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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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