Ghrelin modulates hypothalamic fatty acid-sensing and control of food intake in rainbow trout

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Abstract

There is no information available on fish as far as the possible effects of ghrelin on hypothalamic fatty acid metabolism and the response of fatty acid-sensing systems, which are involved in the control of food intake. Therefore, we assessed in rainbow trout the response of food intake, hypothalamic fatty acid-sensing mechanisms and expression of neuropeptides involved in the control of food intake to the central treatment of ghrelin in the presence or absence of a long-chain fatty acid such as oleate. We observed that the orexigenic actions of ghrelin in rainbow trout are associated with changes in fatty acid metabolism in the hypothalamus and an inhibition of fatty acid-sensing mechanisms, which ultimately lead to changes in the expression of anorexigenic and orexigenic peptides resulting in increased orexigenic potential and food intake. Moreover, the response to increased levels of oleate of hypothalamic fatty acid-sensing systems (activation), expression of neuropeptides (enhanced anorexigenic potential) and food intake (decrease) were counteracted by the simultaneous treatment with ghrelin. These changes provide evidence for the first time in fish of a possible modulatory role of ghrelin on the metabolic regulation by fatty acid of food intake occurring in the hypothalamus.

Key Words

- ▶ trout
- ▶ fish
- ▶ food intake
- ▶ ghrelin
- ▶ fatty acid
- ▶ hypothalamus

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Introduction

In mammals, hypothalamic nuclei from interconnected neuronal circuits react to changes in energy status by regulating the expression of agouti-related protein (AgRP)/neuropeptide Y (NPY) and pro-opio melanocortin (POMC)/cocaine and amphetamine-related transcript (CART), ultimately leading to changes in food intake (Blouet & Schwartz 2010). These neurons integrate information of peripheral signals, such as i) changes in the levels of nutrients/metabolites like glucose, fatty acids

and amino acids that are detected through nutrient-sensor mechanisms (Blouet & Schwartz 2010), and ii) changes in the levels of hormones like leptin, ghrelin (GHRL), insulin, glucagon-like peptide 1, cannabinoids, glucocorticoids and adiponectins that are detected by binding of those hormones to their receptors (Diéguez *et al.* 2009).

The fatty acid-sensing mechanisms detect increases in plasma levels of long-chain fatty acid (LCFA), but not short-chain fatty acid (SCFA) or medium-chain fatty acid

(MCFA) through several mechanisms such as i) fatty acid metabolism by means of inhibition of carnitine palmitoyltransferase 1 (CPT1) to import fatty acid-CoA into the mitochondria for oxidation; ii) binding to fatty acid translocase (FAT/CD36), and further modulation of transcription factors like peroxisome proliferator-activated receptor type alpha (PPAR α), and sterol regulatory element-binding protein type 1c (SREBP1c); iii) activation of protein kinase C- θ ; and iv) mitochondrial production of reactive oxygen species (ROS) by electron leakage, resulting in an inhibition of ATP-dependent inward rectifier potassium channel (K_{ATP}) activity (López *et al.* 2007, Benoit *et al.* 2009, Blouet & Schwartz 2010). The activation of these systems, together with changes in the activity of integrative sensors like 5'-AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1), is associated with the inhibition of the orexigenic factors AgRP and NPY and the enhancement of the anorexigenic factors POMC and CART, ultimately leading to decreased food intake (Migrenne *et al.* 2007, Blouet & Schwartz 2010). Our previous studies in fish have characterized the presence and function of fatty acid-sensing systems in the hypothalamus of rainbow trout (*Oncorhynchus mykiss* Walbaum) (Librán-Pérez *et al.* 2012, 2013a,b; 2014a,b, 2015a,b). These systems respond to changes in the levels not only of LCFA such as oleate but also, unlike mammals, to MCFA like octanoate (Soengas 2014). The activation of these systems is associated with the inhibition of AgRP and NPY and the enhancement of POMC and CART expression, ultimately leading to decreased food intake (Librán-Pérez *et al.* 2012, 2014a). To date, there is almost no evidence in fish concerning the potential endocrine modulation of central fatty acid-sensing systems, with only some preliminary data obtained about insulin (Librán-Pérez *et al.* 2015a,b).

GHRL is a peptide hormone basically synthesized in the gastrointestinal tract that is octanoylated by ghrelin O-acyl transferase. This octanoylation is essential for binding to the growth hormone secretagogue receptor (GHS-R1a) present in several locations, including hypothalamic neurons (Perello & Dickson 2015, Al Massadi *et al.* 2015). The binding elicits an orexigenic response through enhanced stimulation of NPY/AgRP and decreased POMC/CART gene expression (Velásquez *et al.* 2011). This orexigenic effect is mediated by changes in fatty acid metabolism and other parameters related to fatty acid-sensing systems that basically reverse those elicited by raised levels of LCFA (López *et al.* 2008, Sangiao-Alvarellos *et al.* 2010, Martins *et al.* 2013, Gao *et al.* 2013, Stark *et al.* 2015). Besides the role of GHRL

in food intake regulation, this hormone has been also involved in the control of glucose and lipid homeostasis and metabolism (Sangiao-Alvarellos *et al.* 2010, Ratner *et al.* 2015) and modulation of hormone release (Carreira *et al.* 2013), among others (Sato *et al.* 2014, Müller *et al.* 2015). GHRL has been also identified in a number of fish species, including rainbow trout (*Oncorhynchus mykiss*), Japanese eel (*Anguilla japonica*), sea bass (*Dicentrarchus labrax*), Atlantic salmon (*Salmo salar*), zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), tilapia (*Oreochromis mossambicus*) and channel catfish (*Ictalurus punctatus*) (Kaiya *et al.* 2008, Kang *et al.* 2011). It is highly expressed in the gastrointestinal tract, moderately in the brain and to a lower extent in other tissues (Unniappan *et al.* 2002, Unniappan & Peter 2005, Volkoff *et al.* 2005). Once octanoylated, GHRL binds to GHS-R1a, which is also expressed in a panel of fish tissues such as the brain (particularly hypothalamus), gastrointestinal tissue, pancreatic cells and liver (Chang & Cheng 2004, Kaiya *et al.* 2009, Cruz *et al.* 2010). The role of GHRL in fish is far from elucidated though it has been involved in the control of food intake (Unniappan *et al.* 2002, Kang *et al.* 2011, Jönsson 2013), glucose and lipid metabolism (Kaiya *et al.* 2009, Salmerón *et al.* 2015) and the modulation of growth hormone and prolactin release (Kaiya *et al.* 2008). GHRL is accepted as an orexigenic factor in fish (Unniappan *et al.* 2002, 2004, Matsuda *et al.* 2006, Shepherd *et al.* 2007, Miura *et al.* 2006, 2007, Tinoco *et al.* 2014), though changes in the expression of neuropeptides involved in the metabolic regulation of food intake have been scarcely studied (Polakof *et al.* 2011a, Riley 2013, Tinoco *et al.* 2014, Schroeter *et al.* 2015). Furthermore, there is no information available in fish regarding the possible effects of GHRL on hypothalamic fatty acid metabolism and the response of fatty acid-sensing systems. Based on the anorectic actions of LCFA like oleate in rainbow trout (Librán-Pérez *et al.* 2012, 2014a), the orexigenic actions of GHRL in fish (Kang *et al.* 2011), and the fact that plasma GHRL levels increase in rainbow trout after a meal (Pankhurst *et al.* 2008), especially when fish were fed with a lipid-enriched diet (Jönsson *et al.* 2007), we hypothesize that the response to raised levels of fatty acid of food intake, hypothalamic fatty acid-sensing mechanisms and expression of neuropeptides involved in the control of food intake might be counteracted by GHRL treatment in rainbow trout.

Therefore, we aimed to elucidate in rainbow trout i) the effects of GHRL on hypothalamic fatty acid metabolism and the response of fatty acid-sensing systems involved in the control of food intake, and ii) the possible

modulatory effect of GHRL on the effects of raised levels of oleate. Thus, we centrally injected GHRL in the presence or absence of oleate and evaluated food intake, and the mRNA abundance of hypothalamic neuropeptides involved in the nutrient control of food intake, such as AgRP, NPY, POMC and CART as well as CRF, as a peptide involved in the anorectic response to stressful conditions. We also evaluated variables related to putative fatty acid-sensing systems based on i) fatty acid metabolism, such as activities of fatty acid synthase (FAS, *EC* 2.3.1.85), ATP-citrate lyase (ACLY, *EC* 4.1.3.8) and CPT1 (*EC* 2.3.1.21), and mRNA abundance of acetyl-CoA carboxylase (ACC), ACLY, CPT1c and FAS; ii) binding to FAT/CD36 and further modulation of transcription factors, such as mRNA abundance of FAT/CD36, PPAR α and SREBP1c; and iii) mitochondrial activity, such as mRNA abundance of mitochondrial uncoupling protein 2a (UCP2a) and inward rectifier K⁺ channel pore type 6.x-like (Kir6.x-like). Finally, we also evaluated changes in other integrative sensors such as mRNA abundance of AMPK α 1, SIRT1 and lipoprotein lipase (LPL).

Materials and methods

Fish

Rainbow trout were obtained from a local fish farm (A Estrada, Spain). Fish were maintained for 1 month in 100-l tanks under laboratory conditions and a 12 h light:12 h darkness photoperiod (lights on at 0800 h, lights off at 2000 h) in dechlorinated tap water at 15 °C. Fish weight was 100 ± 2 g. Fish were fed once daily (0900 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat and 11.5% ash; 20.2 MJ/kg of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE) and of the Spanish Government (RD 55/2013) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

Experimental design

Following 1 month acclimation period, fish were randomly assigned to 100-l experimental tanks. Fish were fasted for 24 h before treatment to ensure basal hormone levels were achieved. On the day of experiment, fish were lightly anaesthetized with 2-phenoxyethanol (Sigma, 0.2% v/v) and weighed. Intracerebroventricular (ICV) administration was performed as previously described

(Polakof & Soengas 2008). Briefly, fish were placed on a plexiglass board with Velcro straps adjusted to hold them in place. A 29½-gauge needle attached through a polyethylene cannula to a 10 µl Hamilton syringe was aligned with the 6th pre-orbital bone at the rear of the eye socket, and from this point the syringe was moved through the space in the frontal bone into the third ventricle. The plunger of the syringe was slowly depressed to dispense 1 µl/100 g body mass of vehicle (control), or containing 200 ng of rainbow trout octanoylated 23 amino acid GHRL (synthesized by Bachem according to the sequence published by Kaiya *et al.* (2003)), 1 µmol oleate (Sigma Chemical Co.), or GHRL+oleate. The vehicle used, saline-HPB, was a mixture (1:1 in vol) of Hanks' saline and a solution in Hanks' saline of 45% hydroxypropyl-beta-cyclodextrin (HPB) to a final concentration of 17 mM (Morgan *et al.* 2004). We used the HPB fraction to safely deliver oleate, and the saline fraction to dissolve GHRL. No effects of HPB alone were noted for any of the parameters assessed (data not shown).

In the first set of experiments, food intake was registered for 3 days before treatment (to evaluate basal level of food intake) and then 6, 24 and 48 h after ICV treatment with saline-HPB alone (control, $n=10$ for each time point) or containing GHRL ($n=10$ for each time point), oleate ($n=10$ for each time point) or GHRL+oleate ($n=10$ for each time point). After feeding, the uneaten food remaining at the bottom (conical tanks) and feed waste were withdrawn, dried and weighed. The amount of food consumed by all fish in each tank was calculated as previously described as the difference from the feed offered (De Pedro *et al.* 1998, Polakof *et al.* 2008a,b). The experiment was repeated four times. Results are shown as the mean $\pm$ S.E.M. ($n=4$).

In a second set of experiments, fish were ICV injected with saline-HPB alone (control, $n=15$ at 2 h, and $n=15$ at 6 h) or containing GHRL ($n=15$ at 2 h, and $n=15$ at 6 h), oleate ($n=15$ at 2 h, and $n=15$ at 6 h) or GHRL+oleate ($n=15$ at 2 h, and $n=15$ at 6 h) with the same concentrations described above. After 2 or 6 h, fish were lightly anaesthetized with 2-phenoxyethanol (Sigma, 0.2% v/v). Blood was collected by caudal puncture with ammonium-heparinized syringes, and plasma samples were obtained after blood centrifugation, de-proteinized immediately (using 0.6 M perchloric acid) and neutralized (using 1 M potassium bicarbonate) before freezing with liquid nitrogen and storage at -80 °C until further assay. Fish were sacrificed by decapitation and the hypothalami were dissected, snap-frozen and stored at -80 °C. Each time, nine fish per group were used to assess enzyme activities

and metabolite levels whereas the remaining six fish were used for the assessment of mRNA levels by qRT-PCR.

Assessment of metabolite levels and enzyme activities

Levels of fatty acid, total lipid, triglyceride, glucose and lactate in plasma were determined enzymatically using commercial kits (Wako Chemicals, Neuss, Germany, for fatty acid; Spinreact, Barcelona, Spain for total lipid, triglyceride and lactate; Biomérieux, Grenoble, France, for glucose).

Samples used to assess hypothalamic metabolite levels were homogenized immediately by ultrasonic disruption in 7.5 vols of ice-cooled 0.6 M perchloric acid, and neutralized as before. The homogenate was centrifuged (10 000 *g*), and the supernatant used to assay tissue metabolites. Tissue fatty acid, total lipid and triglyceride levels were determined enzymatically using commercial kits as described above for plasma samples.

Samples for enzyme activities were homogenized by ultrasonic disruption with 9 vols of ice-cold-buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4-dithiothreitol and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged (10 000 *g*) and the supernatant used immediately for enzyme assays. Enzyme activities were determined using a microplate reader INFINITE 200 Pro (Tecan, Männedorf, Switzerland). Reaction rates of enzymes were determined by the increase or decrease in the absorbance of NAD(P)H at 340 nm or, in the case of CPT1 activity, of 5,5'-dithiobis(2-nitrobenzoic acid)-CoA complex at 412 nm. The reactions were started by the addition of supernatant (15 μ l) at a pre-established protein concentration, omitting the substrate in control wells (final volume 265–295 μ l), and allowing the reactions to proceed at 20 °C for pre-established times (3–10 min). Enzyme activities were normalized to protein levels (mg). Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method with BSA (Sigma, USA) as standard. Enzyme activities were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations. ACLY, FAS and CPT1 activities were determined following available methods (Alvarez *et al.* 2000, Polakof *et al.* 2011b, Ditlecadet *et al.* 2012 respectively).

mRNA abundance analysis by real-time quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Life Technologies) and subsequently treated with RQ1-DNase (Promega). Two microgram total RNA were

reverse-transcribed using Superscript II reverse transcriptase (Promega) and random hexamers (Promega) to obtain ~20 μ l. Gene expression levels were determined by real-time quantitative RT-PCR (q-PCR) using the iCycler iQ (BIO-RAD). Analyses were performed on 1 μ l cDNA using MAXIMA SYBR Green qPCR Mastermix (Life Technologies), in a total PCR reaction volume of 15 μ l, containing 50–500 nM of each primer. mRNA abundance of ACC, ACLY, AMPK, AgRP, CART, CPT1, FAT/CD36, FAS, Kir6.x-like, LPL, NPY, POMC, PPAR α , SIRT1, SREBP1c and UCP2a were determined as described in the same species (Ducasse-Cabanot *et al.* 2007, Kolditz *et al.* 2008, Polakof *et al.* 2008c, Cruz-Garcia *et al.* 2009, Lansard *et al.* 2009, Conde-Sieira *et al.* 2010, Polakof *et al.* 2010, 2011b; Figueiredo-Silva *et al.* 2012, Librán-Pérez *et al.* 2013b). Sequences of the forward and reverse primers used for each gene expression are shown in Table 1. Relative quantification of the target gene transcript was done using β -actin gene expression as reference, which was stably expressed in this experiment. Thermal cycling was initiated with incubation at 95 °C for 90 s using hot-start iTaq DNA polymerase activation followed by 35 cycles, each one consisting of heating at 95 °C for 20 s, and specific annealing and extension temperatures for 20 s. Following the final PCR cycle, melting curves were systematically monitored (55 °C temperature gradient at 0.5 °C/s from 55 to 94 °C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without RNA were run for each reaction as negative controls. Relative quantification of the target gene transcript with the β -actin reference gene transcript was made following the Pfaffl (2001) method. This mathematical algorithm computes an expression ratio based on q-PCR efficiency and the crossing point deviation of the unknown sample versus a control group:

$$R = \frac{(E_{\text{target gene}})^{\Delta CT_{\text{target gene}} (\text{mean control} - \text{mean unknown sample})}}{(E_{\beta\text{-actin}})^{\Delta CT_{\beta\text{-actin}} (\text{mean control} - \text{mean unknown sample})}}$$

where E is PCR efficiency determined using a standard curve of cDNA serial dilutions (cDNA dilutions from 1/32 up to 1/512) and ΔCT is the crossing point deviation of an unknown sample versus a control.

Statistical analysis

Comparisons among groups were carried out with one-way ANOVA followed by a Student-Newman-Keuls test, and differences were considered statistically significant at $P < 0.05$.

Table 1 Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

Gene	Forward primer	Reverse primer	Annealing temperature (°C)	Data base	Accession number
β-actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59	GenBank	NM_001124235.1
ACC	TGAGGGCGTTTTCACTATCC	CTCGATCTCCCTCTCCACT	59	Signae	tcbk0010c.b.21_5.1.om.4
ACLY	CTGAAGCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	60	GenBank	CA349411.1
AMPK α 1	ATCTTCTTCACGCCCCAGTA	GGGAGCTCATCTTTGAACCA	60	GenBank	HQ40367
AgRP	ACCAGCAGTCCTGTCTGGGTAA	AGTAGCAGATGGAGCCGAACA	60	GenBank	CR376289
CART	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	60	GenBank	NM_001124627
CPT-1c	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	59	GenBank	AJ619768
CRF	ACAACGACTCAACTGAAGATCTCG	AGGAAATTGAGTTCATGTCAGG	60	GenBank	AF296672
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTGATGGTGAGCTGT	59	Signae	tcab0001c.e.06 5.1.s.om.8
FAT/CD36	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCTCCACAGGA	62	DFCI	AY606034.1
Kir6.x-like	TTGGCTCTCTTCGCCATGT	AAAGCCGATGGTGACCTGGA	60	Signae	CA346261.1.s.om.8:1:773:1
LPL	TAATTGGCTGCAGAAAACAC	CGTCAGCAAACCTCAAAGGT	59	GenBank	AJ224693
NPY	CTCGTCTGGACCTTTATATGC	GTTTCATCATATCTGGACTGTG	58	GenBank	NM_001124266
POMC-A1	CTCGTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	60	Tigr	TC86162
PPAR α	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTGTCAGCAGAT	55	GenBank	AY494835
SIRT-1	GCTACTTGGGGACTGTGACG	CTCAAAGTCTCCGCCAAC	57	GenBank	EZ774344.1
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	60	GenBank	CA048941.1
UCP2a	TCCGGTACAGATCCAGG	CTCTCCACAGACCACGCA	57	GenBank	DQ295324

ACC, Acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; AMPK α 1, AMP-activated protein kinase; CART, cocaine- and amphetamine-related transcript; CPT-1, carnitine palmitoyl transferase type 1; CRF, corticotropin-releasing factor; FAS, fatty acid synthetase; FAT/CD36, fatty acid translocase; Kir6.x-like, inward rectifier K⁺ channel pore type 6.-like; LPL, Lipoprotein lipase; NPY, neuropeptide Y; POMC-A1, pro-opio melanocortin A1; PPAR α , peroxisome proliferator-activated receptor type α ; SIRT-1, sirtuin, SREBP1c, sterol regulatory element-binding protein type 1c; UCP2a, mitochondrial uncoupling protein 2a.

Results

Levels of plasma metabolites are shown in Fig. 1. Levels of fatty acid (Fig. 1A), triglyceride (Fig. 1B), glucose (Fig. 1C) and lactate (Fig. 1D) were not affected by treatments. Hypothalamic metabolite levels are shown in Fig. 2. Fatty acid levels (Fig. 2A) in the oleate group increased compared with control (2 h), GHRL (2 and 6 h) and GHRL+oleate (6 h) groups. Triglyceride levels (Fig. 2B) increased in the oleate group at 2 h compared with control and GHRL groups. Total lipid levels (Fig. 2C) increased in the oleate group compared with the other groups after 2 h of treatment.

Enzyme activities in hypothalamus are shown in Fig. 3. CPT1 activity (Fig. 3A) decreased in all treated groups compared with controls either after 2 or 6 h of treatment; the values of the GHRL group were lower than those of oleate group after 2 h. FAS activity (Fig. 3B) after 2 h of treatment was lower in the oleate group than in GHRL and GHRL+oleate groups. ACLY activity (Fig. 3C) was lower in the oleate group than in control (2 and 6 h), GHRL (2 and 6 h) and GHRL+oleate (6 h) groups.

Changes in the mRNA abundance of neuropeptides are shown in Fig. 4. POMC mRNA levels (Fig. 4A) decreased in the GHRL group compared with control (2 and 6 h), oleate (2 and 6 h) and GHRL+oleate (6 h); the values in

the oleate group were also higher than all other groups after 2 (except GHRL+oleate) or 6 h of treatment. CART mRNA abundance (Fig. 4B) decreased in GHRL group compared with control (2 h), oleate (2 and 6 h) and GHRL+oleate (2 h) groups; the values in the oleate group were higher than those of control (2 h), GHRL (2 and 6 h) and GHRL+oleate (6 h) groups. The mRNA abundance of NPY (Fig. 4C) was higher in the GHRL group compared with all other groups after 2 or 6 h of treatment (except GHRL+oleate after 2 h) whereas values of the oleate group after 2 h of treatment were lower than those of the remaining groups. The mRNA abundance of AgRP (Fig. 4D) after 2 h was lower in the oleate group compared with the other groups. Finally, no significant changes were observed for mRNA abundance of CRF (Fig. 4E).

Changes in food intake are shown in Fig. 5. GHRL treatment increased food intake compared with control (24 h), oleate (24 and 48 h) and GHRL+oleate (24 and 48 h) groups. Oleate treatment decreased food intake compared with control and GHRL groups.

Changes in mRNA abundance of parameters related to fatty acid sensing in hypothalamus are shown in Table 2. FAT/CD36 decreased in the GHRL compared with control groups after 2 h whereas the value of the oleate group was higher than that of GHRL group after 6 h. ACC mRNA

Figure 1

Levels of fatty acid (A), triglyceride (B), glucose (C) and lactate (D) in plasma of rainbow trout 2 or 6 h after intracerebroventricular administration of 1 µl/100 g body mass of saline-HPB alone (Control, C) or containing 200 ng of rainbow trout ghrelin (GHRL), or 1 µmol oleate (OL), or GHRL+oleate (GHRL+OL). Each value is the mean + s.e.m. of $n=15$ fish per treatment. Different letters indicate significant differences ($P<0.05$) from different groups.

abundance increased in the GHRL group compared with control (6 h), oleate (2 and 6 h) and GHRL+oleate (6 h) groups whereas the GHRL+oleate group was also higher than oleate group after 2 h. ACLY mRNA abundance was higher in the GHRL than in the oleate group after 2 or 6 h, whereas the value of the oleate group was lower than that of control after 6 h. CPT1c value of the oleate group after 6 h was lower than those of control and GHRL groups. The value of FAS in the GHRL group was higher than those of control (2 and 6 h), oleate (2 and 6 h) and GHRL+oleate (6 h) groups whereas the value of the GHRL+oleate group was higher than those of control (2 h), GHRL (6 h) and oleate (2 and 6 h) groups. The mRNA abundance of UCP2a

after 2 h decreased in GHRL and oleate groups compared with control and GHRL+oleate groups. The value of Kir6.x-like in the oleate group was lower than in the other groups either after 2 or 6 h of treatment (except GHRL+oleate after 6 h). The mRNA abundance of PPAR α after 6 h of treatment was higher in the GHRL group than in the oleate group whereas the value of the oleate group was also lower than that of GHRL+oleate group. The value of SREBP1c after 6 h of treatment was lower in the GHRL and GHRL+oleate groups compared with control group. The mRNA abundance of AMPK α 1 increased in the oleate and GHRL+oleate groups compared with GHRL group after 2 h, whereas the GHRL group was higher than that of control and oleate groups 6 h after treatment. The value of SIRT1 after 2 h was higher in the oleate than in the remaining groups, whereas after 6 h the values of all treated groups were lower than those of controls. Finally, LPL mRNA abundance decreased in all treated groups compared with controls either after 2 or 6 h of treatment.

Figure 2

Levels of fatty acid (A), triglyceride (B) and total lipid (C) in hypothalamus of rainbow trout 2 or 6 h after intracerebroventricular administration of 1 µl/100 g body mass of saline-HPB alone (Control, C) or containing 200 ng of rainbow trout ghrelin (GHRL), or 1 µmol oleate (OL), or ghrelin + oleate (GHRL+OL). Each value is the mean + s.e.m. of $n=9$ fish per treatment. Different letters indicate significant differences ($P<0.05$) from different groups.

Figure 3

Activities of CPT1 (A), FAS (B) and ACLY (C) in hypothalamus of rainbow trout 2 or 6 h after intracerebroventricular administration of 1 μ l/100 g body mass of saline-HPB alone (Control, C) or containing 200 ng of rainbow trout ghrelin (GHRL), or 1 μ mol oleate (OL), or ghrelin + oleate (GHRL + OL). Each value is the mean $\pm$ s.e.m. of $n=9$ fish per treatment. Different letters indicate significant differences ($P<0.05$) from different groups.

Discussion

Oleate treatment activates fatty acid-sensing systems and inhibit food intake

The central treatment with oleate alone resulted in hypothalamic changes of parameters related to fatty acid sensing. These changes include increased levels of fatty acid, triglyceride and total lipid, decreased activities of CPT1 and ACLY, and decreased mRNA abundance of ACLY, CPT1c, UCP2a and Kir6.x-like, and are indicative of the activation of different fatty acid-sensing systems in response to raised levels of oleate. The activation of these systems is also associated with the increased mRNA abundance of the anorexigenic peptides POMC and CART and the decreased mRNA abundance of the orexigenic peptides AgRP and NPY. The resultant increased anorexigenic potential agrees with the decreased food intake observed in oleate-treated fish. All these changes are in agreement with previous studies carried out in rainbow trout (Librán-Pérez *et al.* 2014a), which together with the absence of changes in plasma levels of metabolites (suggesting the absence of stress) validate the experimental design.

GHRL treatment inhibits fatty acid-sensing systems increasing hypothalamic orexigenic potential and stimulating food intake

Central GHRL treatment did not affect levels of metabolites in plasma. This lack of changes is in agreement with the absence of changes in glycaemia in the same

Figure 4

mRNA levels of POMC (A), CART (B), NPY (C), AgRP (D) and CRF (E) in hypothalamus of rainbow trout 2 or 6 h after intracerebroventricular administration of 1 μ l/100 g body mass of saline-HPB alone (Control, C) or containing 200 ng of rainbow trout ghrelin (GHRL), or 1 μ mol oleate (OL), or ghrelin + oleate (GHRL + OL). Each value is the mean $\pm$ s.e.m. of $n=6$ fish per treatment. Gene expression results are referred to control group and are normalized by β -actin expression. Different letters indicate significant differences ($P<0.05$) from different groups.

Figure 5

Food intake in rainbow trout 6, 24 and 48 h after intracerebroventricular administration of 1 μ l/100 g body mass of saline-HPB alone (Control, C) or containing 200 ng of rainbow trout ghrelin (GHRL), or 1 μ mol oleate (OL), or ghrelin + oleate (GHRL + OL). Food intake is displayed as means $\pm$ S.E.M. of the percentage of food ingested with respect to baseline levels (calculated as the average of food intake the 3 days previous to experiment). The results are shown as mean $\pm$ S.E.M. of the results obtained in four different tanks in which ten fish were used per group in each tank.

species after ICV GHRL treatment (Polakof *et al.* 2011a), which further validates the experimental design since treatments are reflecting central and not peripheral mechanisms. Thus, in other studies in fish in which GHRL treatment was through, i.p. injection changes in plasma glucose levels were noted (Schwandt *et al.* 2010). Furthermore, the lack of changes in fatty acid levels is in agreement with that reported in mice under a comparable ICV treatment (Stark *et al.* 2015). GHRL treatment did not affect levels of fatty acid, triglyceride or total lipid in the hypothalamus. This is in contrast to the mammalian model, where ICV GHRL treatment resulted in increased hypothalamic fatty acid levels (Andrews *et al.* 2008).

GHRL treatment did not affect food intake 6 h after ICV treatment, but feeding levels increased 24 h after treatment and this trend remained (though no significance was found) up to 48 h after treatment. The orexigenic effect is in agreement with that observed in most fish species after IP or ICV GHRL treatments (Unniappan *et al.* 2002, 2004, Matsuda *et al.* 2006, Miura *et al.* 2006, 2007, Tinoco *et al.* 2014), and is in line to the effect of this peptide in other vertebrates, including mammals (López *et al.* 2008, Sangiao-Alvarellos *et al.* 2010, Velásquez *et al.* 2011, Martins *et al.* 2013, Stark *et al.* 2015). It is surprising that GHRL did not also increase food intake

6 h after treatment. We have no explanation for this lack of effect, which could relate to the fact that food intake was measured per group and not individually. Our results differ from those reported previously in rainbow trout showing an anorectic effect of ICV-administrated rainbow trout GHRL (Jönsson *et al.* 2010). In their study, Jönsson *et al.* (2010) attributed the anorectic effects of GHRL to the observed increase in the mRNA abundance of hypothalamic CRF. In our study, we have not observed any change in the hypothalamic CRF expression. We do not have an explanation for this controversial finding, but increased CRF levels in the study of Jönsson *et al.* (2010) suggest an activation of the HPI axis. Therefore, the result observed by Jönsson *et al.* (2010) could result from an interaction between GHRL treatment and central stress response. Accordingly, tilapia treated with cortisol showed a decrease in plasma GHRL levels and food intake, demonstrating an interaction between stress and GHRL (Upton & Riley 2013).

Considering the changes observed in the mRNA abundance of neuropeptides involved in the metabolic control of food intake, we observed that GHRL induced decreased expression levels of the anorexigenic peptides POMC and CART and increased values of the orexigenic peptides AgRP and NPY. These changes are indicative of an orexigenic response in agreement with the changes observed in food intake. In previous studies in fish, only NPY and POMC responses to GHRL treatment have been studied. Results reported controversial conclusions showing GHRL-induced increase (Riley 2013), decrease (Polakof *et al.* 2011a) or no effects (Tinoco *et al.* 2014) on central NPY expression, whereas no changes were observed in POMC hypothalamic expression in channel catfish after IP GHRL treatment (Schroeter *et al.* 2015). This is the first study in which the GHRL effect on the four main neuropeptides involved in the metabolic regulation of food intake have been assessed simultaneously. The observed changes are in concordance with those previously observed in mammals in which central GHRL administration resulted in consistent increases of NPY and AgRP expression (Andrews *et al.* 2008, Sangiao-Alvarellos *et al.* 2010, Martins *et al.* 2013, Stark *et al.* 2015) and, occasionally, in decreased POMC and CART mRNA levels (Velásquez *et al.* 2011).

Effects of GHRL on central lipid metabolism or fatty acid sensing in fish remains unexplored. Our results shows differential effects depending on the fatty acid-sensing mechanism studied. The fatty acid-sensing system related to fatty acid metabolism displayed changes opposed to those previously described after central oleate or octanoate

Table 2 mRNA levels in hypothalamus of rainbow trout 2 or 6 h after intracerebroventricular administration of 1 µl/100 g body mass of saline-HPB alone (Control, C) or containing 200 ng of rainbow trout ghrelin (GHRL), or 1 µmol oleate (OL), or ghrelin + oleate (GHRL + oleate) (GHRL + OL).

	2 h			6 h				
	C	GHRL	OL	GHRL+OL	C	GHRL	OL	GHRL+OL
Fatty acid transport								
FAT/CD36	1.00±0.05*	0.82±0.08 [†]	1.00±0.11* [†]	1.02±0.15* [†]	1.00±0.11* [†]	1.29±0.09*	0.90±0.05 [†]	1.07±0.10* [†]
Fatty acid metabolism								
ACC	1.00±0.11* [†]	1.27±0.04 [†]	0.81±0.06*	1.23±0.12 [†]	1.00±0.09*	1.46±0.13 [†]	0.86±0.12*	0.96±0.14*
ACLY	1.00±0.17* [†]	1.22±0.19*	0.77±0.08 [†]	0.93±0.15* [†]	1.00±0.11*	0.98±0.09*	0.70±0.06 [†]	0.82±0.08* [†]
CPT1c	1.00±0.12	0.93±0.22	1.26±0.17	0.97±0.12	1.00±0.08* [†]	1.11±0.05	0.72±0.06 [†]	0.82±0.07 [†] *
FAS	1.00±0.08*	1.47±0.12 [†]	0.73±0.18*	1.34±0.17 [†]	1.00±0.07* [†] *	1.55±0.11 [†]	0.84±0.13*	1.25±0.08 [†]
Mitochondrial uncoupling								
UCP2a	1.00±0.17*	0.55±0.05 [†]	0.46±0.11 [†]	0.60±0.20*	1.00±0.02	1.00±0.04	0.99±0.02	0.97±0.30
K _{ATP} channel								
Kir6.x-like	1.00±0.10*	1.25±0.07*	0.65±0.14 [†]	1.18±0.24*	1.00±0.02*	1.10±0.15*	0.56±0.04 [†]	0.78±0.15* [†]
Transcription factors								
PPARα	1.00±0.14	1.03±0.09	0.87±0.10	0.91±0.17	1.00±0.13* [†]	1.35±0.12 [†] *	0.91±0.09*	1.24±0.08 [†]
SREBP1c	1.00±0.10	1.25±0.06	1.07±0.14	1.05±0.08	1.00±0.06*	0.78±0.11 [†]	0.85±0.08* [†]	0.68±0.06 [†]
Integrative sensors								
AMPKαpha1	1.00±0.10* [†]	0.90±0.03*	1.25±0.12 [†]	1.25±0.12 [†]	1.00±0.06*	1.39±0.08 [†]	0.70±0.05*	0.82±0.08* [†]
SIRT-1	1.00±0.11*	1.07±0.12*	1.39±0.11 [†]	0.97±0.05*	1.00±0.16*	1.69±0.06 [†]	1.12±0.09*	1.09±0.21*
LPL	1.00±0.20*	0.36±0.12 [†]	0.30±0.06 [†]	0.32±0.05 [†]	1.00±0.11*	0.31±0.15 [†]	0.22±0.03 [†]	0.23±0.12 [†]

Data represent mean ± S.E.M. of six measurements. Gene expression results are referred to control group and are normalized by β-actin expression. Different symbols indicate significant differences ($P < 0.05$) from different groups.

administration in the same species (Librán-Pérez *et al.* 2014a), i.e., increased ACC and FAS expression but absence of changes in FAS and ACLY activities, suggesting a desensitization of this fatty acid-sensing mechanism. Central administration of GHRL also increases ACC expression in the rat brain but decreases FAS expression (López *et al.* 2008, Gao *et al.* 2013), although extended GHRL treatment results in decreased CPT1 activity and no changes in FAS hypothalamic activity (Sangiao-Alvarellos *et al.* 2010). In mammals, the orexigenic effect of GHRL seems to be mediated by increasing AMPK expression that results in decreased hypothalamic concentration of malonyl CoA and stimulation of hypothalamic CPT1 activity (Andrews *et al.* 2008, López *et al.* 2008). We did observe an increase in mRNA abundance of AMPKα1 after 6 h but not the changes predicted in CPT1 expression, suggesting that the action of GHRL could follow a different mechanism than that reported in mammalian species. Moreover, GHRL treatment increases SIRT1 expression after 6 h as it made in rats (Martins *et al.* 2013). These changes in AMPK and SIRT1 could result in inhibition of *de novo* lipogenesis and increased fatty acid oxidation, as described in mammals (Martins *et al.* 2013).

The mechanism related to binding of fatty acid to FAT/CD36 and subsequent modulation of transcription factors was also affected by GHRL treatment inducing decreased mRNA abundance of FAT/CD36 (2 h) and SREBP1c (6 h), i.e., changes again opposed to those described in response to raised levels of oleate or octanoate suggesting an inhibition of this fatty acid-sensing mechanism. In mammals, ICV-injected GHRL also resulted in decreased SREBP1 expression (López *et al.* 2008).

The mechanism related to mitochondrial activity displayed few changes after GHRL treatment since no or only moderate effects were observed on Kir6.x-like and UCP2a expression respectively. In the mammalian hypothalamus, GHRL is known to increase the production of ROS and subsequently induce UCP2a expression (Andrews *et al.* 2008, Gao *et al.* 2013), thus suggesting again a different functional mechanism.

GHRL treatment in the presence of oleate counteracts the effects of oleate on the activation of fatty acid-sensing systems, the expression of hypothalamic neuropeptides and food intake

We hypothesized that the effects of oleate treatment on hypothalamic function and food intake might be counteracted by the simultaneous treatment with GHRL, indicating a modulatory role of this peptide in the metabolic

regulation of food intake. Indeed, the oleate-induced inhibition of food intake levels is counteracted by GHRL administration. To our knowledge, this is the first demonstration for a role of GHRL in fatty acid regulation of feeding. The effects of the joint treatment on food intake are in line with those observed in the expression of hypothalamic neuropeptides. Overall, oleate increased anorexigenic potential by decreasing NPY and AgRP hypothalamic expression and increasing mRNA abundance of POMC and CART.

GHRL treatment also counteracted changes elicited by oleate treatment in several of the fatty acid-sensing mechanisms. Thus, in the fatty acid-sensing mechanism based on fatty acid metabolism, oleate treatment increased levels of fatty acid, triglyceride and total lipid, and this effect was antagonized by GHRL treatment. A similar counteractive effect of GHRL was also observed when studying ACC, ACLY and FAS expression. However, the responses of the parameters related to the fatty acid-sensing systems based on FAT/CD36 were not affected by the simultaneous treatment. The system related to mitochondrial activity displayed in the fish treated with GHRL and oleate values similar to those of controls and different to those observed in the group treated only with oleate, as observed in mRNA abundance of UCP2a and Kir6.x-like. These changes are effectively suggesting that GHRL counteracts the response elicited by oleate at least in two of the fatty acid-sensing systems assessed. As far as we are aware, this kind of experimental approach had not been conducted before, even in mammalian models. Therefore, these results bring up interesting questions regarding the mechanisms through which GHRL binding to GHS-R1a modulates the changes in cellular signalling pathways previously induced by activation of fatty acid-sensing systems. In this way, we recently demonstrated that feeding rainbow trout with a lipid-enriched diet resulted in hypothalamus in the activation of AMPK, mTOR and Akt (Librán-Pérez *et al.* 2015b). These molecules, among others, could be involved in the changes in cellular signalling elicited by fatty acid-sensing systems, which could be also modulated by GHRL action. These counteractive effects of GHRL and fatty acid-sensing mechanisms deserve further study.

In summary, we have demonstrated, for the first time in a non-mammalian vertebrate, that the orexigenic actions of GHRL in fish are associated with changes in fatty acid metabolism and an inhibition of fatty acid-sensing systems in the hypothalamus, which ultimately lead to changes in the expression of anorexigenic and orexigenic peptides, resulting in increased orexigenic

potential and food intake. Moreover, the response to increased levels of oleate of hypothalamic fatty acid-sensing systems (activation), expression of neuropeptides (enhanced anorexigenic potential) and food intake (decrease) were counteracted by the simultaneous treatment with GHRL. These changes provide evidence, again for the first time, of a possible modulatory role of GHRL on the metabolic regulation of food intake occurring in the hypothalamus of fish. The mechanisms observed are similar to those known in mammals in some cases but not in others, suggesting a new model of hypothalamic integration of peripheral signals like levels of metabolites (fatty acid) and hormones (GHRL) involved in the regulation of food intake. Further studies are necessary to elucidate how these peripheral signals are integrated in hypothalamus.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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