temperature cycles as zeitgebers of zebrafish (*Danio rerio*) circadian activity rhythms. Chronobiol. Int. 23, 537–550. https://doi.org/10.1080/07420x520600651065.

Mang, G.M., la Spada, F., Emmenegger, Y., Chappuis, S., Ripperger, J.A., Albrecht, U., Franken, P., 2016. Altered sleep homeostasis in REV-ERBα knockout mice. Sleep 39, 589–601. https://doi.org/10.5665/sleep.5534.

Mashhour, A., Al Mansour, Z., al Hallaj, A.S., Ali, R., Trivilegio, T., Boudjelal, M., 2018. P19 cells as a model for studying the circadian clock in stem cells before and after cell differentiation. J. Circadian Rhythms 16, 1–13. https://doi.org/10.5334/jcr.157.

Mendoza, J., 2007. Circadian clocks: setting time by food. J. Neuroendocrinol. 19, 127–137. https://doi.org/10.1111/j.1365-2826.2006.01510.x.

Meng, Q.J., McMaster, A., Beesley, S., Lu, W.Q., Gibbs, J., Parks, D., Collins, J., Farrow, S., Donn, R., Ray, D.W., Loudon, A., 2008. Ligand modulation of REV-ERBα function resets the peripheral circadian clock in a phasic manner. J. Cell Sci. 121, 3629–3635. https://doi.org/10.1242/jcs.035048.

Mistlberger, R.E., 2006. Circadian rhythms: perturbing a food-entrained clock. Curr. Biol. 16 (22), R968–R969. https://doi.org/10.1016/j.cub.2006.10.020.

Mosure, S.A., Strutzenberg, T.S., Shang, J., Munoz-Tello, P., Solt, L.A., Griffin, P.R., Kojetin, D.J., 2021. Structural basis for heme-dependent NCoR binding to the transcriptional repressor REV-ERBβ. Sci. Adv. 7, 6479–6506. https://doi.org/10.1126/SCIADV.ABC6479.

Murray, M.H., Valfort, A.C., Koelblen, T., Ronin, C., Ciesielski, F., Chatterjee, A., Veerakanellore, G.B., Elgendy, B., Walker, J.K., Hegazy, L., Burris, T.P., 2022. Structural basis of synthetic agonist activation of the nuclear receptor REV-ERB. Nat. Commun. 13 (1), 7131. https://doi.org/10.1038/S41467-022-34892-4.

Nisembaum, L.G., Velarde, E., Tinoco, A.B., Azpeleta, C., de Pedro, N., Alonso-Gómez, A. L., Delgado, M.J., Isorna, E., 2012. Light-dark cycle and feeding time differentially entrains the gut molecular clock of the goldfish (*Carassius auratus*). Chronobiol. Int. 29 (6), 665–673. https://doi.org/10.3109/07420528.2012.686947.

Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S., Hogenesch, J.B., 2002. Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109, 307–320. https://doi.org/10.1016/S0092-8674(02)00722-5.

Patke, A., Young, M.W., Axelrod, S., 2020. Molecular mechanisms and physiological importance of circadian rhythms. Nat. Rev. Mol. Cell Biol. 21 (2), 67–84. https://doi.org/10.1038/s41580-019-0179-2.

Pett, J.P., Kondoff, M., Bordyugov, G., Kramer, A., Herzel, H., 2018. Co-existing feedback loops generate tissue-specific circadian rhythms. Life Sci. Alliance 1 (3), e201800078. https://doi.org/10.26508/lsa.201800078.

Preitner, N., Damiola, F., Luis-Lopez-Molina, Zakany, J., Duboule, D., Albrecht, U., Schibler, U., 2002. The orphan nuclear receptor REV-ERBα controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110, 251–260. https://doi.org/10.1016/S0092-8674(02)00825-5.

Raghuram, S., Stayrook, K.R., Huang, P., Rogers, P.M., Nosie, A.K., McClure, D.B., Burris, L.L., Khorasanizadeh, S., Burris, T.P., Rastinejad, F., 2007. Identification of heme as the ligand for the orphan nuclear receptors REV-ERBα and REV-ERBβ. Nat. Struct. Mol. Biol. 14, 1207–1213. https://doi.org/10.1038/nsmb1344.

Refinetti, R., 2015. Comparison of light, food, and temperature as environmental synchronizers of the circadian rhythm of activity in mice. J. Physiol. Sci. 2015 (65), 359–366. https://doi.org/10.1007/S12576-015-0374-7.

Relógio, A., Westermark, P.O., Wallach, T., Schellenberg, K., Kramer, A., Herzel, H., 2011. Tuning the mammalian circadian clock: robust synergy of two loops. PLoS Comput. Biol. 7, e1002309 https://doi.org/10.1371/journal.pcbi.1002309.

Roenneberg, T., Merrow, M., 2003. The network of time: understanding the molecular circadian system. Curr. Biol. 13 (5), R198R–R207. https://doi.org/10.1016/S0960-9822(03)00124-6.

Rogers, P.M., Ying, L., Burris, T.P., 2008. Relationship between circadian oscillations of Rev-erbα expression and intracellular levels of its ligand, heme. Biochem. Biophys. Res. Commun. 368, 955–958. https://doi.org/10.1016/J.BBRC.2008.02.031.

Saiz, N., Gómez-Boronat, M., De Pedro, N., Delgado, M.J., Isorna, E., 2021. The lack of light-dark and feeding-fasting cycles alters temporal events in the goldfish (*Carassius auratus*) stress axis. Animals 11, 1–19. https://doi.org/10.3390/ani11030669.

Saiz, N., Herrera-Castillo, L., Isorna, E., Delgado, M.J., Conde-Sieira, M., Soengas, J.L., de Pedro, N., 2022. REV-ERBα agonist SR9009 promotes a negative energy balance in goldfish. Int. J. Mol. Sci. 23, 2921. https://doi.org/10.3390/ijms23062921.

Sánchez-Bretaño, A., Alonso-Gómez, Á.L., Delgado, M.J., Isorna, E., 2015. The liver of goldfish as a component of the circadian system: integrating a network of signals. Gen. Comp. Endocrinol. 221, 213–216. https://doi.org/10.1016/J.YGCEN.2015.05.001.

Sánchez-Bretaño, A., Callejo, M., Montero, M., Alonso-Gómez, Á.L., Delgado, M.J., Isorna, E., 2016. Performing a hepatic timing signal: glucocorticoids induce *gper1a* and *gper1b* expression and repress *gclock1a* and *gbmal1a* in the liver of goldfish. J. Comp. Physiol. B. 186, 73–82. https://doi.org/10.1007/s00360-015-0936-2.

Sánchez-Vázquez, F.J., Aranda, A., Madrid, J.A., 2001. Differential effects of meal size and food energy density on feeding entrainment in goldfish. J. Biol. Rhythm. 16, 58–65. https://doi.org/10.1177/074873040101600107.

Sen, S., Dumont, S., Sage-Ciocca, D., Reibel, S., de Goede, P., Kalsbeek, A., Challet, E., 2018. Expression of the clock gene Rev-erbα in the brain controls the circadian organisation of food intake and locomotor activity, but not daily variations of energy metabolism. J. Neuroendocrinol. 30, e12557 https://doi.org/10.1111/jne.12557.

Solt, Laura A., Wang, Y., Banerjee, S., Hughes, T., Douglas, J., Lundasen, T., Shin, Y., Liu, J., Cameron, M.D., Yoo, S., Takahashi, J.S., Butler, A.A., Theodore, M., Burris, T. P., 2012. Regulation of circadian behavior and metabolism by sinthetic REV-ERB agonists, 485, 62–68. https://doi.org/10.1038/nature11030.

Stratmann, M., Schibler, U., 2012. REV-ERBs: more than the sum of the individual parts. Cell Metab. 15 (6), 791–793. https://doi.org/10.1016/j.cmet.2012.05.006.

Sundvold, H., Lien, S., 2001. Identification of a novel peroxisome proliferator-activated receptor (PPAR) gamma promoter in man and transactivation by the nuclear receptor RORalpha1. Biochem. Biophys. Res. Commun. 287, 383–390. https://doi.org/10.1006/BBRC.2001.5602.

Tahara, Y., Otsuka, M., Fuse, Y., Hirao, A., Shibata, S., 2011. Refeeding after fasting elicits insulin-dependent regulation of Per2 and Rev-erbα with shifts in the liver clock. J. Biol. Rhythm. 26, 230–240. https://doi.org/10.1177/0748730411405958.

Takahashi, J.S., 2015. Molecular components of the circadian clock in mammals. Diabetes Obes. Metab. 17 (1), 6–11. https://doi.org/10.1111/dom.12514.

Takeda, Y., Jothi, R., Birault, V., Jetten, A.M., 2012. RORγ directly regulates the circadian expression of clock genes and downstream targets in vivo. Nucleic Acids Res. 40, 8519–8535. https://doi.org/10.1093/nar/gks630.

Ueda, H.R., Hayashi, S., Chen, W., Sano, M., Machida, M., Shigeyoshi, Y., Iino, M., Hashimoto, S., 2005. System-level identification of transcriptional circuits underlying mammalian circadian clocks. Nat. Genet. 37, 187–192. https://doi.org/10.1038/ng1504.

Vatine, G., Vallone, D., Gothilf, Y., Foulkes, N.S., 2011. It's time to swim! Zebrafish and the circadian clock. FEBS Lett. 585, 1485–1494. https://doi.org/10.1016/j.febslet.2011.04.007.

Wang, J., Liu, C., Ma, F., Chen, W., Liu, J., Hu, B., Zheng, L., 2014. Circadian clock mediates light/dark preference in zebrafish (*Danio rerio*). Zebrafish 11, 115–121. https://doi.org/10.1089/zeb.2013.0929.

Wang, S., Li, F., Lin, Y., Wu, B., 2020. Targeting REV-ERBα for therapeutic purposes: promises and challenges. Theranostics 10, 4168–4182. https://doi.org/10.7150/thno.43834.

Yang, X., Downes, M., Yu, R.T., Bookout, A.L., He, W., Straume, M., Mangelsdorf, D.J., Evans, R.M., 2006. Nuclear receptor expression links the circadian clock to metabolism. Cell 126, 801–810. https://doi.org/10.1016/J.CELL.2006.06.050.

Yin, L., Lazar, M.A., 2005. The orphan nuclear receptor Rev-erbα recruits the N-CoR/histone deacetylase 3 corepressor to regulate the circadian Bmal1 gene. Mol. Endocrinol. 19, 1452–1459. https://doi.org/10.1210/me.2005-0057.

Yin, L., Wu, N., Curtin, J.C., Qatanani, M., Szwergold, N.R., Reid, R.A., Waitt, G.M., Parks, D.J., Pearce, K.H., Wisely, G.B., Lazar, M.A., 2007. Rev-erbα, a heme sensor that coordinates metabolic and circadian pathways. Science 318, 1786–1789. https://doi.org/10.1126/SCIENCE.1150179.