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RESEARCH ARTICLE

Ceramide counteracts the effects of ghrelin on the metabolic control of food intake in rainbow trout

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ABSTRACT

In mammals, ceramides are involved in the modulation of the orexigenic effects of ghrelin (GHRL). We previously demonstrated in rainbow trout that intracerebroventricular (ICV) treatment with ceramide (2.5 µg/100 g fish) resulted in an anorexigenic response, i.e. a response opposed to that described in mammals, where ceramide treatment is orexigenic. Therefore, we hypothesized that the putative interaction between GHRL and ceramide must be different in fish. Accordingly, in a first experiment, we observed that ceramide levels in the hypothalamus of rainbow trout did not change after ICV treatment with GHRL. In a second experiment, we assessed whether the effects of GHRL treatment on the regulation of food intake in rainbow trout changed in the presence of ceramide. Thus, we injected ICV GHRL and ceramide alone or in combination to evaluate in hypothalamus and hindbrain changes in parameters related to the metabolic control of food intake. The presence of ceramide generally counteracted the effects elicited by GHRL on fatty acid-sensing systems, the capacity of integrative sensors (AMPK, mTOR and SIRT-1), proteins involved in cellular signalling pathways (Akt and FoxO1) and neuropeptides involved in the regulation of food intake (AgRP, NPY, POMC and CART). The results are discussed in the context of regulation of food intake by metabolic and endocrine inputs.

KEY WORDS: *Oncorhynchus mykiss*, Anorexigenic neuropeptide, Orexigenic neuropeptide, Hypothalamus, Hindbrain

INTRODUCTION

The regulation of food intake occurring in specific brain regions, mainly the hypothalamus, is a complex process in which changes in circulating levels of metabolites and hormones as well as nervous signals are integrated. The result of integration is the production or inhibition of anorexigenic and orexigenic neuropeptides ultimately regulating food intake (Morton et al., 2014). This is a conserved mechanism in vertebrates, as described in mammals (Blouet and Schwartz, 2010) and fish (Volkoff, 2016). The information provided by nutrient-sensing systems is one of the main processes involved in hypothalamic integration (Efeyan et al., 2015), and the mechanisms involved have been partially characterized in fish (Soengas, 2014; Conde-Sieira and Soengas, 2017).

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In previous studies in fish, we demonstrated that in rainbow trout (Librán-Pérez et al., 2012, 2013, 2014, 2015; Velasco et al., 2016a) and Senegalese sole (Conde-Sieira et al., 2015) the hypothalamus detects changes in the levels of specific long-chain fatty acids (LCFAs) through fatty acid-sensing mechanisms. A schematic drawing summarizing five main fatty acid-sensing systems in fish including the enzymes involved (Soengas, 2014; Conde-Sieira and Soengas, 2017) and their effects on food intake is shown in Fig. 1. In response to raised LCFA levels, these systems respond as follows. (1) Increased levels of malonyl-CoA inhibit carnitine palmitoyl transferase-1 (CPT-1), resulting in the inability of mitochondria to import fatty acid-CoA for oxidation. (2) Increased capacity of fatty acid translocase (FAT/CD36) results in changes in the expression of several transcription factors. (3) Activation of specific isoforms of protein kinase C (PKC) results in the inhibition of inward rectifier ATP-dependent K^+ channels (K_{ATP}). (4) Increased capacity of mitochondria to produce reactive oxygen species (ROS) inhibits K_{ATP}. (5) Enhanced lipoprotein lipase (LPL) activity stimulates G-protein-coupled receptors 40 and 120. These mechanisms are, in general, comparable to those described in mammals (Blouet and Schwartz, 2010; Lipina et al., 2014; Morton et al., 2014; Magnan et al., 2015) with the notable exception of the ability of fish systems to detect changes in the levels of not only LCFAs but also medium-chain fatty acids like octanoate (rainbow trout) and polyunsaturated fatty acids like α-linolenate (Senegalese sole). The activation of these systems results in an increase in the anorexigenic potential (balance between mRNA abundance of anorexigenic and orexigenic neuropeptides) through increased production of the anorexigenic peptides pro-opio melanocortin (POMC) and cocaine- and amphetamine-related transcript (CART) and decreased production of the orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), ultimately leading to decreased food intake (Librán-Pérez et al., 2012, 2014; Velasco et al., 2016a).

The function of nutrient-sensing systems and their impact on food intake regulation can be modulated by the action of peripheral hormones in mammals (Blouet and Schwartz, 2010; Morton et al., 2014) and fish (Soengas, 2014; Conde Sieira and Soengas, 2017). One of these hormones is the gastrointestinal peptide ghrelin (GHRL), which binds to mammalian hypothalamus, eliciting an orexigenic response. This response is mediated by changes in fatty acid-sensing systems and neuropeptide expression, reversing changes elicited by raised LCFA levels (Sangiao-Alvarellos et al., 2010; Velásquez et al., 2011; Stark et al., 2015). In fish, GHRL is clearly involved in the control of food intake as an orexigenic factor, as demonstrated in several fish species (Unniappan et al., 2002; Kang et al., 2011; Jönsson, 2013) including rainbow trout (Velasco et al., 2016a). However, other studies suggested an anorexigenic effect of GHRL in rainbow trout (Jönsson et al., 2007, 2010), which could relate to the different time period assessed (1 versus 6–24 h) as well as the increased mRNA abundance of corticotropin-releasing

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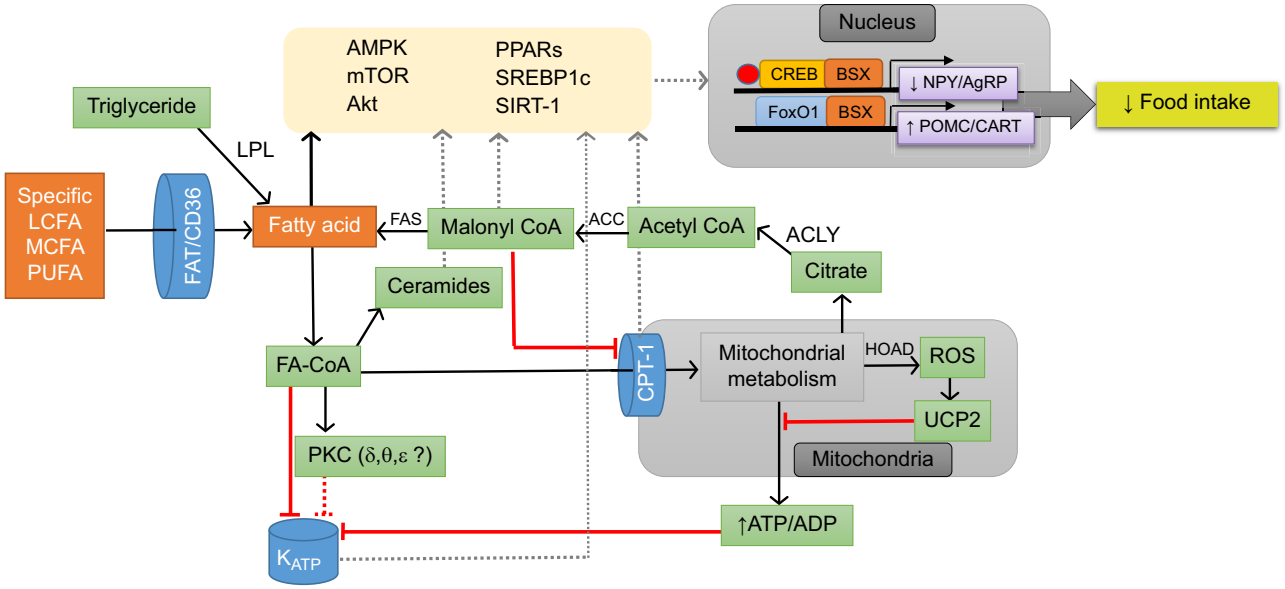

Fig. 1. Fatty acid-sensing systems involved in the control of food intake in fish brain. Black line, activation; grey dotted line, hypothetical activation; red line, inhibition; red dotted line, hypothetical inhibition; ACC, acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; AgRP, agouti-related peptide; Akt, protein kinase B; AMPK, AMP-activated protein kinase; BSX, brain homeobox transcription factor; CART, cocaine- and amphetamine-related transcript; CPT-1, carnitine palmitoyl transferase type 1; CREB, cAMP response element binding protein; FA, fatty acid; FAS, fatty acid synthase; FAT/CD36, fatty acid translocase; FoxO1, forkhead box protein O1; HOAD, hydroxyacyl-CoA dehydrogenase; K_{ATP}, inward rectifier ATP-dependent K^+ channel; LCFA, long-chain fatty acid; LPL, lipoprotein lipase; MCFA, medium-chain fatty acid; mTOR, target of rapamycin; NPY, neuropeptide Y; POMC, pro-opio melanocortin; PPARs, peroxisome proliferator-activated receptors; PKC, protein kinase C; PUFA, poly-unsaturated fatty acid; ROS, reactive oxygen species; SIRT-1, sirtuin 1; SREBP1c, sterol regulatory element binding protein type 1c; UCP2, uncoupling protein 2.

factor observed by Jönsson et al. (2010). Moreover, GHRL is also involved in the control of glucose and lipid metabolism in fish (Kaiya et al., 2009; Salmerón et al., 2015). In a previous study in rainbow trout, we demonstrated that intracerebroventricular (ICV) GHRL treatment counteracts the effects in hypothalamus of raised levels of a LCFA-like oleate on the activity of fatty acid-sensing systems, the expression of neuropeptides and food intake (Velasco et al., 2016a).

In mammals, ceramides – lipids synthesized from fatty acid and sphingosine whose levels increase after feeding on high fat diets (Borg et al., 2012) as well as in obesity (Yang et al., 2009; Aburasayn et al., 2016) – are involved in the control of food intake (Contreras et al., 2014). The orexigenic effect of ceramides is mediated by changes in fatty acid-sensing mechanisms (Gao et al., 2013; Lipina et al., 2014; Picard et al., 2014), integrative sensors such as AMP-activated protein kinase (AMPK) and sirtuin-1 (SIRT-1) (Gao et al., 2011; Ramírez et al., 2013), and cellular signalling pathways such as those mediated by protein kinase B (Akt), target of rapamycin (mTOR) and forkhead box protein O1 (FoxO1) (Bikman and Summers, 2011; Lipina et al., 2014). In fish, the few studies available demonstrated the presence of ceramides in brain (Duan et al., 2010) and their role in development (Fenderson et al., 1992) and apoptosis (Yabu et al., 2001). As for their possible involvement in food intake regulation, in a previous study we demonstrated anorexigenic effects for ceramide through changes in the mRNA abundance of neuropeptides as well as decreased food intake with few changes in the activity of fatty acid-sensing systems (Velasco et al., 2016b).

GHRL treatment in mammals enhances ceramide levels in the hypothalamus (Ramírez et al., 2013), suggesting that ceramides are involved in the modulation of the orexigenic effects of GHRL. As the effects of ceramide in fish were not comparable to those described in mammals (Velasco et al., 2016b), we hypothesize that the putative interaction between GHRL and ceramide must be different in fish. We used rainbow trout as a model as in this species we previously demonstrated the effects of GHRL and ceramides on lipid metabolism and the regulation of food intake (Velasco et al., 2016a,b). Accordingly, in a first experiment, we observed that ceramide levels in the hypothalamus and hindbrain of rainbow trout did not change after ICV treatment with GHRL. In a second experiment, we assessed whether the presence of ceramides might alter the effects of GHRL treatment, through joint treatment of GHRL with ceramide. In the different groups, we evaluated in the hypothalamus and hindbrain changes in parameters related to fatty acid sensing, neuropeptide mRNA abundance and cellular signalling pathways, all of which are involved in the control of food intake in fish (Conde-Sieira and Soengas, 2017). The results obtained suggest a differential pattern of response of these factors compared with the known mammalian models.

MATERIALS AND METHODS

Fish

Rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792), were obtained from a local fish farm (A Estrada, Spain). Fish were maintained for 1 month in 100 l tanks under laboratory conditions and 12 h light:12 h dark photoperiod (lights on at 08:00 h, lights off at 20:00 h) in dechlorinated tap water at 15°C. Fish mass was 98±2 g. Fish were fed once daily (10:00 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^{-1} 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.

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Experimental design

Experiment 1: effects of ICV administration of oleate and/or GHRL on levels of ceramide in fish brain areas

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 the experiment, fish were lightly anaesthetized with 2-phenoxyethanol (Sigma Chemical Co., St Louis, MO, USA; 0.2% v/v), and weighed to carry out ICV administration as previously described (Polakof and Soengas, 2008). Briefly, fish were placed on a Plexiglas board with Velcro straps adjusted to hold them in place. A 29.5 gauge needle attached through a polyethylene cannula to a 10 µl Hamilton syringe was aligned with the 6th preorbital 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^{-1} body mass of vehicle alone (control, n=7) or containing 200 ng of rainbow trout octanoylated 23 amino acid GHRL (n=7, synthesized by Bachem, Bubendorf, Switzerland, according to the sequence published by Kaiya et al., 2003), 1 µmol oleate (n=7, Sigma Chemical Co.) or GHRL+oleate (n=7). The vehicle used, saline–hydroxypropyl-β-cyclodextrin (HPB), was a mixture (1:1 in vol.) of Hanks' saline and a solution of 45% HPB in Hanks' saline to a final concentration of 17 mmol l^{-1} (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). After 2 h, fish were lightly anaesthetized with 2-phenoxyethanol (0.2% v/v) and killed by decapitation. The hypothalamus, hindbrain and midbrain were taken, snap-frozen, and stored at −80°C for further quantification of ceramide levels (see below).

Experiment 2: effects of ICV administration of GHRL, ceramide and GHRL+ceramide on parameters related to regulation of food intake by lipid metabolism

Fish were fasted for 24 h before treatment to ensure basal hormone levels were achieved. On the day of the experiment, fish were lightly anaesthetized with 2-phenoxyethanol (0.2% v/v), weighed and ICV injected as described above with 1 µl 100 g^{-1} body mass of DMSO-saline (1:3) alone (control, n=21) or containing 200 ng of rainbow trout octanoylated 23 amino acid GHRL, 2.5 µg of C6:0 ceramide (*N*-hexanoyl-D-sphingosine; Sigma; n=21) or GHRL+ceramide (n=21). After 6 h, fish were anaesthetized with 2-phenoxyethanol (0.2% v/v). The time and doses of GHRL and ceramide were selected based on previous studies carried out in rainbow trout (Velasco et al., 2016a,b). Blood was collected by caudal puncture with ammonium-heparinized syringes, and plasma samples were obtained after blood centrifugation, deproteinized immediately (using 0.6 mol l^{-1} perchloric acid) and neutralized (using 1 mol l^{-1} potassium bicarbonate) before freezing in liquid nitrogen and storage at −80°C until further assay. Fish were killed by decapitation, and the hypothalamus and hindbrain were taken, snap-frozen and stored at −80°C. Nine fish per group were used to assess enzyme activities and metabolite levels, six fish per group were used for the assessment of mRNA levels by reverse transcription-quantitative PCR (RT-qPCR), and the remaining six fish per group were used in western blots to assess changes in the levels of proteins involved in cellular signalling.

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, glucose and lactate).

Samples used to assess metabolite levels in brain areas were homogenized immediately by ultrasonic disruption in 7.5 vol. of ice-cooled 0.6 mol l^{-1} perchloric acid, and neutralized as described above for plasma samples. 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 used to assess enzyme activity were homogenized by ultrasonic disruption with 9 vol. of ice-cold buffer consisting of 50 mmol l^{-1} Tris (pH 7.6), 5 mmol l^{-1} EDTA, 2 mmol l^{-1} 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 activity was determined using a microplate reader (INFINITE 200 Pro, Tecan, Männedorf, Switzerland). Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm or, in the case of CPT-1 activity, of 5,5′-dithiobis(2-nitrobenzoic acid)-CoA complex at 412 nm. The reactions were started by the addition of supernatant (15 µl) at a pre-established protein concentration (Polakof et al., 2008; Librán-Pérez et al., 2012), omitting the substrate in control wells (final volume 265–295 µl), and reactions were allowed to proceed at 20°C for pre-established times (3–10 min). Enzyme activity was normalized to protein levels (mg). Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method with bovine serum albumin (Sigma) as standard. ATP-citrate lyase (ACLY, EC 4.1.3.8), hydroxyacil-CoA dehydrogenase (HOAD, EC 1.1.1.35), fatty acid synthase (FAS, EC 2.3.1.85) and CPT-1 (EC 2.3.1.21) activity was determined following available methods (Alvarez et al., 2000; Kolditz et al., 2008; Polakof et al., 2011; Ditlecadet and Driedzic, 2013, respectively). Briefly, ACLY activity was assessed in a Tris-HCl buffer (50 mmol l^{-1}, pH 7.8) containing 100 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} citrate, 10 mmol l^{-1} β-mercaptoethanol, 5 mmol l^{-1} ATP, 0.3 mmol l^{-1} NADH, 7 U ml^{-1} malate dehydrogenase and 50 µmol l^{-1} coenzyme A (omitted for controls). HOAD activity was assessed in imidazole buffer (50 mmol l^{-1}, pH 7.6) containing 0.15 mmol l^{-1} NADH and 3.5 mmol l^{-1} acetoacetyl-CoA (omitted for controls). FAS activity was assessed in a phosphate buffer (100 mmol l^{-1}, pH 7.6) containing 0.1 mmol l^{-1} NADPH, 25 µmol l^{-1} acetyl-CoA and 30 µmol l^{-1} malonyl-CoA (omitted for controls). CPT-1 activity was assessed in a Tris-HCl buffer (75 mmol l^{-1}, pH 8.0) containing 1.5 mmol l^{-1} EDTA, 0.25 mmol l^{-1} DTNB, 35 µmol l^{-1} palmitoyl CoA and 0.7 mmol l^{-1} L-carnitine (omitted for controls).

mRNA abundance analysis by RT-qPCR

Total RNA was extracted using Trizol reagent (Life Technologies, Grand Island, NY, USA) and subsequently treated with RQ1-DNAse (Promega, Madison, WI, USA). A 2 µg sample of total RNA was reverse transcribed using Superscript II reverse transcriptase (Promega) and random hexamers (Promega) in a volume of ∼20 µl. Gene expression levels were determined by RT-qPCR using the iCycler iQ (Bio-Rad, Hercules, CA, USA). Analyses were performed on 1 µl cDNA using MAXIMA SYBR Green qPCR Mastermix (Life Technologies), in a total PCR reaction volume of 15 µl, containing 50–500 nmol l^{-1} of each primer. mRNA abundance of transcripts was determined as previously described in the same species by Leder and Silverstein (2006; POMCa1), Kolditz et al. (2008; FAS, LPL and PPARα), Polakof et al. (2010; ACLY),

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Conde-Sieira et al. (2010; CART, NPY), Polakof et al. (2011; CPT1c), Sánchez-Gurmaches et al. (2012; FAT/CD36 and PPARγ), Figueiredo-Silva et al. (2012; UCP2a), Craig and Moon (2013; AMPKα1), MacDonald et al. (2014; AgRP) and Rolland et al. (2015; mTOR). 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 a 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 PCR 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^{-1} 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.

Western blot analysis

The expression of selected phosphorylated and unphosphorylated proteins was analysed in the hypothalamus and hindbrain of fish in experiment 2. Frozen samples (20 mg) were homogenized in 1 ml of buffer containing 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA (pH 7.4), 100 mmol l^{-1} sodium fluoride, 4 mmol l^{-1} sodium pyrophosphate, 2 mmol l^{-1} sodium orthovanadate, 1% Triton X-100, 0.5% NP40-IGEPAL and 1.02 mg ml^{-1} protease inhibitor cocktail (Sigma). Tubes were kept on ice during the whole process to prevent protein denaturation. Homogenates were centrifuged at 1000 ***g*** for 15 min at 4°C, and supernatants were again centrifuged at 20,000 ***g*** for 30 min. The resulting supernatants were recovered and stored at −80°C. The concentration of protein in each sample was determined using the Bradford assay with bovine serum albumin as standard. Hypothalamus and hindbrain protein lysates (20 µg) were used in western blotting with appropriate antibodies. Anti-phospho Akt (Ser473), anti-carboxyl terminal Akt, anti-phospho AMPK (Thr172), anti-AMPK, anti-phospho-FoxO1 (Thr24) and anti-FoxO1 (L27) antibodies were used (Cell Signaling Technology, Saint Quentin Yvelings, France). All these antibodies cross-react successfully with rainbow trout proteins of interest (Skiba-Cassy et al., 2009; Kamalam et al., 2012; Velasco et al., 2016b). After washing, membranes were incubated with an IgG-HRP secondary antibody (Bio-Rad) and spots were quantified by Image Lab software version 5.2.1 (Bio-Rad).

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

	Forward/reverse primer	Annealing temp. (°C)	Database	Accession no.
Housekeeping				
β-actin	F: GATGGGCCAGAAAGACAGCTA R: TCGTCCCAGTTGGTGACGAT	59	GenBank	NM_ 001124235.1
Fatty acid sensing				
ACLY	F: CTGAAGCCCAGACAAGGAAG R: CAGATTGGAGGCCAAGATGT	60	GenBank	CA349411.1
CPT-1c	F: CGCTTCAAGAATGGGGTGAT R: CAACCACCTGCTGTTTCTCA	59	GenBank	AJ619768
FAS	F: GAGACCTAGTGGAGGCTGTC R: TCTTGTTGATGGTGAGCTGT	59	Sigenae	tcab0001c.e.06 5.1.s.om.8
FAT/CD36	F: CAAGTCAGCGACAAACCAGA R: ACTTCTGAGCCTCCACAGGA	62	DFCI	AY606034.1
LPL	F: TAATTGGCTGCAGAAAACAC R: CGTCAGCAAACTCAAAGGT	59	GenBank	AJ224693
PPARα	F: CTGGAGCTGGATGACAGTGA R: GGCAAGTTTTTGCAGCAGAT	55	GenBank	AY494835
PPARγ	F: GACGGCGGGTCAGTACTTTA R: ATGCTCTTGGCGAACTCTGT	60	DFCI	CA345564
UCP2a	F: TCCGGCTACAGATCCAGG R: CTCTCCACAGACCACGCA	57	GenBank	DQ295324
Integrative sensors				
AMPKα1	F: ATCTTCTTCACGCCCCAGTA R: GGGAGCTCATCTTTGAACCA	60	GenBank	HQ40367
mTOR	F: ATGGTTCGATCACTGGTCATCA R: TCCACTCTTGCCACAGAGAC	60	GenBank	EU179853
SIRT-1	F: GCTACTTGGGGACTGTGACG R: CTCAAAGTCTCCGCCCAAC	57	GenBank	EZ774344.1
Neuropeptides				
AgRP	F: ACCAGCAGTCCTGTCTGGGTAA R: AGTAGCAGATGGAGCCGAACA	60	GenBank	CR376289
CART	F: ACCATGGAGAGCTCCAG R: GCGCACTGCTCTCCAA	60	GenBank	NM_001124627
NPY	F: CTCGTCTGGACCTTTATATGC R: GTTCATCATATCTGGACTGTG	58	GenBank	NM_001124266
POMC-A1	F: CTCGCTGTCAAGACCTCAACTCT R: GAGTTGGGTTGGAGATGGACCTC	60	Tigr	TC86162

ACLY, ATP-citrate lyase; AMPKα1, AMP-activated protein kinase α1; AgRP, agouti-related peptide; CART, cocaine- and amphetamine-related transcript; CPT-1c, carnitine palmitoyl transferase type 1c; FAS, fatty acid synthetase; FAT/CD36, fatty acid translocase; LPL, lipoprotein lipase; mTOR, mammalian target of rapamycin; NPY, neuropeptide Y; POMC-A1, pro-opio melanocortin A1; PPARα, peroxisome proliferator-activated receptor type α; PPARγ, peroxisome proliferator-activated receptor type γ; SIRT-1, sirtuin 1; UCP2a, mitochondrial uncoupling protein 2a.

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Ceramide analysis using UHPLC-MS/MS

Ceramides were extracted from tissues (~20 mg) using a two-phase extraction method (Folch et al., 1957) and C18 ceramide levels were quantified via UHPLC-MS/MS. Water and methanol (LC/MS grade) were purchased from Panreac (Barcelona, Spain). Formic acid (98–100% purity) and ammonium formate (≥99% purity) and C18 ceramide (*N*-stearoyl-D-sphingosine) were purchased from Sigma.

Chromatographic separation was carried out using a 1290 Infinity UHPLC system coupled to an Agilent G6460A triple quadrupole mass spectrometer equipped with a Jet Stream ESI source (Agilent Technologies, Germany). Ceramide was separated using a Zorbax SB-C8 Rapid Resolution High Definition (Agilent Technologies; 2.1×50 mm, 1.8 µm) at 40°C. Mobile phase A was methanol:water: formic acid (74:25:1 v/v) and phase B was methanol:formic acid (99:1 v/v); both contained 2 mmol l^{-1} ammonium formate. A gradient programme with a flow rate of 0.4 ml min^{-1} was run starting with 2 min isocratic at 50% B, followed by a linear gradient to 56% B in 7 min and then an isocratic at 100% B in 3 min. After an isocratic hold time of 3 min at 50% B in order to equilibrate the column at initial chromatographic conditions prior to the next injection, a 5 µl amount of the diluted sample extract was injected into UHPLC-MS/MS System. The samples in the autosampler were cooled to 4°C.

The MS system source and interface were operated in positive ionization mode. The capillary voltage was set to 5 kV. A drying gas flow of 10 l min^{-1} at a temperature of 300°C, a nebulizer gas pressure of 50 psi (Nitrogen Generator System, Zefiro40, Evry, France) and a sheath gas (nitrogen 99.999% pure, Airliquide, Porriño, Spain) flow of 11 l min^{-1} at a temperature of 400°C were chosen. Three multiple reaction monitoring transitions were set up for ceramide analysis (*m*/*z* 566.6→548.6; 566.6→530.6; 566.6→254.6) with a collision energy of 8, 12 and 24 eV, respectively, a fragmentor voltage of 100 V and a dwell time of 20 ms. The transition with the higher intensity (*m*/*z* 566.6→264.4) was used for quantification, while the transition with the lower intensity (566.6→530.6) was used for confirmation purposes.

Statistics

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

RESULTS

Experiment 1

In the hypothalamus (Fig. 2A), levels of ceramide decreased in groups treated with oleate and GHRL+oleate compared with controls. In the hindbrain (Fig. 2B), levels of ceramide were lower in fish treated with oleate than in fish treated with GHRL. No significant changes were observed in the midbrain (Fig. 2C).

Experiment 2

No significant effects were observed in plasma levels of fatty acid, triglyceride, total lipid, glucose and lactate after any treatment (data not shown).

Levels of metabolites in the hypothalamus and hindbrain are shown in Fig. 3. Levels of fatty acid in the hypothalamus increased after treatment with ceramide and GHRL+ceramide compared with the control group (Fig. 3A). Triglyceride levels in the hypothalamus increased in fish treated with GHRL+ceramide compared with control and GHRL groups; levels in fish treated with ceramide were also higher than those in fish treated with GHRL (Fig. 3B). Total lipid levels in the hypothalamus (Fig. 3C) increased in fish treated with ceramide or GHRL+ceramide compared with the remaining groups, whereas levels decreased in the GHRL-treated group compared with controls. In the hindbrain, no significant changes were noted in fatty acid (Fig. 3D) and total lipid (Fig. 3F) levels, whereas triglyceride levels in the hindbrain were higher in fish treated with ceramide than in fish treated with GHRL (Fig. 3E).

Enzyme activities are shown in Fig. 4. CPT-1 activity in the hypothalamus decreased in GHRL and GHRL+ceramide groups compared with the control group (Fig. 4A). FAS activity in the hypothalamus was lower in the GHRL-treated group compared with the other groups (Fig. 4B). No significant changes occurred in ACLY activity in the hypothalamus (Fig. 4C). In the hindbrain, CPT-1 activity (Fig. 4D) decreased in the GHRL-treated group compared with the control and ceramide groups. FAS activity in the hindbrain decreased in the GHRL+ceramide group compared with the other groups (Fig. 4E). ACLY activity in the hindbrain (Fig. 4F) was lower in the GHRL group compared with the other groups and higher in the ceramide-treated group compared with the control and GHRL groups. Finally, HOAD activity in the hindbrain (Fig. 4G) was lower in all treated groups than in controls.

The mRNA abundance of neuropeptides is shown in Fig. 5. POMC-A1 mRNA levels in the hypothalamus (Fig. 5A) were

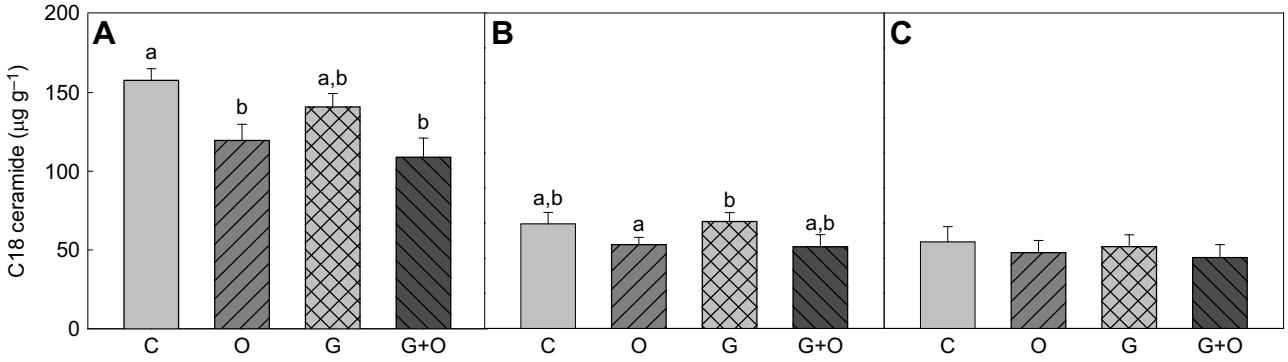

Fig. 2. Ceramide levels in brain of rainbow trout after ghrelin (GHRL) and/or oleate treatment. Levels of C18 ceramide in the hypothalamus (A), hindbrain (B) and midbrain (C) of rainbow trout 2 h after intracerebroventricular (ICV) administration of 1 µl 100 g^{-1} body mass of saline–hydroxypropyl-β-cyclodextrin (HPB) alone (control, C) or containing 200 ng of rainbow trout GHRL (G) or 1 µmol oleate (O), or GHRL+oleate (G+O). Each value is the mean+s.e.m. of $n=7$ fish per treatment. Different letters indicate significant differences ($P<0.05$) between groups.

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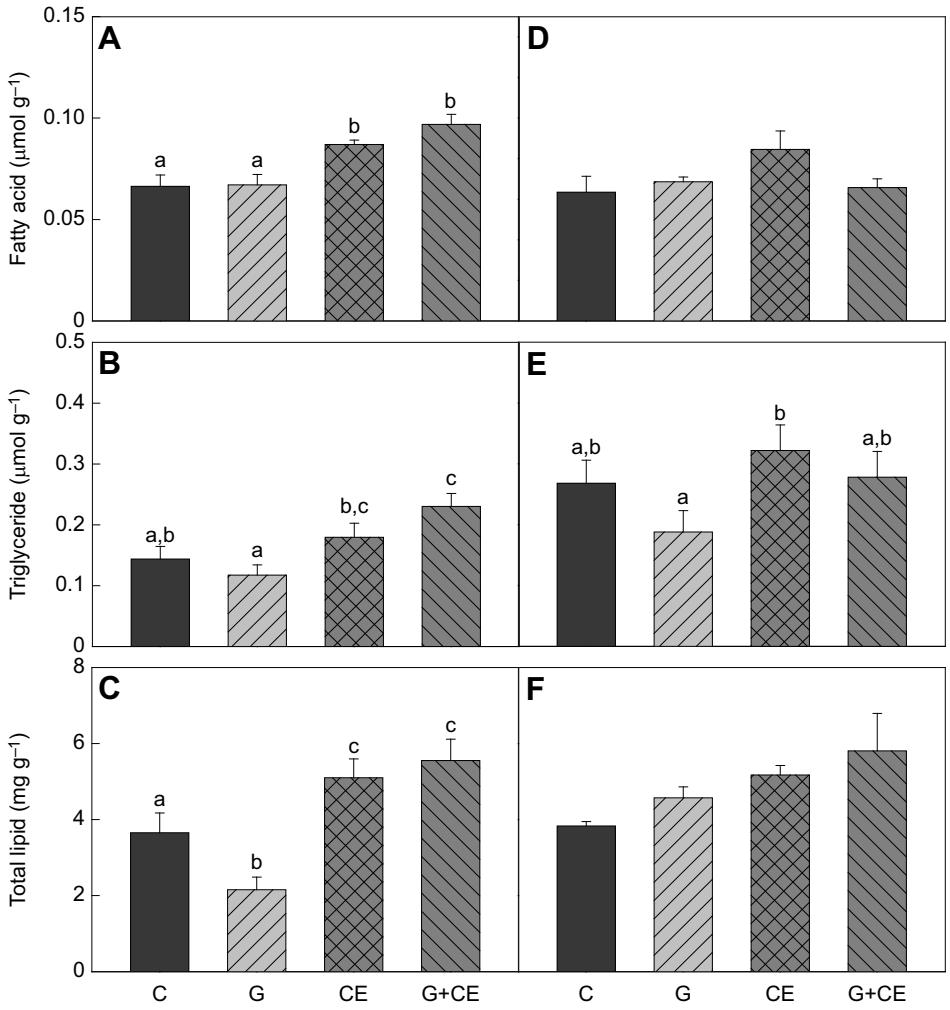

Fig. 3. Metabolite levels in the hypothalamus and hindbrain of rainbow trout after GHRL and/or ceramide treatment. Levels of fatty acid (A,D), triglyceride (B,E) and total lipid (C,F) in the hypothalamus (A–C) and hindbrain (D–F) of rainbow trout 6 h after ICV administration of 1 µl 100 g^{-1} body mass of DMSO-saline (1:3) alone (control, C) or containing 200 ng of rainbow trout GHRL (G), 2.5 µg of C6:0 ceramide (CE), or GHRL+ceramide (G+CE). Each value is the mean+s.e.m. of n=9 fish per treatment. Different letters indicate significant differences (P<0.05) between groups.

higher in the ceramide-treated group than in the other groups; the levels of GHRL-treated groups (alone or in the presence of ceramide) were lower than in the other groups. CART mRNA levels in the hypothalamus were lower in the GHRL group than in the control and ceramide groups, and the levels in the ceramide group were higher than in the other groups (Fig. 5B). AgRP mRNA levels in the hypothalamus (Fig. 5C) were higher in the GHRL+ceramide group than in all other groups; the levels in the GHRL group were also higher than those of the control and ceramide groups. NPY mRNA levels in the hypothalamus (Fig. 5D) increased in the GHRL-treated group compared with the other groups, whereas the levels in the ceramide group were lower than those in control. In the hindbrain, levels of POMC-A1 mRNA (Fig. 5E) decreased in the GHRL group compared with the control and ceramide groups, and levels in the ceramide group were higher than those of the other groups. CART mRNA levels in the hindbrain (Fig. 5F) decreased in the group treated with GHRL compared with the control and ceramide groups, and levels in the ceramide group were higher than those in the other groups. AgRP mRNA levels in the hindbrain (Fig. 5G) increased in GHRL-treated fish compared with control and ceramide groups. Finally, NPY mRNA levels in the hindbrain (Fig. 4H) were higher in GHRL-treated groups than in the other groups and levels in the GHRL+ceramide group were lower than those of control.

The mRNA abundance of parameters related to fatty acid transport and metabolism is shown in Fig. 6. FAT/CD36 mRNA levels in the hypothalamus were higher in the GHRL group than in control (Fig. 6A). No significant changes were observed in ACLY mRNA levels in the hypothalamus (Fig. 6B) or hindbrain (Fig. 6F). The mRNA levels of CPT-1c in the hypothalamus (Fig. 6C) decreased in fish treated with ceramide compared with fish treated with GHRL. The mRNA levels of FAS in the hypothalamus (Fig. 6D) in GHRL and GHRL+ceramide groups were higher than those of the ceramide group. FAT/CD36 mRNA levels in the hindbrain of the ceramide and GHRL+ceramide groups were lower than those of the control and GHRL groups (Fig. 6E). mRNA levels of CPT-1c in the hindbrain (Fig. 6G) decreased in GHRL, ceramide and GHRL+ceramide groups compared with controls. FAS values in the hindbrain decreased in the GHRL-treated group compared with the control group (Fig. 6H).

The mRNA abundance of additional parameters involved in lipid sensing is shown in Fig. 7. The mRNA levels of LPL in the hypothalamus (Fig. 7A) decreased in fish treated with GHRL compared with all other groups, whereas values in fish treated with ceramide were higher than in the other groups. The mRNA levels of UCP2a in the hypothalamus increased in the ceramide-treated group compared with the control and GHRL groups (Fig. 7B). The mRNA levels of PPARα in the hypothalamus increased in the GHRL +ceramide group compared with control (Fig. 7C). No significant changes occurred in PPARγ mRNA levels in the hypothalamus (Fig. 7D). LPL mRNA levels in the hindbrain (Fig. 7E) decreased in the GHRL group compared with the control and ceramide groups;

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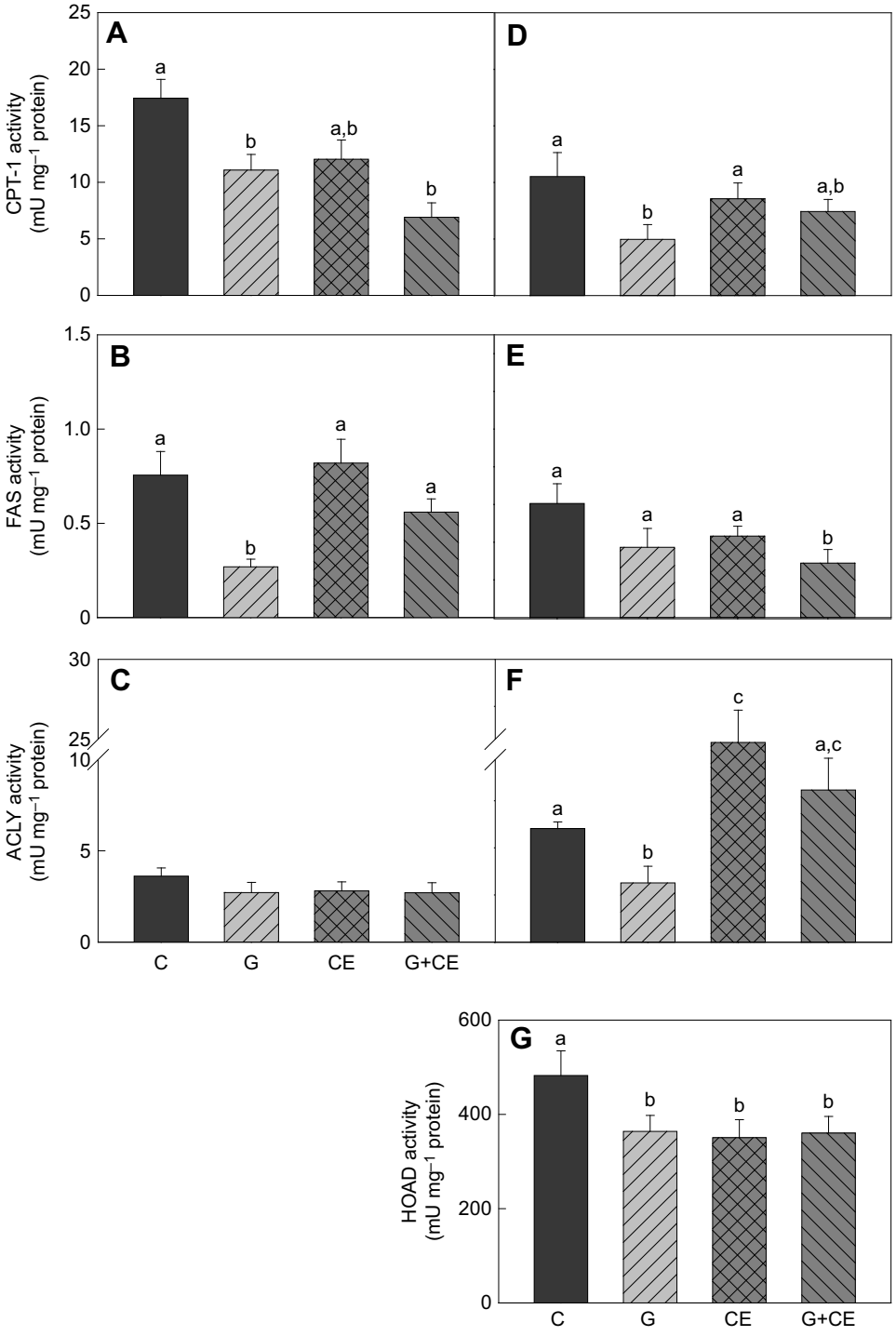

Fig. 4. Enzyme activity in the hypothalamus and hindbrain of rainbow trout after GHRL and/or ceramide treatment. Activity of CPT-1 (A,D), FAS (B,E), ACLY (C,F) and HOAD (G) in the hypothalamus (A–C) and hindbrain (D–G) of rainbow trout 6 h after ICV administration of 1 µl 100 g^{-1} body mass of DMSO-saline (1:3) alone (control, C), or containing 200 ng of rainbow trout GHRL (G), 2.5 µg of C6:0 ceramide (CE) or GHRL+ceramide (G+CE). Each value is the mean+s.e.m. of n=9 fish per treatment. Different letters indicate significant differences (P<0.05) between groups.

furthermore, mRNA levels of the ceramide group were higher than those of the other groups. The mRNA levels of UCP2a in the hindbrain (Fig. 7F) in the GHRL+ceramide group were higher than those of the control and GHRL group; mRNA levels of the ceramide group were higher than those of control. No significant changes occurred in mRNA levels of PPARα in the hindbrain (Fig. 7G). Finally, mRNA levels of PPARγ in the hindbrain (Fig. 7H) decreased in fish treated with ceramide compared with fish treated with GHRL.

The mRNA abundance of integrative sensors is shown in Fig. 8. The AMPKα1 mRNA levels in the hypothalamus (Fig. 8A) increased in the GHRL group compared with the control and ceramide groups. No significant changes were observed in SIRT-1 mRNA in the hypothalamus (Fig. 8B). The mRNA levels of mTOR in the hypothalamus (Fig. 8C) of the ceramide-treated group were higher than those of the control and GHRL groups. The levels of AMPKα1 mRNA in hindbrain (Fig. 8D) decreased in the group treated with ceramide compared with the control and GHRL groups. The mRNA levels of SIRT-1 in the hindbrain (Fig. 8E) decreased in the ceramide group compared with the control group. Finally, the mRNA levels of mTOR in the hindbrain were lower in the GHRL+ceramide group than in controls (Fig. 8F).

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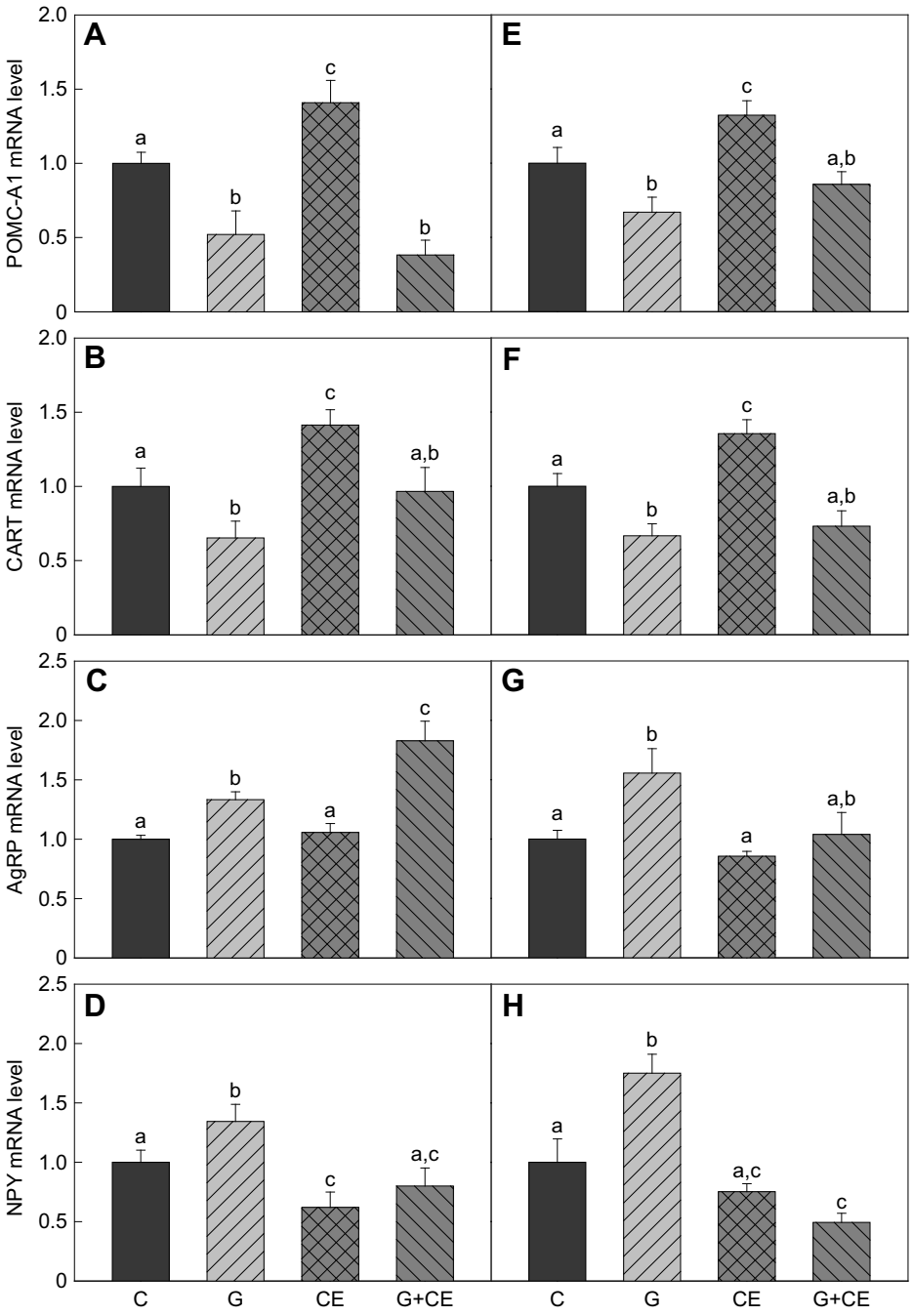

Fig. 5. mRNA levels of neuropeptides in the hypothalamus and hindbrain of rainbow trout after GHRL and/or ceramide treatment. Levels of POMC-A1 (A,E), CART (B,F), AgRP (C,G) and NPY (D,H) in the hypothalamus (A–D) and hindbrain (E–H) of rainbow trout 6 h after ICV administration of 1 µl 100 g^{-1} body mass of DMSO-saline (1:3) alone (control, C) or containing 200 ng of rainbow trout GHRL (G), 2.5 µg of C6:0 ceramide (CE) or GHRL+ceramide (G+CE). Each value is the mean+s.e.m. of n=6 fish per treatment. Gene expression results are referred to the control group and are normalized by β-actin expression. Different letters indicate significant differences (P<0.05) between groups.

The phosphorylation state of proteins is shown in Fig. 9. In the hypothalamus, the phosphorylation state of Akt (Fig. 9A) was lower in the GHRL group than in the control and ceramide groups, whereas the value for the ceramide group was also higher than that of control. The phosphorylation state of AMPK (Fig. 9B) was lower in the GHRL group than in the ceramide group. FoxO1 phosphorylation state in the hypothalamus (Fig. 9C) was lower in the GHRL group than in the ceramide group and the value in the ceramide-treated group was also higher than that of the control group. In the hindbrain, the phosphorylation state of AMPK (Fig. 9E) of the GHRL-treated group was lower than that of the control and ceramide-treated group, whereas the value of the ceramide-treated group was also higher than that of the control group. No significant changes were observed in the phosphorylation state of Akt (Fig. 9D) and FoxO1 (Fig. 9F) in the hindbrain.

DISCUSSION

GHRL treatment does not affect ceramide levels in brain lipid-sensing areas

Ceramide levels decreased after treatment with oleate in the hypothalamus whereas a non-significant decrease occurred in the hindbrain. In contrast, no significant changes occurred in the midbrain. We had selected the midbrain as a negative control because this region in mammals is not involved in the metabolic control of food intake (Blouet and Schwartz, 2010) and the results obtained support such a contention. The effects of oleate in the hypothalamus have never been observed before in any fish species and support the role of monounsaturated fatty acids like oleate in decreasing the production of lipid derivatives such as ceramides (Bergouignan et al., 2009). No changes in ceramide levels occurred in any of the regions assessed in rainbow trout after GHRL treatment

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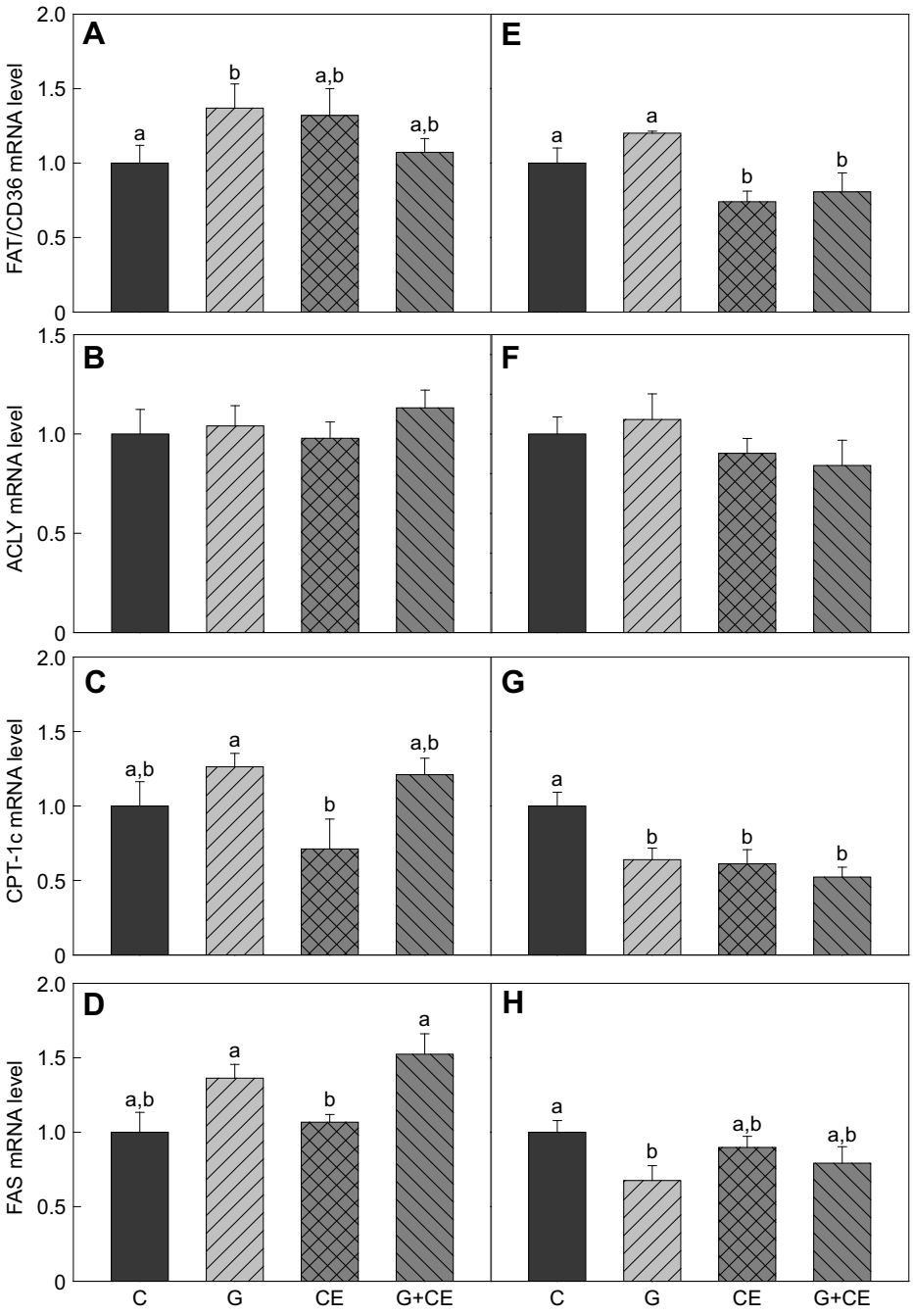

Fig. 6. mRNA levels of parameters related to fatty acid transport and metabolism in the hypothalamus and hindbrain of rainbow trout after GHRL and/or ceramide treatment. Levels of FAT/CD36 (A,E), ACLY (B,F), CPT-1c (C,G) and FAS (D,H) in the hypothalamus (A–D) and hindbrain (E–H) of rainbow trout 6 h after IVC administration of 1 µl 100 g^{-1} body mass of DMSO-saline (1:3) alone (control, C) or containing 200 ng of rainbow trout GHRL (G), 2.5 µg of C6:0 ceramide (CE) or GHRL +ceramide (G+CE). Each value is the mean+s.e.m. of n=6 fish per treatment. Gene expression results are referred to the control group and are normalized by β-actin expression. Different letters indicate significant differences (P<0.05) between groups.

in the present study. In fact, it is presumably the presence of oleate that induced the decrease in ceramide levels in the hypothalamus of the GHRL+oleate group. These results suggest a different relationship between ceramide and GHRL in fish compared with mammals (Ramírez et al., 2013), such that ceramide in fish is presumably not involved in mediating the effects of GHRL. We have no explanation for this differential behaviour, which could relate to the different life history of fish.

The effect of GHRL in rainbow trout is not mediated by changes in cellular signalling comparable to those characterized in mammals

The treatment with GHRL alone induced in the hypothalamus the activation of different fatty acid-sensing systems related to fatty acid metabolism (decreased activity of FAS and CPT-1), transport through FAT/CD36 (increased mRNA abundance) and LPL (decreased mRNA abundance). These changes support the activation of fatty acid-sensing systems by GHRL treatment, and most of them are in agreement with a previous study carried out in the same species under similar experimental conditions (Velasco et al., 2016a). The activation of these systems is also in agreement with the increased orexigenic potential observed simultaneously, as supported by decreased mRNA abundance of POMC and CART and increased mRNA abundance of NPY and AgRP, in a way comparable to that reported in mammals (Ramírez et al., 2013). This orexigenic potential is also in agreement with similar changes observed in a previous study in which increased food intake occurred in rainbow trout after ICV GHRL treatment (Velasco et al., 2016a).

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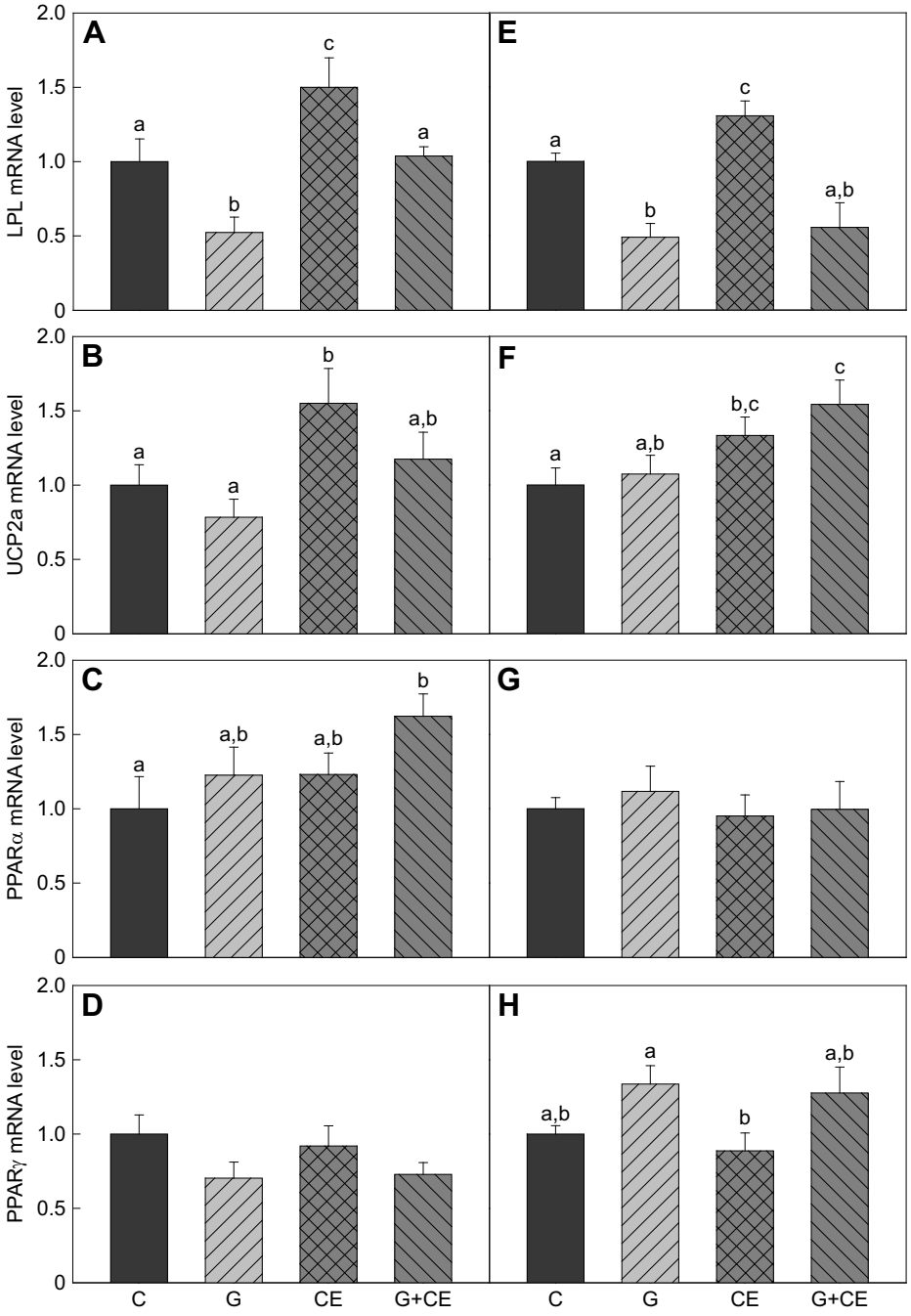

Fig. 7. mRNA levels of additional parameters involved in lipid sensing in the hypothalamus and hindbrain of rainbow trout after GHRL and/or ceramide treatment. Levels of LPL (A,E), UCP2a (B,F), PPARα (C,G) and PPARγ (D,H) in the hypothalamus (A–D) and hindbrain (E–H) of rainbow trout 6 h after ICV administration of 1 µl 100 g^{-1} body mass of DMSO-saline (1:3) alone (control, C) or containing 200 ng of rainbow trout GHRL (G), 2.5 µg of C6:0 ceramide (CE) or GHRL+ ceramide (G+CE). Each value is the mean+ s.e.m. of *n*=6 fish per treatment. Gene expression results are referred to the control group and are normalized by β-actin expression. Different letters indicate significant differences (P<0.05) between groups.

We also assessed the impact of GHRL on the function of integrative sensors, such as AMPK, mTOR and SIRT-1 (Florant and Healy, 2012; Hardie and Ashford, 2014; Nillni, 2016). The increase in mRNA abundance of AMPKα1 in the hypothalamus is comparable to that observed before (Velasco et al., 2016a) but we had not previously assessed the effects of GHRL on the phosphorylation state of AMPK. The absence of changes in phosphorylation state is different from the increase observed in mammals after GHRL treatment (Ramírez et al., 2013; Müller et al., 2015). Moreover, in mammals, activated AMPK decreases FAS mRNA abundance in the hypothalamus (Diéguez et al., 2009). In the present study, the absence of changes in AMPK phosphorylation state also coincided with the lack of changes in FAS mRNA abundance. As far as we are aware, this is the first study assessing in fish the effects of GHRL on other integrative sensors such as mTOR and SIRT-1, and we observed no changes in their mRNA abundance in the hypothalamus after GHRL treatment. This is in contrast with the increase observed in mammals (Velásquez et al., 2011; Martins et al., 2012).

We also assessed, for the first time in fish, changes in the phosphorylation state of two proteins involved in cellular signalling pathways: Akt and FoxO1. The absence of changes in FoxO1 is clearly different from the increase observed in mammalian hypothalamus after GHRL treatment (Ramírez et al., 2013). As for Akt, there is no evidence available in mammalian hypothalamus regarding the effects of GHRL on phosphorylation state but in other tissues an increase has been reported (Soares et al., 2012; Liang et al., 2013). Considering that FoxO1 is involved in the modulation of neuropeptide transcription (Diéguez et al., 2011), it is very

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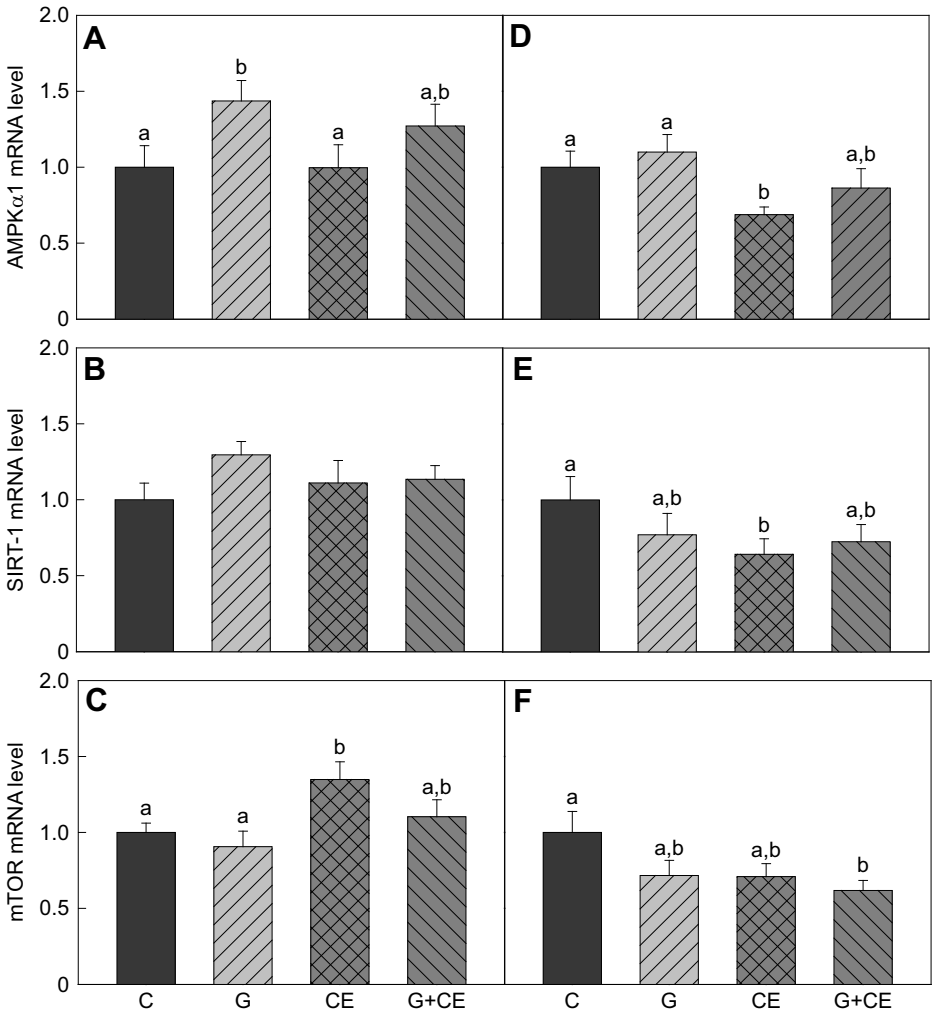

Fig. 8. mRNA levels of integrative sensors in the hypothalamus and hindbrain of rainbow trout after GHRL and/or ceramide treatment. Levels of AMPKα1 (A,D), SIRT-1 (B,E) and mTOR (C,F) in the hypothalamus (A–C) and hindbrain (D–F) of rainbow trout 6 h after ICV administration of 1 µl 100 g^{-1} body mass of DMSO-saline (1:3) alone (control, C) or containing 200 ng of rainbow trout octanoylated 23 amino acid GHRL (G), 2.5 µg of C6:0 ceramide (CE) or GHRL+ceramide (G+CE). Each value is the mean+s.e.m. of n=6 fish per treatment. Gene expression results are referred to the control group and are normalized by β-actin expression. Different letters indicate significant differences (P<0.05) between groups.

interesting that the effects of GHRL on neuropeptide mRNA abundance (increased orexigenic potential) occurred both in mammals and in rainbow trout despite the fact that changes in signalling pathways were different.

There is no available evidence, even in mammalian models, about the action of GHRL in brain areas other than the hypothalamus that are possibly involved in the metabolic control of food intake, such as the hindbrain. In the present study, we observed in GHRL-treated fish responses in the hindbrain in parameters involved in different fatty acid-sensing systems such as those mediated by fatty acid metabolism (decreased activity of CPT-1 and ACLY and decreased mRNA abundance of CPT1c and FAS) and LPL (decreased mRNA abundance). These changes are comparable to those observed in the hypothalamus. The mRNA abundance of neuropeptides in the hindbrain after GHRL treatment also displayed changes comparable to those in the hypothalamus, supporting an orexigenic response. A clear decrease in the phosphorylation state of AMPK occurred in the hindbrain but not in the hypothalamus, whereas the decrease in phosphorylation state of Akt in the hypothalamus was not observed in the hindbrain. As a whole, comparable changes in the activity of fatty acid-sensing systems, but not at the level of integrative sensors and cellular signalling pathways, elicit a similar orexigenic response in the hypothalamus and hindbrain. The differences between the two areas may arise because the hindbrain, besides being involved in the control of food intake, is also involved in the integration of peripheral metabolic information related to other energy balance mechanisms (Zheng and Berthoud, 2008).

The presence of ceramide counteracts several of the effects of GHRL

The treatment with ceramide alone induced changes in several parameters in the hypothalamus and hindbrain indicative of increased anorexigenic potential (increased mRNA abundance of POMC and CART and decreased mRNA abundance of NPY). These changes are comparable to those observed in a previous study in rainbow trout in which decreased food intake occurred after ICV ceramide treatment (Velasco et al., 2016b). Comparable results also occurred in the activity of integrative sensors and cellular signalling pathways (increased phosphorylation state of FoxO1 in the hypothalamus or AMPK in the hindbrain) and the activity of fatty acid-sensing systems (few changes but indicative of an inactivation). It is interesting to mention, however, that the effects of ceramide treatment are different to those known in mammals regarding changes in food intake, mRNA abundance of neuropeptides and activity of cellular signalling pathways (Yang et al., 2009; Gao et al., 2011, 2013; Ramírez et al., 2013).

The mRNA abundance of the four neuropeptides assessed was affected by combined treatment with GHRL and ceramide such that the presence of ceramide generally counteracts the effects elicited by GHRL, as occurred for POMC (hindbrain), CART (hypothalamus

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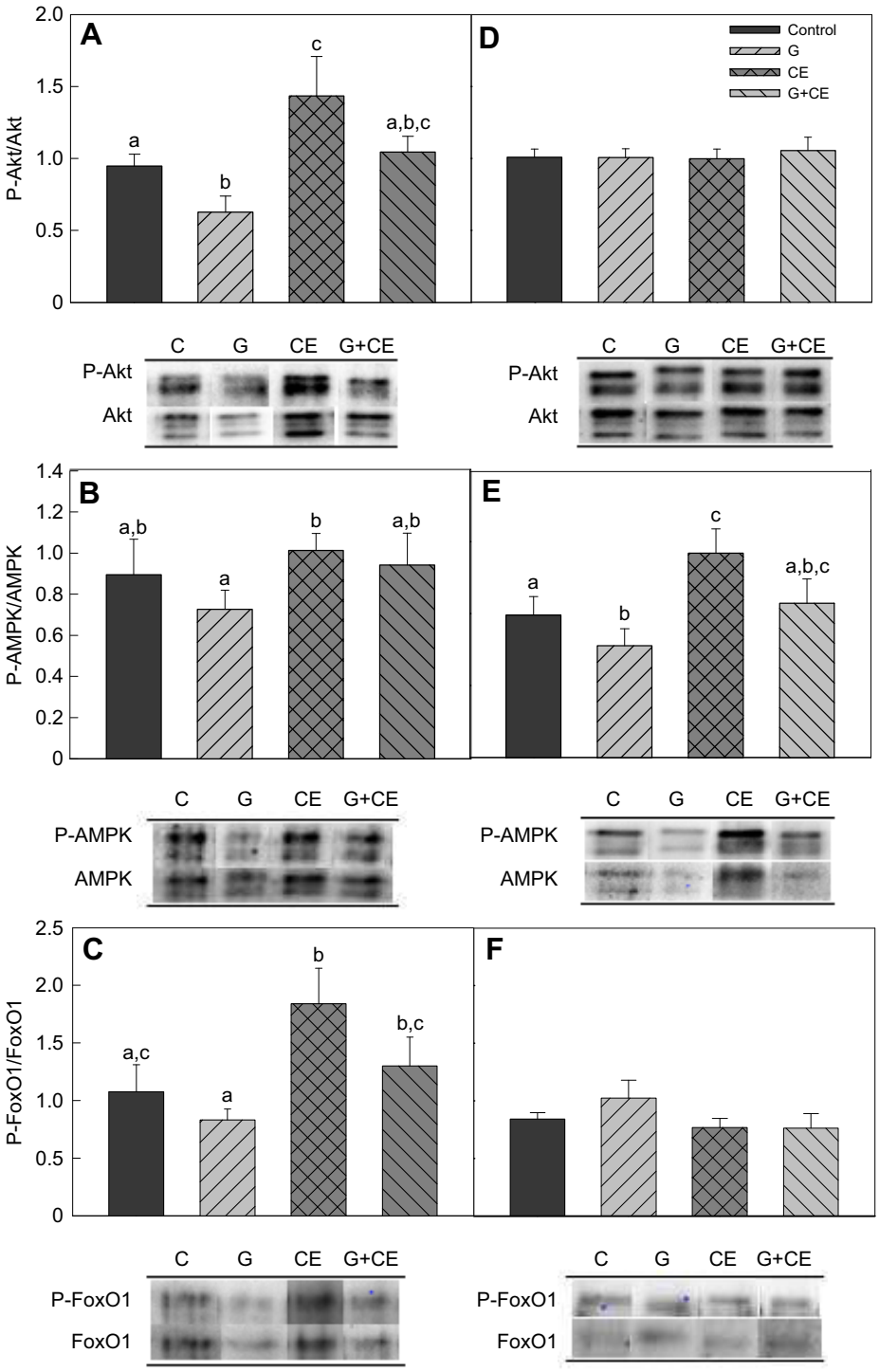

Fig. 9. Phosphorylation state of proteins in the hypothalamus and hindbrain of rainbow trout after GHRL and/or ceramide treatment. Western blot analysis of Akt (A,D), AMPK (B,E) and FoxO1 (C,F) phosphorylation status in the hypothalamus (A–C) and hindbrain (D–F) of rainbow trout 6 h after ICV administration of 1 µl 100 g^{-1} body mass of DMSO-saline (1:3) alone (control, C), or containing 200 ng of rainbow trout GHRL (G), 2.5 µg of C6:0 ceramide (CE) or GHRL+ceramide (G+CE). A 20 µg sample of total protein was loaded on the gel per lane. Western blots were performed on 6 individual samples per treatment and one representative blot per treatment is shown here. Graphs represent the ratio between the phosphorylated protein (P) and the total amount of the target protein. Each value is the mean+s.e.m. of n=6 fish per treatment. Different letters indicate significant differences (P<0.05) between groups. The values were log-transformed prior to statistical analysis.

and hindbrain), AgRP (hindbrain) and NPY (hypothalamus and hindbrain). There are some exceptions, however, such as POMC and AgRP in the hypothalamus. This counteractive effect is opposite to that known in mammals where the action of GHRL on expression of neuropeptides is favoured by the production of ceramides (Ramírez et al., 2013). The reason may relate to those factors involved in the modulation of neuropeptide expression including fatty acid-sensing systems, integrative sensors and cellular signalling pathways as discussed below.

The presence of ceramide counteracted the effects of GHRL on parameters related to fatty acid-sensing mechanisms. These included mechanisms related to fatty acid metabolism (activity of FAS and mRNA abundance of CPT-1c in the hypothalamus, and the activity of CPT-1 and ACLY and mRNA abundance of FAS and PPARγ in the hindbrain), transport through FAT/CD36 (mRNA abundance in the hypothalamus and hindbrain), LPL (mRNA abundance in the hypothalamus and hindbrain), and mitochondrial activity (UCP2a mRNA abundance in the hypothalamus). This is very interesting because GHRL and ceramide alone had opposite effects on food intake (Velasco et al., 2016a,b) and on the expression of neuropeptides (Velasco et al., 2016a,b; this study). These opposite effects were not so clear for fatty acid-sensing

systems as GHRL inactivated those systems (Velasco et al., 2016a; this study) but ceramide had few effects (Velasco et al., 2016b; this study). Thus, presumably the effects of ceramide counteracting the actions of GHRL on food intake are dependent not only on the effects of hormone on the activity of fatty acid-sensing systems but also on the effects of ceramide on other actors. These may include integrative sensors and cellular signalling pathways. Thus, GHRL treatment affected AMPK (mRNA abundance in the hypothalamus and phosphorylation state in the hindbrain), and this effect was clearly counteracted by the presence of ceramide. The presence of ceramide also counteracted the decrease induced by GHRL in the phosphorylation state of Akt and FoxO1 in the hypothalamus (but not in the hindbrain). As these parameters relate to the control of food intake (Blanco de Morentín et al., 2011; Diéguez et al., 2011), the counteractive action of ceramide may contribute to the effects observed in the mRNA abundance of anorexigenic and orexigenic peptides. This interaction again is different from that known in mammalian models where the effects of GHRL are favoured, not counteracted, by ceramide.

Conclusions

We have obtained novel information regarding the effects of GHRL treatment on metabolic regulation of food intake in fish hypothalamus, demonstrating in rainbow trout the effects of GHRL on the phosphorylation state of proteins involved in cellular signalling, with responses quite different from those reported in mammals. Moreover, we have demonstrated that GHRL treatment induced comparable changes in the hindbrain to those previously reported in the hypothalamus in the mRNA abundance of neuropeptides involved in the metabolic regulation of food intake as well as in the function of fatty acid-sensing systems, but not at the level of energy sensors and cellular signalling pathways. Because in mammals GHRL effects on the control of food intake in the hypothalamus are mediated by ceramide, we assessed such a possibility in rainbow trout. The results are different from those obtained in mammals as in rainbow trout, ceramide levels did not change after GHRL treatment, in contrast with the mammalian model where the orexigenic effect of GHRL is mediated by ceramide. This differential behaviour is further supported by the finding that ceramide generally counteracted the effects elicited by GHRL. The reasons behind this difference might rely on the differences observed in the mechanisms involved in the modulation of neuropeptide expression including fatty acid-sensing systems, integrative sensors, and proteins involved in cellular signalling pathways. The biological reason of the different behaviour between fish and mammals probably arises from a specific feature of ceramides in fish as, in general (with exceptions), the effects of GHRL are more comparable to those of mammals, whereas those of ceramide were quite different. This could relate to the clearly different life history and the differences in lipid metabolism between the two groups. The assessment of these differential mechanisms is likely to result in better knowledge of food intake regulation in fish with a subsequent improvement of feeding practices in aquaculture.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.L.S., C.V., G.M., M.C.-S., J.M.L., J.M.M.; Methodology: J.L.S., C.V., G.M., M.C.-S., J.M.L., J.M.M.; Software: C.V., G.M., J.M.L.; Validation: C.V., G.M., J.M.L.; Formal analysis: C.V., G.M., M.C.-S., J.M.L.; Investigation: J.L.S., C.V., G.M., M.C.-S.; Resources: J.L.S.; Data curation: C.V., G.M., J.M.L.; Writing - original draft: J.L.S., C.V., G.M., M.C.-S., J.M.L., J.M.M.; Writing - review & editing: J.L.S., C.V., M.C.-S., J.M.M.; Visualization: C.V., M.C.-S., J.M.M.; Supervision: J.L.S., J.M.M.; Project administration: J.L.S.; Funding acquisition: J.L.S.

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