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Crosstalking between the "gut-brain" hormone ghrelin and the circadian system in the goldfish. Effects on clock gene expression and food anticipatory activity

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ABSTRACT

Ghrelin is a potent orexigenic signal mainly synthesized in the stomach and foregut of vertebrates. Recent studies in rodents point out that ghrelin could also act as an input for the circadian system and/or as an output of peripheral food-entrainable oscillators, being involved in the food anticipatory activity (FAA). In this study we pursue the possible interaction of ghrelin with the circadian system in a teleost, the goldfish (*Carassius auratus*). First, we analyzed if ghrelin is able to modulate the core clock functioning by regulating clock gene expression in fish under a light/dark cycle 12L:12D and fed at 10 am. As expected the acute intraperitoneal (IP) injection of goldfish ghrelin ($gGRL_{[1-19]}$, 44 pmol/g bw) induced the expression of hypothalamic orexin. Moreover, ghrelin also induced (~2-fold) some *Per* clock genes in hypothalamus and liver. This effect was partially counteracted in liver by the ghrelin antagonist ([D-Lys^3]-GHRP-6, 100 pmol/g bw). Second, we investigated if ghrelin is involved in daily FAA rhythms. With this aim locomotor activity was studied in response to IP injections (5–10 days) of $gGRL_{[1-19]}$ and [D-Lys^3]-GHRP-6 at the doses above indicated. Ghrelin and saline injected fish showed similar 24 h activity patterns. However, ghrelin antagonist treatment abolished the FAA in schedule fed fish under 24 h light, suggesting the involvement of the endogenous ghrelin system in this pre-feeding activity. Altogether these results suggest that ghrelin could be acting as an input for the entrainment of the food-entrainable oscillators in the circadian organization of goldfish.

1. Introduction

Ghrelin (GRL) is a peptide identified for the first time by Kojima and coworkers (1999) in the stomach of rats. At present it is known that the gastrointestinal tract is the main system for the synthesis of this hormone in vertebrates (Kaiya et al., 2008), although it has been also detected in other peripheral and central locations (Cowley et al., 2003). In fish, the highest levels of GRL mRNA occur in the stomach or the foregut, depending on the species (Breves et al., 2009; Kaiya et al., 2003; Parhar et al., 2003; Unniappan et al., 2002). Lower mRNA expression is detected in other peripheral tissues and also in the brain (Amole and Unniappan, 2009; Piccinetti et al., 2010; Unniappan et al., 2002). Specifically in the fish hypothalamus, the main integration center for food intake regulation (Kang et al., 2011; Unniappan et al., 2004), ghrelin mRNA has been detected in all studied species, including goldfish (*Carassius auratus*; Jönsson, 2013; Unniappan et al., 2002).

The first reported effect of GRL was the stimulation of GH (growth hormone) release from the pituitary gland, as a growth hormone secretagogue (Kojima et al., 1999). Later, subsequent studies identified other functions of GRL, such as orexigenic action (Nakazato et al., 2001). Moreover, GRL levels are modified in response to the nutritional state, with increases in GRL mRNA expression in mice under fasting conditions (Toshinai et al., 2001). In addition, a preprandial rise (Cummings et al., 2001) followed by a postprandial decrease in circulating GRL (Tschöp et al., 2001) was also observed in mammals, indicating that this hormone is a signal of meal initiation. In some fish species, as goldfish and zebrafish (*Danio rerio*), GRL mRNA expression in brain and gut is also modified as GRL serum levels by the nutritional state, increasing under fasting conditions and decreasing after feeding (Amole and Unniappan, 2009; Unniappan et al., 2004). In accordance with GRL modifications by feeding status, intracerebroventricular (ICV) and/or intraperitoneal (IP) administration of this

Abbreviations: GRL, ghrelin; 24L, constant light; FAA, food anticipatory activity; FEO, food entrainable oscillator; LD cycle, light-dark cycle.

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hormone usually increases food intake in the studied fish species (Jönsson, 2013). Moreover, interactions between GRL and some central feeding regulators have been shown. Thus, GRL activates other orexigenic systems, such as the neuropeptide Y (NPY) and orexin in fish (Miura et al., 2006, 2007), suggesting that these peptides could mediate the GRL-induced food intake stimulation.

Besides feeding regulation, GRL modifies locomotor activity in a few vertebrate species. Some studies in rodents (Keen-Rhinehart and Bartness, 2005) and goldfish (Kang et al., 2011; Matsuda et al., 2006; Yahashi et al., 2012) show an increment of locomotor activity induced by GRL administration, although a decrease of locomotor activity was also described in rodents (Tang-Christensen et al., 2004), goldfish (Kang et al., 2011; Yahashi et al., 2012) and chicken (*Gallus gallus*) (Carvajal et al., 2009).

Because of the above mentioned dual role of GRL, as a regulator of food intake and locomotor activity, it is proposed that this peptide could be involved in the generation of the food anticipatory activity (FAA), *i.e.* the increase in locomotor activity observed just 3–4 h before food supply in scheduled fed animals, including goldfish (Sánchez-Vázquez et al., 1997). The FAA is related to food reward (Challet and Mendoza, 2010), and GRL has been also involved in the brain reward circuitry (Perello et al., 2010). FAA is controlled by the named food entrainable oscillators (FEOs), a network of oscillators located at both, central and peripheral level (Albrecht, 2012; Escobar et al., 2009). In this sense, some studies in mammals suggested the involvement of GRL in the circadian system. Thus, GRL produces phase advances in the electric activity and PER2 expression in the suprachiasmatic nucleus explants of mice (Yannielli et al., 2007), and it could be acting as an output of a FEO located in the oxyntic cells of the stomach in mice (LeSauter et al., 2009). In addition, IP injections of the growth hormone releasing protein-6, a synthetic analog of GRL, also induced a phase advance in daily locomotor activity of animals food deprived (Yannielli et al., 2007). The recently demonstrated activation of hypothalamic neurons by GRL during the FAA in mammals also supports this putative role of GRL as a synchronizer of FEOs (Van der Plasse et al., 2013).

To date, nothing is known about possible crosstalking between GRL and the circadian system in fish. Daily rhythms in clock genes expression have been reported in various central and peripheral locations of goldfish, including the liver and the hypothalamus (Feliciano et al., 2011; Nisembaum et al., 2012; Velarde et al., 2009), that also express GRL receptors (Kaiya et al., 2010). Thus, the aim of the present study was to identify the possible role of GRL as an input to the circadian system in goldfish and its relationship with the FAA. First, the acute effects of a synthetic GRL ($gGRL_{[1-19]}$) and a GRL receptor antagonist ([D-Lys^3]-GHRP-6) on clock genes (*Per1a*, *Per2a*, *Per3*, *Cry3* and *Bmal1a*) and hypothalamic feeding regulators (NPY and orexin) expression were investigated. Second, sub-chronic effects of these GRL agonist and antagonist on daily locomotor activity rhythms were studied.

2. Material and methods

2.1. Acclimation conditions

Goldfish (9 ± 0.2 g of body weight (bw) were purchased from a local supplier (ICA, Spain), and maintained in 60-l tanks (n = 6–9 fish/tank) with constant flow of filtered water in a temperature-controlled room (22 ± 1 °C). Before starting the experiments, fish were acclimated under a 12 h light and 12 h dark photoperiod (12L:12D), with lights onset at 08:00 h, and a scheduled feeding regime at 10:00 h (SF10) with a food ratio of 1% bw for at least 2 weeks. The tanks walls were covered with opaque paper to minimize external interferences during the experiments. All experimental protocols were approved by the Animal Experimentation Committee of Complutense University of Madrid, and comply with current laws in Spain (RD53/2013) and the European Directive 2010/63/EU.

2.2. Drugs and intraperitoneal injections

For the IP injections fish were anesthetized in water containing tricaine methanesulphonate (MS-222, 0.1 g/l, Sigma Chemical, Madrid, Spain). Immediately after loss of equilibrium, fish were weighed and injected using 1 ml syringes and 0.3 mm Microlance needles (Lab-Center, Spain), close to the ventral midline posterior to the pelvic fins. All animals were injected with 10 μl/g bw of teleost saline (20 mg Na_2CO_3 per 100 ml of 0.6% NaCl) alone or containing the drugs. Ghrelin ($gGRL_{[1-19]}$, Ser-(decanoyl^3)-Ghrelin-19-goldfish-acetate, Bachem, Switzerland; 44 pmol/g bw) and the ghrelin antagonist [D-Lys^3]-GHRP-6, (BACHEM, Switzerland; 100 pmol/g bw) were IP injected. These doses were chosen based on previous reports in goldfish (Kaiya et al., 2010; Miura et al., 2007; Unniappan et al., 2004) and summer flounder (*Paralichthys dentatus*; Breves et al., 2009). Immediately after the injections, fish were transferred to anesthetic-free water where swimming activity and equilibrium were recovered within 1–2 min.

2.3. Effect of GRL on clock genes, orexin-A, and NPY expression

Four groups of fish (under 12L:12D and SF10) received two sequential IP injections separated by 10 min: (1) control group that received two injections of teleost saline; (2) GRL group, injected with teleost saline followed by $gGRL_{[1-19]}$; (3) GRL antagonist group, injected with [D-Lys^3]-GHRP-6 followed by teleost saline; and (4) antagonist plus GRL group, injected with [D-Lys^3]-GHRP-6 followed by $gGRL_{[1-19]}$. Fish were injected at midday (14:00 h) and at 1 and 3 h post-injection fish (n = 7/group/sampling time) were anesthetized, sacrificed, and the hypothalamus, pituitary and liver were quickly sampled and stored at −80 °C until used. Clock gene expression was quantified in the three collected tissues, and orexin and NPY expression was analyzed in the hypothalamus.

2.4. Effect of GRL and GRL antagonist on locomotor activity rhythms

Firstly, goldfish (n = 7–9 fish/tank, 6 tanks) acclimated for 2 weeks as above described were food deprived for 5 days, and under these conditions, they were IP injected with teleost saline (control fish, 3 tanks), or with $gGRL_{[1-19]}$ (GRL treated fish, 3 tanks). Locomotor activity was continuously recorded before, during, and after injections. At the end of injection period, animals were still food deprived for 4 days in order to test if the putative rhythmic locomotor activity was maintained.

Secondly, four tanks of fish (n = 6–9 fish/tank) were acclimated for 17 days under constant light (24L) and randomly fed twice in 24 h (each time with 1% bw). Under such conditions, the daily locomotor activity rhythm was lost. Then, a scheduled feeding protocol at 16:00 h was imposed to induce a FAA. Under these conditions, goldfish were IP injected at 13:00 h during 10 days with teleost saline (control fish, 2 tanks) or with a GRL antagonist ([D-Lys^3]-GHRP-6 group, 2 tanks). Locomotor activity was continuously recorded before, during, and after treatment to analyze the effect of the GRL antagonist on the FAA. At the end of the injection period, animals were food deprived during 4 additional days to observe the putative maintenance of the possible FAA.

2.5. Gene expression analysis by quantitative Real Time PCR (qRT-PCR)

Total RNA from goldfish tissues was extracted with Trizol (TRI® Reagent method, Sigma Chemical, Madrid, Spain) and treated with

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DNase (Promega, Madison, USA) at 37 °C for 40 min to eliminate genomic DNA. Then, 0.5 μg of RNA was retro-transcribed using the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, USA). The mRNA expression of clock genes (*gPer1a*, *gPer2a*, *gPer3*, *gCry3* and *gBmal1a*) and the two orexigenic hormones, orexin-A and NPY, was quantified by qRT-PCR in a CFX96TM Real-Time System (Biorad Laboratories, Hercules, USA). The PCR reactions were performed in a 20 μl final volume using 10 μl iTaqTM SYBR® Green Supermix (Biorad Laboratories, Hercules, USA), specific primers (0.4 μM; Sigma Chemical, Madrid, Spain; see Table 1) and 1 μl cDNA. The protocol for measuring mRNA expressions was: 1 cycle at 95 °C for 3 min and 40 cycles consisting in 95 °C for 10 s, 58 °C for 30 s and 72 °C for 45 s, for the three *Per* genes and *gCry3*, varying the annealing temperature for *gBmal1a* (55 °C), *gOx* (65 °C), *gNpy* (64 °C) and for *β-actin* expression (60 °C), the last one used as reference gene. All samples were analyzed in duplicated and the relative mRNA expression was determined by the ΔΔ*Ct* method (Livak and Schmittgen, 2001), represented by fold change. The efficiency of the amplification for all genes studied was around 100%. The specificity of the amplification reactions was confirmed

Table 1
Sequence of primers used for quantitative Real Time-PCR.

Target gene	Accession number		Primer sequences 5′ → 3′	Product (pb)
gPer1a	EF690698	Forward	AGCGCCACTTCCTCCTCTGA	130
		Reverse	TGAAGACCTGCTGTCCGTTGG	
gPer2a	EF690697	Forward	TTTGTCAATCCCTGGAGCCGC	116
		Reverse	AAGGATTTGCCCTCAGCCACG	
gPer3	EF690699	Forward	GGCTATGGCAGTCTGGCTAGTAA	130
		Reverse	CAGCACAAAACCGCTGCAATGTC	
gCry3	EF690702	Forward	GGTGAGACAGAAGCCCTGGAA	102
		Reverse	GCTTGCGAACAGTGATTGAGCG	
gBmal1a	KF840401	Forward	ATCGATGAGTCGTTCCCGTG	161
		Reverse	AGATTCTGTTCGTCTCGGAG	
gNpy	M87297	Forward	TTCGTCTGCTTGGGAACTCT	151
		Reverse	TGGACCTTTTGCCATACCTC	
gOX	DQ923590	Forward	ACTGCACAGCCAAGAGAGTTCA	188
		Reverse	GTTATTAAAGCGGCCGATATGC	
gβ-actin	AB039726	Forward	GGCCTCCCTGTCTATCTTCC	156
		Reverse	TTGAGAGGTTTGGGTTGGTC	

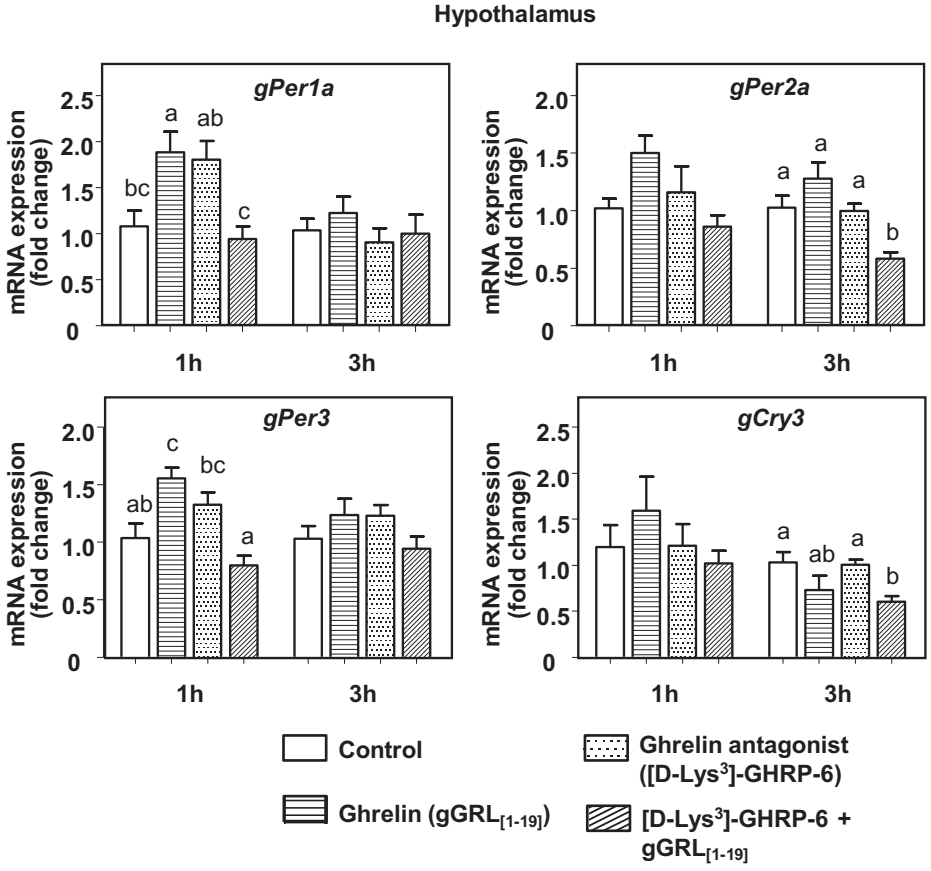

Fig. 1. Expression of clock genes (*gPer1a*, *gPer2a*, *gPer3*, *gCry3*) in hypothalamus of goldfish maintained under 12L:12D photoperiod, scheduled feeding at 10:00 h and injected at 14:00 h. Data are shown in relative units (ΔΔ*Ct* method) as the mean ± standard error ($n = 6/7$), 1 and 3 h post-injection. Different letters indicate differences among the groups (ANOVA and SNK, $p < 0.05$).

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by the melting temperature in each sample and by the size of the obtained PCR products in an agarose gel.

2.6. Recording of locomotor activity

Locomotor activity was recorded by using infrared photocells (OMRON E3S-AD12, Japan) fixed on different parts of the aquaria wall (one under the food dispenser in the upper area, another in the center of the aquaria wall, and the last one at the bottom). The photocells continuously emitted an infrared light beam, which was interrupted each time fish swam in that zone, generating an output signal. The number of light beam interruptions was automatically counted and stored every 10 min by specific software (Micronec, Spain). The locomotor activity recorded was analyzed and represented as actograms and periodograms obtained with the chronobiology software "*El temps* ®".

2.7. Statistics

The statistical analyses were performed using the Statgraphics software (StatPoint Technologies, Warrenton, VA, version 5.1.) Statistical differences in gene expression among the different sampling times were determined by one-way analysis of variance (ANOVA) followed by a posthoc (Student Newman Keuls, SNK). In the case of *gPer2a* and *gBmal1a* in liver, and *gOx* in hypothalamus, values were transformed (logarithmic transformation) to obtain a normal distribution and homogeneity of variances. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Effects of GRL and GRL antagonist on clock genes, orexin-A, and NPY expression

The acute treatment with $gGRL_{[1-19]}$ induced the expression of the three *Per* clock genes in the hypothalamus and liver of goldfish (around 2–3-fold; Figs. 1 and 2), but not in the pituitary (data not shown), while *gCry3* (Figs. 1 and 2) and *gBma1a* (Fig. 3) were not affected by the IP injection of this peptide. This *Per* induction was observed only at 1-h, but not at 3-h post-injection. In the hypothalamus, *gPer1a* and *gPer3a* increased with $gGRL_{[1-19]}$ injection, while *gPer2a* showed an increment but not statistically significant. The increase of *gPer1a* and *gPer3a* expression levels in $gGRL_{[1-19]}$ treated fish was counteracted by the treatment with the ghrelin antagonist [D-Lys^3]-GHRP-6 (Fig. 1). However, a tendency (but not statistically significant) to induce *Per* genes can be observed in fish treated with the GRL antagonist alone. The expression of *gCry3* in the hypothalamus was not modified at any time of treatment, except at 3-h after the double treatment ([D-Lys-3]-GHRP-6 and $gGRL_{[1-19]}$), when the expression was lower than in the control group (Fig. 1). In the liver, the induction of *Per* genes caused by $gGRL_{[1-19]}$ injection was partially blocked by the antagonist [D-Lys^3]-GHRP-6, that had no effect by itself on the expression of these genes (Fig. 2). The *gCry3* expression was not modified by any treatment neither at 1 nor at 3-h post-injection (Fig. 2).

In both, hypothalamus and liver, the expression of *gBmal1a* was not modified by GRL at any times post-injection (Fig. 3). The

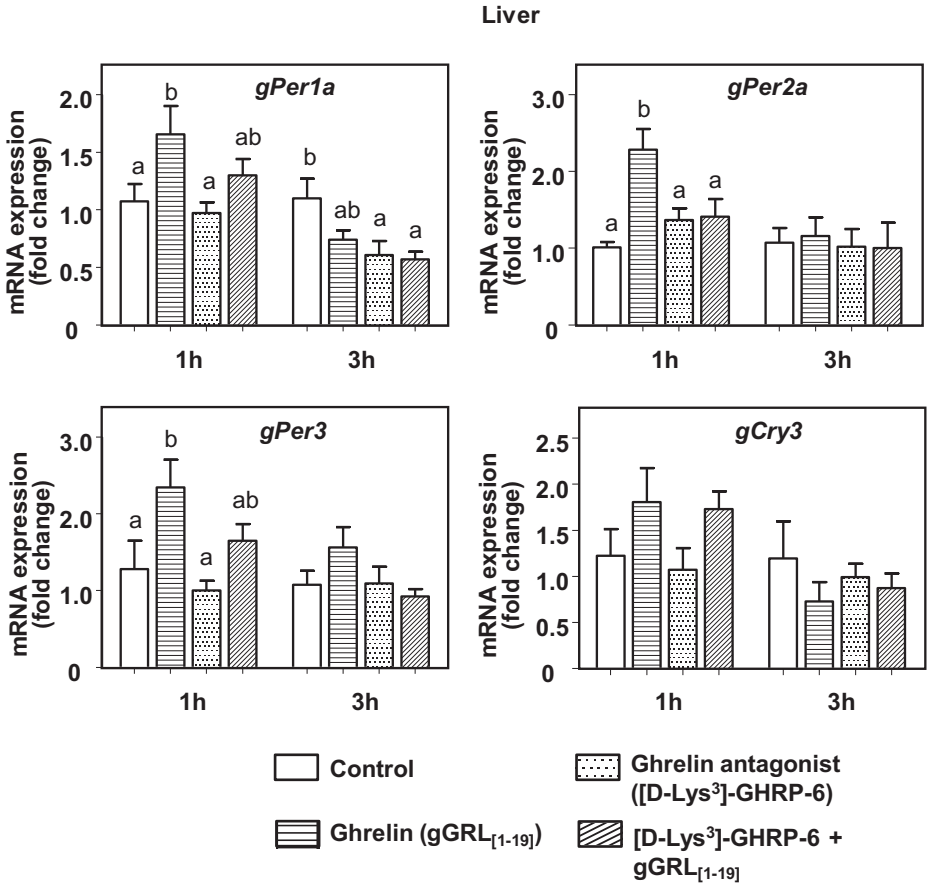

Fig. 2. Expression of clock genes (*gPer1a*, *gPer2a*, *gPer3*, *gCry3)* in liver of goldfish maintained under 12L:12D photoperiod, scheduled feeding at 10:00 h and injected at 14:00 h. Data are shown in relative units ($\Delta\Delta Ct$ method) as the mean ± standard error ($n = 6/7$), 1 and 3 h post-injection. Different letters indicate differences among the groups (ANOVA and SNK, $p < 0.05$).

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antagonist [D-Lys^3]-GHRP-6 by itself does not affect *gBmal1a* expression, which decreased in the hypothalamus 3-h after the double treatment with gGRL$_{[1-19]}$ and its antagonist (Fig. 3).

The Fig. 4 shows the effects of GRL and the GRL antagonist on NPY and orexin expression in the hypothalamus. The gGRL$_{[1-19]}$ increased (2-fold) the expression of orexin at 3-h post-injection, but not before. The [D-Lys^3]-GHRP-6 induced a similar stimulation to that induced by the agonist, either by itself or in combination with gGRL$_{[1-19]}$.The NPY expression was not modified by any treatment neither at 1 or at 3-h post-injections (Fig. 4).

3.2. Effects of GRL and GRL antagonist on locomotor activity daily rhythms

The IP sub-chronic treatment with gGRL$_{[1-19]}$ was used to investigate its putative role as an endogenous synchronizer signal that generates a locomotor anticipatory activity similar to the FAA. In the first experiment, under 12L:12D photoperiod and fasting conditions, both groups of fish (saline and gGRL$_{[1-19]}$ injected) showed a daily locomotor activity rhythm, with a period around 24 h (Fig. 5). During the 4 days post-injection, the anticipatory activity remained with a periodicity around 24 h in all tanks of gGRL$_{[1-19]}$ injected fish (3 tanks, data not shown), in contrast to the control group, that any tank maintained such 24 h period (Fig. 5). In the second experiment, under 24L conditions and scheduled fed at 16:00 h, fish injected with [D-Lys^3]-GHRP-6 at 13:00 h did not show a FAA (Fig. 6). The control group exhibited a FAA 7 days after the scheduled feeding regime. These daily activity profiles accord with their periodograms, which indicated a 24-h significant rhythm only in the control group. The locomotor activity in food deprived fish during the 4 days after the injection period showed a periodicity around 24-h in the control group. By contrast, the circadian period of the daily activity profile in the [D-Lys^3]-GHRP-6 injected group was not statistically significant (Fig. 6), and then, it cannot be considered as a significant rhythm.

4. Discussion

4.1. GRL and clock genes in goldfish

Present results shows for the first time in vertebrates that the acute treatment of GRL is able to induce clock gene expression at central (hypothalamus) and peripheral (liver) locations. The induction of *gPer1a*, *gPer2a* and *gPer3* genes by gGRL$_{[1-19]}$ suggest that physiological changes in endogenous GRL could alter the clockwork in these tissues. GRL could modify the sensitivity of these oscillators to ulterior inputs, and to a lesser extent to promote phase shifts. It is well documented that a direct light induction of *per2* in zebrafish is the mechanism by which clock gene rhythms are synchronized to the LD cycle in this species (Vatine et al.,

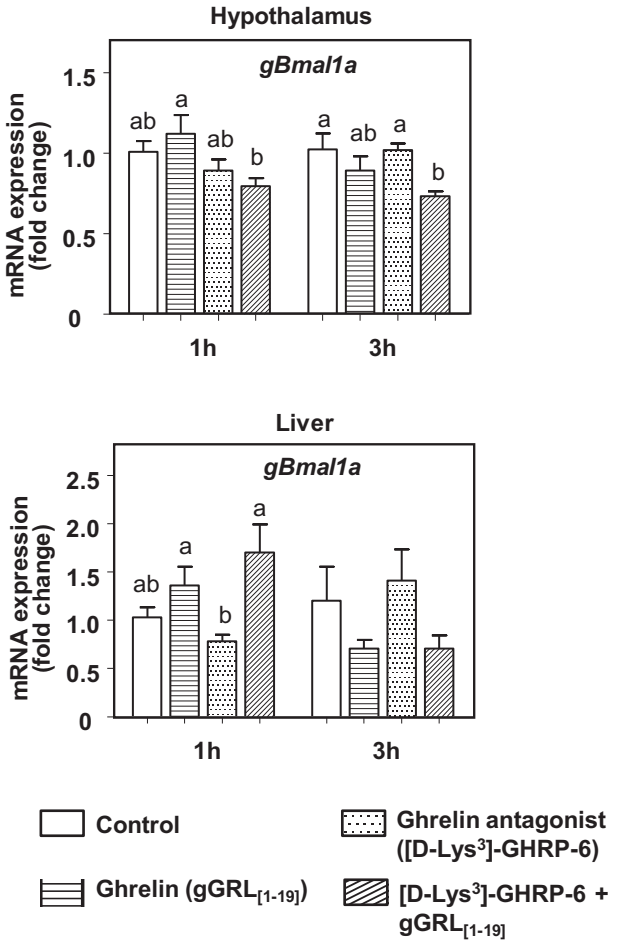

Fig. 3. Expression of *gBmal1a* in hypothalamus and liver of goldfish maintained under 12L:12D photoperiod, scheduled feeding at 10:00 h and injected at 14:00 h. Data are shown in relative units ($\Delta\Delta Ct$ method) as the mean ± standard error (n = 6/7), 1 and 3 h post-injection. Different letters indicate differences among the groups (ANOVA and SNK, $p < 0.05$).

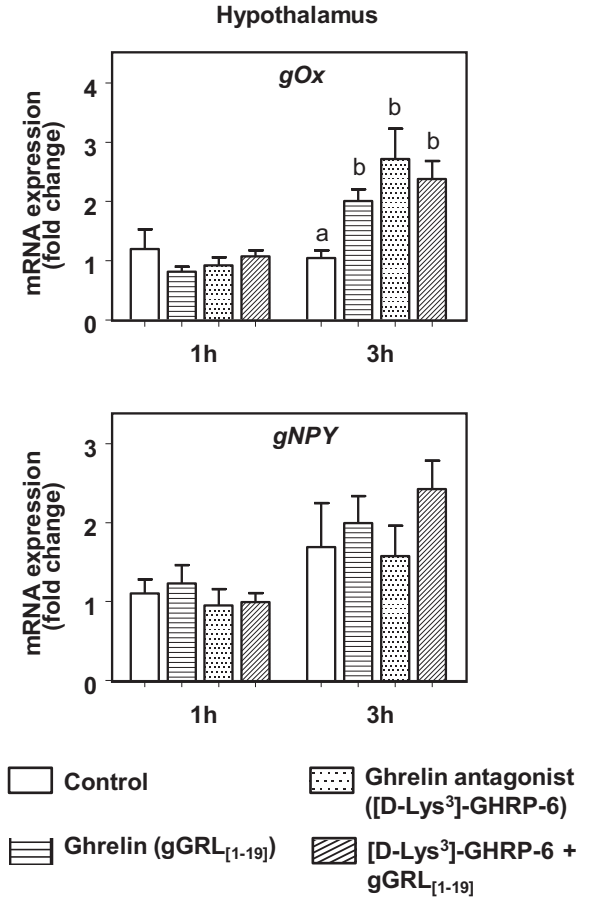

Fig. 4. Expression of feeding regulators *gOx* and *gNpy* in the hypothalamus of goldfish maintained under 12L:12D photoperiod, scheduled feeding at 10:00 and injected at 14:00 h. Data are shown in relative units ($\Delta\Delta Ct$ method) as the mean ± standard error (n = 6/7), 1 and 3 h post-injection. Different letters indicate differences among the groups (ANOVA and SNK, $p < 0.05$).

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2011). This also occurs for other hormones that are candidates to act as "internal *zeitgebers*" in some mammalian species (Albrecht, 2012; Dickmeis et al., 2013). The most studied are glucocorticoids, that in mammals synchronize peripheral oscillators (Dickmeis et al., 2013) probably via *Per1* induction (Yamamoto et al., 2005).

Interestingly it seems that the family of *Per* genes are usually the targets of different synchronizing molecules in mammals (Oike et al., 2011). This could be also the case in goldfish, where it is reported the GRL induction of *Per* genes (present results), changes in *Per* gene expression by orexin (Nisembaum et al., 2014), by cortisol (unpublished own results), light (*Per2a*; Azpeleta et al., 2012; Velarde et al., 2009), and food availability (Nisembaum et al., 2012). Moreover, in this teleost daily rhythms in *Per* transcripts have higher amplitudes than other clock genes (particularly *Per1* and *Per3*; Feliciano et al., 2011; Nisembaum et al., 2012; Velarde et al., 2009). In the present study, neither *gCry3* (other gene from the negative loop) nor *gBmal1a* (a gene from the positive loop of the molecular clock) were modified by GRL treatment, indicating that GRL effect is likely specific for *Per* family clock genes, and supporting the proposal that *Per* genes are more sensitive to the environmental regulation than other clock genes. Thus, they are probably targets for different inputs to the goldfish circadian system.

It is known that *gPer2a* expression is induced by light in goldfish central tissues, but not in peripheral ones (Azpeleta et al., 2012; Feliciano et al., 2011; Velarde et al., 2009), where it is more sensitive to feeding cues (Feliciano et al., 2011; Nisembaum et al., 2012).This fact could explain the significant stimulation of *gPer2a* expression found in the liver, but not in the hypothalamus. Similarly, a different response of *gPer2a* in foregut and hypothalamus after orexin ICV administration has been also reported in goldfish (Nisembaum et al., 2014), reinforcing the different tissue sensitivity of this gene to external signals (Azpeleta et al., 2012; Feliciano et al., 2011; Nisembaum et al., 2014). The lack of changes in clock genes expression in the pituitary by GRL seems to indicate that the effects of GRL on this gland are rather related with gonadotropins stimulation (Grey et al., 2010; Unniappan and Peter, 2004) than to the FEO mechanism.

The effects of GRL on clock genes expression in liver are probably mediated by specific GRL receptors since they are counteracted by a GRL antagonist. Three of the four described subtypes of GRL receptors are functional in goldfish (GHS-R1a-1, GHS-R1a-2, GHS-R2a-1) with differential tissue distribution (Kaiya et al., 2010). Both GHS-R1 subtypes are expressed in higher levels in liver than in the diencephalon, while GHS-R2a-1 is highly expressed in the diencephalon, and undetected in liver of goldfish (Kaiya et al., 2010). This distribution could explain the different effects induced by the [D-Lys^3]-GHRP-6 in the hypothalamus and the liver in the present study. The surprisingly induction of PER and orexin by the GRL antagonist in the hypothalamus needs to be clarified, but it agrees with recent results in chicken where a similar double effect of [D-Lys^3]-GHRP-6 has been described, *i.e.* [D-Lys^3]-GHRP-6 acts

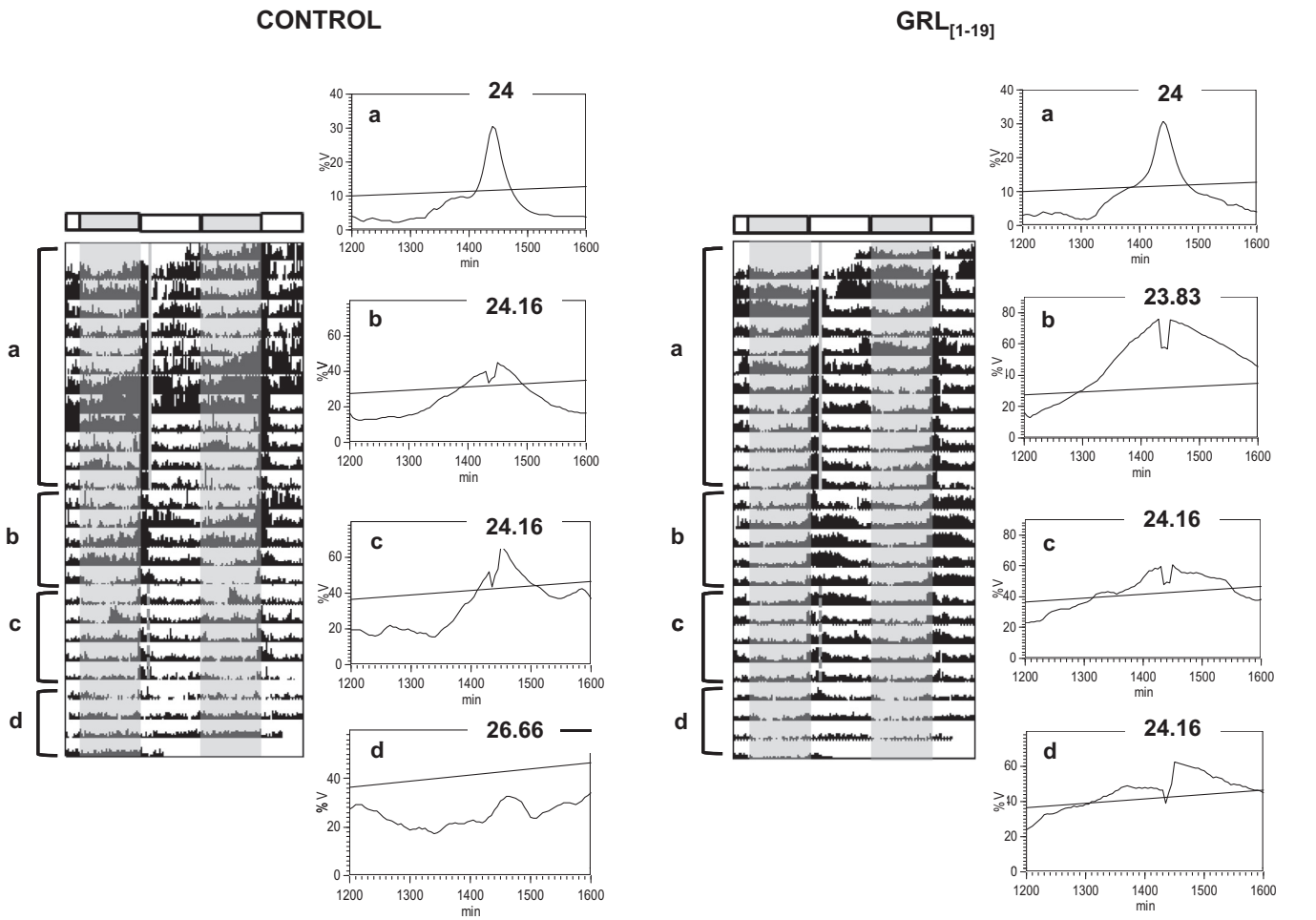

Fig. 5. Representative actograms of locomotor activity of control and gGRL$_{[1-19]}$ treated goldfish maintained under 12L:12D photoperiod and the following experimental conditions: scheduled feeding at 10:00 (a); fasting (b); injection at 10:00 h under fasting conditions (c); and fasting after injection period (d). The grey squares indicate the dark phase of the photocycle. The continuous line indicates the feeding time and the dashed line indicates the injection time. Actograms are double plotted (48 h time scale). Peaks over the threshold line in the periodograms indicate a significant rhythm. The period value of each condition is represented on the top of each periodogram.

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by itself as an agonist while its combination with GRL counteracts the GRL effects (Sirotkin et al., 2013).

4.2. Crosstalking among central and peripheral feeding regulators

The expected increase in hypothalamic orexin transcripts by the IP administration of GRL in the present study demonstrates the effectiveness of $gGRL_{[1-19]}$ treatment, and supports the existence of an interaction among peripheral and central orexigenic signals in this teleost, as previously suggested (Miura et al., 2007; Nisembaum et al., 2014). Besides orexin induction it is reported that central, but not peripheral, GRL administration induces NPY mRNA expression at 2-h post-injection (Miura et al., 2006). The lack of effect of GRL on NPY mRNA expression in goldfish hypothalamus (present results) agrees with those from Miura and co-workers, and confirms such central regulatory effects of GRL.

This crosstalking between central and peripheral areas involved in the food intake control could be also important in the interaction of feeding regulators with the circadian system. Besides the *Per* induction by GRL showed here, it was recently reported the induction of some *Per* genes by orexin in the goldfish hypothalamus and the foregut at 3-h post-injection, but not before (Nisembaum et al., 2014). A possible interaction between both orexigenic peptides (orexin and GRL) for the stimulation of hypothalamic *Per* expression cannot be excluded, since IP injected GRL increases hypothalamic orexin expression (present results), similarly to the effect of ICV GRL injections described in goldfish diencephalon (Miura et al., 2007). In this sense, orexin ICV injected also increases GRL expression in goldfish foregut (Nisembaum et al., 2014).

4.3. GRL and daily locomotor activity rhythms

The sub-chronic treatment with the GRL antagonist prevented the FAA under a scheduled feeding protocol and 24L conditions, supporting the involvement of GRL in the establishment of FAA in goldfish. Under such environmental conditions, saline injected goldfish exhibited a marked anticipatory activity as expected (Feliciano et al., 2011; Sánchez-Vázquez et al., 1997). This is the first report in fish that involves GRL in the FAA, as it was suggested in mammals based on the reduction in duration or amplitude of FAA in GRL-R knock-out mice (Blum et al., 2009; LeSauter et al., 2009). However, the specific requirement of GRL to enable the FAA is not clear to date. In fact, recent reports demonstrated that GRL-R knock-out mice were able to anticipate restricted feeding time under 24L conditions (Lamont et al., 2013).

In the presence of LD lighting conditions, fasted goldfish exhibited 24 h locomotor activity rhythms when injected with GRL. However, this activity rhythm cannot be totally ascribed to GRL since under a LD cycle goldfish tends to increase their activity during the light phase (Iigo and Tabata, 1996), as it is observed in control group (present study) that also exhibited daily activity rhythms with periods close to 24 h. Nevertheless, it cannot be discarded that control goldfish (saline-injected) might have increased circulating GRL, since it is reported that several days fasting increases GRL expression in peripheral and central tissues in fish (Amole and Unniappan, 2009; Unniappan et al., 2004). Besides, tissue sensitivity to GRL could be enhanced during food deprivation, as up-regulation of GRL receptors under fasting conditions has been shown in mammals (Nogueiras et al., 2004; Tups et al., 2004) and fish (Zhang et al., 2008; Kaiya et al., 2010).

Present study was planned to avoid the influence of synchronizing cues as food (first experiment, fasting conditions) and L/D photocycle (second experiment under 24L), since previous studies shown that the effectiveness of exogenous GRL, or its agonist GHRP-6, is better observed when other potent *zeitgebers* are absent (mice, Yannielli et al., 2007; brown trout, *Salmo trutta*, Tinoco et al., 2014). This could agree with the consideration of the FEO in mammals as a net of redundant systems, with the interaction of central and peripheral signals (Albrecht, 2012; Escobar et al., 2009), and in which humoral factors could influence timing signalization in

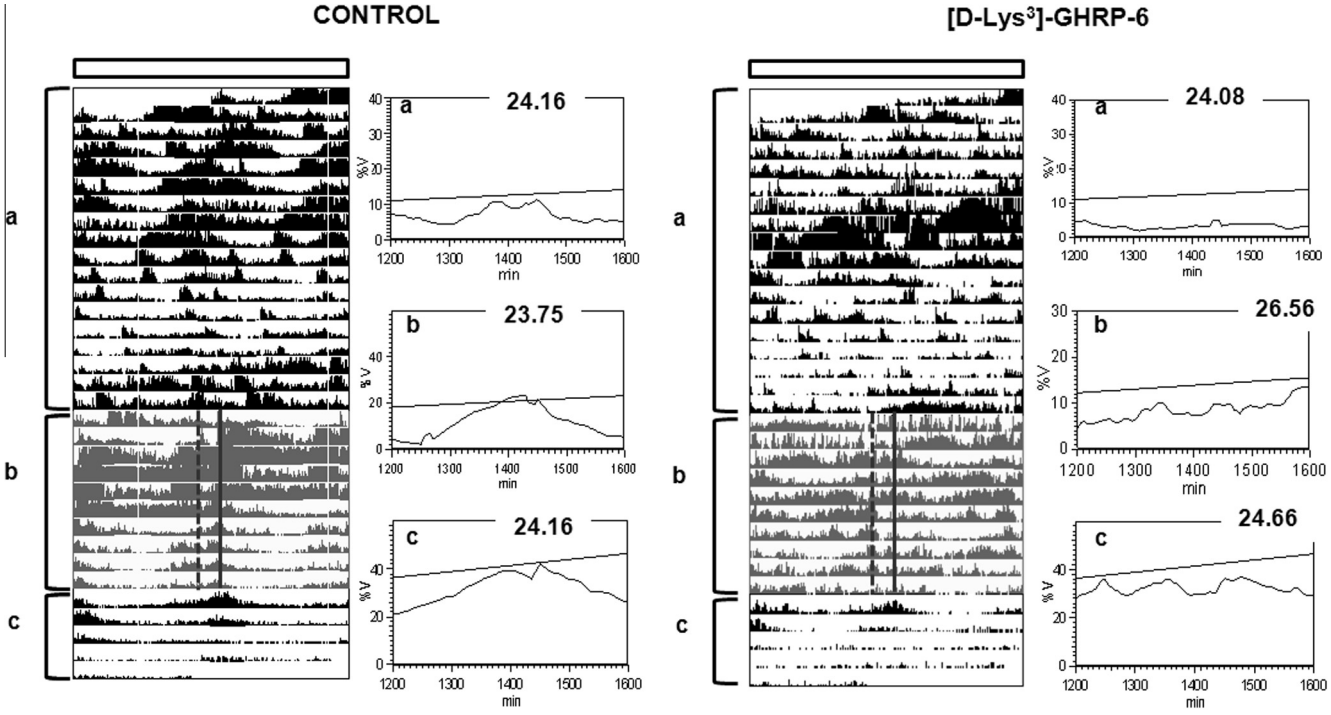

Fig. 6. Representative actograms of locomotor activity of control and ghrelin antagonist [D-Lys^3]-GHRP-6 treated goldfish maintained under 24L and the following experimental conditions: random feeding twice a day (a); injection at 13:00 h and scheduled feeding at 16:00 h (b); and fasting after injection period (c). The continuous line indicates the feeding time and the dashed line indicates the injection time. The white square indicates the injection period. Actograms are double plotted (48 h time scale). Peaks over the threshold line in the periodogram indicate a significant rhythm. The period value of each condition is represented on the top of each periodogram.

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food-entrained brain areas (Carneiro and Araujo, 2009, 2012). This proposal could be also adequate in fish, where a less hierarchical circadian system is probably present (Feliciano et al., 2011; Noche et al., 2011), and central and peripheral oscillators are synchronized by food and LD cycle (Feliciano et al., 2011; Nisembaum et al., 2012). In fact, orexin in goldfish also promotes daily activity rhythms in the absence of other inputs (Nisembaum et al., 2014), suggesting that the FEO is also organized under a redundant net of signals. Finally, the involvement of the reward system in FAA, that also includes orexigenic hormones such as GRL and orexin (Patton and Mistlberger, 2013) remains to be elucidated in fish.

In conclusion, the present study shows an interaction between GRL and the circadian system in goldfish. GRL could be an input of the circadian system acting as an entraining signal of feeding status.

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