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RESEARCH ARTICLE

Role of the G protein-coupled receptors GPR84 and GPR119 in the central regulation of food intake in rainbow trout

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ABSTRACT

We evaluated the role of the G protein-coupled receptors GPR84 and GPR119 in food intake regulation in fish using rainbow trout (*Oncorhynchus mykiss*) as a model. In the first experiment, we assessed the effects on food intake of intracerebroventricular treatment with agonists of these receptors. In the second experiment, we assessed the impact of the same treatments on mRNA abundance in the hypothalamus and hindbrain of neuropeptides involved in the metabolic control of food intake (*npy*, *agrp1*, *pomca1* and *cartpt*) as well as in changes in parameters related to signalling pathways and transcription factors involved in the integrative response leading to neuropeptide production. Treatment with both agonists elicited an anorectic response in rainbow trout attributable to changes observed in the mRNA abundance of the four neuropeptides. Changes in neuropeptides relate to changes observed in mRNA abundance and phosphorylation status of the transcription factor FOXO1. These changes occurred in parallel with changes in the phosphorylation status of AMPKα and Akt, the mRNA abundance of mTOR as well as signalling pathways related to PLCβ and IP3. These results allow us to suggest that (1) at least part of the capacity of fish brain to sense medium-chain fatty acids such as octanoate depends on the function of GPR84, and (2) the capacity of fish brain to sense *N*-acylethanolamides or triglyceride-derived molecules occurs through the binding of these ligands to GPR119.

KEY WORDS: Free fatty acids, Anorectic response, GPCR, Neuropeptides, Hypothalamus, Hindbrain, Fish, Lipid sensing

INTRODUCTION

Free fatty acids (FFAs) are ligands for a group of G protein-coupled receptors (GPCRs). These include FFA receptors (FFARs) and other GPCRs not classified as FFARs. Each FFAR can act as sensor with selectivity for a particular fatty acid carbon chain length (Husted et al., 2017). Thus, FFA1 and FFA4 sense long-chain fatty acids (LCFA, more than 12 carbons) while FFA2 and FFA3 sense short-chain fatty acids (SCFA, fewer than 6 carbons). In brain regions such as the hypothalamus and hindbrain, the sensing of fatty acids (FAs) through FFAR relates to regulation of food intake and energy homeostasis (Hara et al., 2014; Husted et al., 2017; Kimura et al., 2020). However, most available information about FA sensing in brain relates to other mechanisms (Magnan et al., 2015; Efeyan et al., 2015; Bruce et al., 2017). All these mechanisms detect changes in FAs to relate them to the modulation of food intake through changes in the mRNA abundance of two pairs of co-expressed neuropeptides: agouti-related protein (AgRP)/neuropeptide Y (NPY) and pro-opio melanocortin (POMC)/cocaine and amphetamine-related transcript (CART) (Blouet and Schwartz, 2010). The connection between FA-sensing systems and neuropeptide expression is not clear though two steps are apparent (Morton et al., 2014). In a first step, nutrient-sensing activation induces changes in the level and/or phosphorylation status of different molecules involved in signalling pathways such as AMP-activated protein kinase (AMPK), mechanistic target of rapamycin (mTOR) and protein kinase B (Akt). In a second step, the signalling pathways induce changes in the levels and/or phosphorylation status of transcription factors (López et al., 2007; Diéguez et al., 2011) such as phosphorylated cAMP response-element binding protein (CREB), brain homeobox transcription factor (BSX), and forkhead box protein O1 (FOXO1).

Other GPCRs not classified as FFARs sense changes in the levels of FFAs or FFA-related molecules (Kimura et al., 2020). Thus, GPR84 is responsive to medium-chain fatty acids (MCFA, between 6 and 12 carbons) (Luscombe et al., 2020). GPR84 is mainly found in immune-related tissues such as splenic T and B cells and bone marrow cells (Venkataraman and Kuo, 2005), and circulating granulocytes, monocytes and macrophages (Wang et al., 2006), evidencing that GPR84 predominantly relates to the immune system and the inflammatory response. However, other tissues including the brain express GPR84 (Wang et al., 2006; Wittenberger et al., 2001; Falomir-Lockhart et al., 2019). An increase in *Gpr84* mRNA expression occurred in the adipose tissue of mice fed a fat-enriched diet (Nagasaki et al., 2012). Thus, GPR84 plays a role in metabolism though no available study supports its involvement in central mechanisms involved in food intake control. In contrast, GPR119 is responsive to *N*-acylethanolamides (NAEs) such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) as well as to 2-monoacylglycerols (2-MAGs) resulting from triglyceride hydrolysis such as 2-oleolyglycerol (Hansen et al., 2012). GPR119 is predominantly expressed in enteroendocrine and pancreatic β cells, but also in other tissues including the brain (Yang et al., 2018; Falomir-Lockhart et al., 2019). Available studies evidence that endogenous ligands of GPR119 such as OEA reduce food intake and body weight gain in mammals (Azari et al., 2014; Romano et al., 2015). Besides a direct central action, this might also occur through modulation of release of gastrointestinal hormones such as glucagon-like peptide-1 (Hansen et al., 2012; Ekberg et al., 2016).

In fish, several studies demonstrated the presence of central FA-sensing mechanisms, as observed in rainbow trout (Librán-

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Pérez et al., 2012, 2013, 2014, 2015; Velasco et al., 2016, 2020; Roy et al., 2020), zebrafish (Liu et al., 2017), grass carp (Li et al., 2016; Tian et al., 2017), blunt snout bream (Dai et al., 2018), Senegalese sole (Conde-Sieira et al., 2015) and Chinese perch (Luo et al., 2020). These mechanisms are comparable to those observed in mammals (Lipina et al., 2014; Magnan et al., 2015), though fish are also sensitive to MCFA such as octanoate (C8) and poly-unsaturated FA such as α-linolenate. In a recent study in rainbow trout (Velasco et al., 2020), we obtained evidence supporting the involvement of hypothalamic FFA1 and FFA4 in the anorectic response to LCFA. Evidence in fish is scarce regarding GPR84 (Huang et al., 2014; Wang et al., 2019) and GPR119 (Fredriksson et al., 2003). However, their presence and functioning in brain areas involved in food intake control such as the hypothalamus and hindbrain is a reasonable hypothesis. In the case of GPR84, this is supported by the fact that octanoate (a MCFA) treatment in rainbow trout elicited an anorectic response, which occurred in parallel with activation in the hypothalamus and hindbrain of nutrient-sensing systems and integrative mechanisms eliciting changes in neuropeptide expression (Librán-Pérez et al., 2012, 2014). Indirect evidence supporting a role for GPR84 in metabolic regulation comes from zebrafish, where GPR84 is involved in the accumulation of lipid droplets and increased mRNA abundance of *gpr84* occurred in the intestine during food deprivation (Huang et al., 2014). In the case of GPR119, its role is supported by the anorectic response elicited in goldfish by treatment with OEA (Gómez-Boronat et al., 2016) or PEA (Gómez-Boronat et al., 2020).

In a preliminary study, we carried out amino acid sequence alignment for GPR84 and GPR119 comparing sequences available from rainbow trout (GPR84: XM_021609929.2 and GPR119: XM_036989345.1), mouse (GPR84: NM_030720.2 and GPR119: NM_181751.2) and human (GPR84: NM_020370.3 and GPR119: NM_178471.3). We also validated mRNA expression of *gpr84* and *gpr119* in hypothalamus and hindbrain of rainbow trout as shown in Fig. S1. These results allowed us to suggest the presence of these receptors in brain areas of rainbow trout. Therefore, we aimed to elucidate the role of GPR84 and GPR119 in the modulation of food intake in fish. First, we evaluated whether intracerebroventricular (i.c.v.) treatment of rainbow trout with agonists of these receptors elicits an anorectic response. Then, we assessed the impact of the same treatments on the mRNA abundance in the hypothalamus and hindbrain of the four neuropeptides involved in the metabolic control of food intake as well as in changes in parameters related to signalling pathways and transcription factors involved in the integrative response leading to neuropeptide production (Soengas, 2021).

MATERIALS AND METHODS

Fish

Immature rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792), obtained from a local fish farm (A Estrada, Spain) were maintained for 1 month in 100 litre tanks under laboratory conditions, and a 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 97±2 g. Fish were fed once daily (10:00 h) to satiety with commercial dry fish pellets (proximate food analysis: 44% crude protein, 2.5% carbohydrates, 21% crude fat and 17% ash; 20.2 MJ kg^{-1} of feed; Biomar, Dueñas, Spain). 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 (ref. 00013-19JLSF).

Experimental design

Experiment 1: effects of i.c.v. administration of GPR84 and GPR119 agonists on food intake

Following the 1 month acclimation period, fish were randomly assigned to 100 litre experimental tanks. On the day of the experiment, fish were anaesthetized with 2-phenoxyethanol (0.02% v/v; Sigma Chemical Co., St Louis, MO, USA), and weighed to carry out i.c.v. 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 29½ gauge needle attached through a polyethylene cannula to a 10 µl Hamilton syringe was aligned with the sixth 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 DMSO-saline (1:3) alone (control), or containing 2 mmol l^{-1} 3,3′-diindolylmethane (GPR84 agonist, Sigma) or 2 mmol l^{-1} AR231453 (GPR119 agonist, Abcam, Cambridge, UK). No effects occurred as a result of the vehicle alone (data not shown). The agonists and their doses were selected based on studies carried out in mammals for GPR84 (Mahmud et al., 2017; Chen et al., 2020) and GPR119 (Tough et al., 2018; Bashetti et al., 2019; Panaro et al., 2020). We registered food intake for 7 days before treatment (to define baseline data) and then 2, 6 and 24 h after treatment. After feeding, the uneaten food at the bottom of the tank (conical tanks) and feed waste were withdrawn, dried and weighed. We calculated the amount of food consumed by all fish in each tank as previously described as the difference from the amount of feed offered (De Pedro et al., 1998; Polakof et al., 2008a,b). We used 10 fish per treatment and repeated the same experiment 3 times (n=30 fish per treatment).

Experiment 2: effects of i.c.v. administration of GPR84 and GPR119 agonists on mechanisms involved in food intake control

Following acclimation, fish were randomly assigned to 100 litre experimental tanks (8 fish per tank), and fasted for 24 h before treatment to ensure basal hormone and metabolite levels were achieved. On the day of the experiment, fish were anaesthetized in their tanks with 2-phenoxyethanol (0.02% v/v; Sigma-Aldrich), weighed and i.c.v. injected as described above with DMSO-saline (1:3) alone (control, n=8) or containing the GPR84 agonist (n=8) or GPR119 agonist (n=8), using the same concentrations as described above. After 2 h (time selected based on prior studies on FA sensing in the same species; see Conde-Sieira and Soengas, 2017) fish were anaesthetized in tanks with 2-phenoxyethanol (0.02% v/v; Sigma-Aldrich). 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 and storage at −80°C until further assay. We killed fish by decapitation. The hypothalamus and hindbrain were taken, snap-frozen and stored at −80°C. We used the hypothalamus and hindbrain to assess changes in the levels of proteins of interest by western blot and for the assessment of mRNA abundance of transcripts by quantitative real-time PCR (RT-qPCR).

Assessment of metabolite levels

Levels of metabolites in plasma were enzymatically assessed using commercial kits adapted to microplate format from Spinreact (Barcelona, Spain) for glucose, lactate and triglyceride, and from Fuji (Neuss, Germany) for FA levels.

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Western blot analysis

Total protein of samples was extracted using Trizol reagent (Life Technologies, Grand Island, NY, USA) following the manufacturer's protocol for protein isolation. The protein obtained was solubilized in 100 µl 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-Aldrich). We kept tubes on ice during the whole process to prevent protein denaturation, and stored them at −80°C at the end of the process. 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 western blotted using the following antibodies: 1:1000 anti-phospho Akt (Ser473, reference #4060), 1:1000 anti-carboxyl terminal Akt (reference #9272), 1:250 anti-phospho AMPKα (Thr172, reference #2531), 1:250 anti-AMPKα (reference #2532), 1:500 anti-phospho CREB (Ser133, reference #9198), 1:500 anti-CREB (reference #9197), 1:250 anti-phospho-FOXO1 (Thr24, reference #9464), 1:250 anti-FOXO1 (reference #9454) and 1:1000 anti-β-tubulin (reference #2146) (all from Cell Signaling Technology, Leiden, The Netherlands) and 1:500 anti-BSX (reference #56092; Abcam). All these antibodies cross-react successfully with rainbow trout proteins of interest (Skiba-Cassy et al., 2009; Kamalam et al., 2012; Velasco et al., 2016; Conde-Sieira et al., 2018). After washing, membranes were incubated with an IgG-HRP secondary antibody (reference #2015718; Abcam) and bands were quantified by Image Lab software version 5.2.1 (Bio-Rad, Hercules, CA, USA) in a Chemidoc Touch imaging system (Bio-Rad). Bands were assessed by LC-MS/MS in CACTI (Universidade de Vigo), and then compared with available sequences using Uniprot software.

mRNA abundance analysis by RT-qPCR

Total RNA was extracted using Trizol reagent (Life Technologies) and subsequently treated with RQ1-DNAse (Promega, Madison, WI, USA), 2 µg total RNA was then reverse transcribed using Superscript II reverse transcriptase (Promega) and random hexamers (Promega) in a final volume of 20 µl. Gene expression

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

Gene	Forward/reverse primer	Annealing temp. (°C)	Amplicon size (bp)	Database/accession no.
actb	F: GATGGGCCAGAAAGACAGCTA R: TCGTCCCAGTTGGTGACGAT	59	105	GenBank/NM_ 001124235.1
agrp1	F: ACCAGCAGTCCTGTCTGGGTAA R: AGTAGCAGATGGAGCCGAACA	60	87	GenBank/NM_001146677
bsx	F: CATCCAGAGTTACCCGGCAAG R: TTTTCACCTGGGTTTCCGAGA	60	169	GenBank/MG310161
cartpt	F: ACCATGGAGAGCTCCAG R: GCGCACTGCTCTCCAA	60	275	GenBank/NM_001124627
creb1	F: CGGATACCAGTTGGAGGAGGA R: AGCAGCAGCACTCGTTTAGGC	60	124	GenBank/MG310160
eefla1	F: TCCTCTTGGTCGTTTCGCTG R: ACCCGAGGGACATCCTGTG	59	188	GenBank/AF498320
foxo1	F: AACTCCCACAGCCACAGCAAT R: CGATGTCCTGTTCCAGGAAGG	60	118	GenBank/MG310159
gpr84	F: GTTTCGTGGGCTGTTTTGTC R: CTGTTGAGCCAGGTGAGGTT	57.5	109	GeneBank/XM_021609929.2
gpr119	F: CCTCATCATCTCCACCAACC R: ACGAAGCACCAGCTCTGACT	57.8	84	GenBank/XM_036989345.1
itpr3	F: GCAGGGGACCTGGACTATCCT R: TCATGGGGCACACTTTGAAGA	60	64	GenBank/XM_021616029.1
mtor	F: ATGGTTCGATCACTGGTCATCA R: TCCACTCTTGCCACAGAGAC	60	81	GenBank/EU179853
npy	F: CTCGTCTGGACCTTTATATGC R: GTTCATCATATCTGGACTGTG	58	247	GenBank/NM_001124266
plcb1	F: GGAGTTGAAGCAGCAGAAGG R: GGTGGTGTTTCCTGACCAAC	60	83	GenBank/XM_021611355.1
plcb2	F: GGATTGCTGGAAGGGAAAACC R: CGGGGTACTGTGACGTCTTGA	60	134	GenBank/XM_021584705.1
plcb3	F: ATAGTGGACGGCATCGTAGC R: TGTGTCAGCAGGAAGTCCAA	62	120	GenBank/XM_021577635.1
plcb4	F: ACCTCTCTGCCATGGTCAAC R: CGACATGTTGTGGTGGATGT	60	89	GenBank/XM_021600840.1
pomca1	F: CTCGCTGTCAAGACCTCAACTCT R: GAGTTGGGTTGGAGATGGACCTC	60	118	Tigr/TC86162
prkaa1	F: ATCTTCTTCACGCCCCAGTA R: GGGAGCTCATCTTTGAACCA	60	131	GenBank/HQ40367
prkaa2	F: GGGCTACCATTAAAGACATTAGGG R: ACTCGGTGCTCTCAAACTTG	58	147	GenBank/HQ403673.1

actb, beta-actin; *agrp1*, agouti-related protein 1; *bsx*, brain homeobox transcription factor; *cartpt*, cocaine- and amphetamine-related transcript; *creb1*, cAMP response-element-binding protein; *eefla1*, elongation factor 1α; *foxo1*, forkhead boxO1; *gpr84*, G protein-coupled receptor 84; *gpr119*, G protein-coupled receptor 119; *itpr3*, inositol 1,4,5-trisphosphate receptor type 3; *mtor*, mechanistic target of rapamycin; *npy*, neuropeptide y; *plcb1*, phospholipase C β1; *plcb2*, phospholipase C β2; *plcb3*, phospholipase C β3; *plcb4*, phospholipase C β4; *pomca1*, pro-opio melanocortin a1; *prkaa1*, protein kinase AMP-activated catalytic subunit α1; *prkaa2*, protein kinase AMP-activated catalytic subunit α2.

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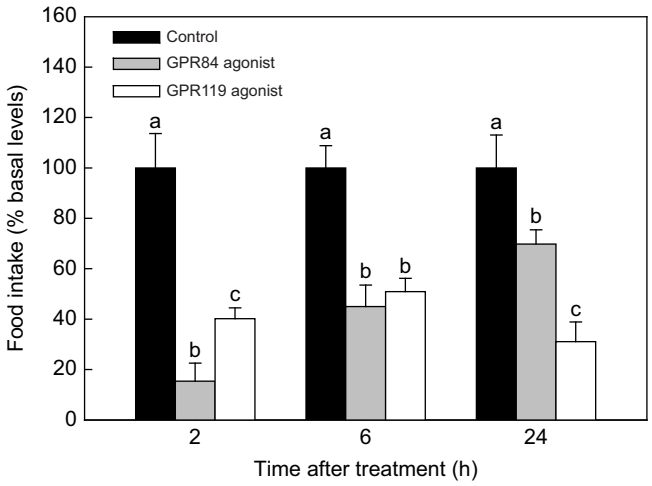

Fig. 1. Average food intake in rainbow trout after GPR84 and GPR119 agonist treatment. Food intake in rainbow trout 2, 6 and 24 h after i.c.v. administration of 1 µl 100 g^{-1} body mass of DMSO-saline alone (control) or containing 2 mmol l^{-1} 3,3′-diindolylmethane (GPR84 agonist) or 2 mmol l^{-1} AR231453 (GPR119 agonist). Food intake is displayed as the percentage of food ingested with respect to baseline levels (calculated as the average food intake in the 7 days prior to the experiment) and was normalized to the control group intake (100%). Data are shown as means±s.e.m. of the results obtained in three different experiments in which 10 fish were used per group in each tank (n=30). Different letters indicate significant differences (P<0.05) between treatments at the same time point.

levels were determined by qPCR 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 using the iCycler iQ (Bio-Rad). mRNA abundance of transcripts was determined as previously described in the same species (Panserat et al., 2000; Geurden et al., 2007; Kolditz et al., 2008; Lansard et al., 2009; Wacyk et al., 2012; Velasco et al., 2020). A fragment of each sequence containing the amplicon was amplified by conventional PCR and run on a 1.2% agarose gel. The corresponding bands were cut from the gel, purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced in an Applied Biosystems 3130 Genetic Analyzer (Foster City, CA, USA) by Servicio de Determinación Estructural, Proteómica y Genómica (CACTI-Universidade de Vigo). The obtained sequences satisfactorily matched the reference GenBank sequences. Forward and reverse primers used for expression analysis of each gene are shown in Table 1. For *gpr84* and *gpr119* (Fig. S1), thermal cycling was initiated with incubation at 95°C for 180 s using hot-start iTaq DNA polymerase activation followed by 40 cycles of heating at 95°C for 30 s, specific annealing for 30 s and extension at 72°C for 1 min. For the remaining transcripts, relative quantification of the target gene transcript was done using *actb* (β-actin) and *eef1a1* (elongation factor 1α) gene expression as a reference, which were 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 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 *actb* and *eef1a1* reference gene transcripts was made following the Pfaffl (2001) method.

Statistical analysis

In the first experiment, comparisons among groups were carried out using two-way ANOVA with treatment and time as main factors. In the second experiment, comparisons were carried out using one-way ANOVA. Shapiro–Wilk and Levene tests were used to confirm normality and homoscedasticity of the data, respectively.

When necessary, data were transformed to logarithmic or square root scale to fulfil the conditions of normality and homoscedasticity. The Bonferroni correction method was used. In the case of a significant effect (P<0.05), *post hoc* comparisons using Student–Newman–Keuls (SNK) test were employed. Comparisons were carried out with the SigmaStat (Systat Software, San José, CA, USA) statistical package.

RESULTS

Experiment 1

Central administration of GPR84 and GPR119 agonists resulted in a significant decrease of food intake post-treatment after 2 h (84.6% and 59.8%), 6 h (55% and 49.1%) and 24 h (30.2% and 68.9%), compared with the control group (Fig. 1).

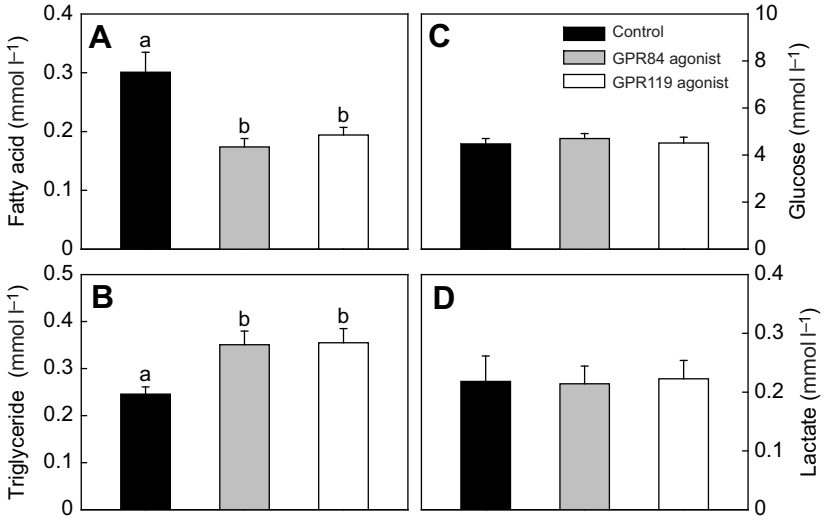

Fig. 2. Metabolite levels in the plasma of rainbow trout after GPR84 and GPR119 agonist treatment. Plasma fatty acid (A), triglyceride (B), glucose (C) and lactate (D) levels in plasma of rainbow trout 2 h after i.c.v. administration of 1 µl 100 g^{-1} body mass of DMSO-saline alone (control) or containing 2 mmol l^{-1} 3,3′-diindolylmethane (GPR84 agonist) or 2 mmol l^{-1} AR231453 (GPR119 agonist). Data are means±s.e.m. of n=8 fish per treatment. Different letters indicate significant differences (P<0.05) between treatments.

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Experiment 2

Plasma FA levels (Fig. 2A) decreased in GPR84 and GPR119 agonists groups compared with control, whereas triglyceride levels (Fig. 2B) increased in the agonist groups. Glucose (Fig. 2C) and lactate (Fig. 2D) levels did not display any significant variation.

The mRNA abundance of neuropeptides is displayed in Fig. 3. *pomca1* mRNA abundance increased after treatment with GPR84 and GPR119 agonists in the hypothalamus (Fig. 3A). *cartpt* mRNA abundance increased after treatment with the GPR84 agonist in the hypothalamus (Fig. 3B) and hindbrain (Fig. 3F) and after treatment with the GPR119 agonist in the hypothalamus (Fig. 3B). *npy* mRNA abundance decreased after treatment with the GPR84 agonist in both the hypothalamus (Fig. 3C) and hindbrain (Fig. 3G). *agrp1* mRNA abundance decreased after treatment with the GPR84 agonist in the hypothalamus (Fig. 3D).

Fig. 4 depicts the mRNA abundance of transcription factors following i.c.v. administration of specific agonists of GPR84 and GPR119. Agonist treatment did not alter the mRNA abundance of *creb1* (Fig. 4A,D). Both treatments increased mRNA abundance of *foxo1* in the hypothalamus (Fig. 4B) and hindbrain (Fig. 4E). *bsx* levels decreased in the hindbrain after treatment with the GPR119 agonist (Fig. 4F).

Fig. 5 shows levels and phosphorylation status of transcription factors. The original western blots are shown in Fig. S2. The i.c.v. administration of GPR84 and GPR119 agonists resulted in a significant decrease in the phosphorylation status of FOXO1 in the hypothalamus (Fig. 5C). No significant changes occurred for BSX or CREB.

The mRNA abundance of molecules involved in cellular signalling is shown in Fig. 6. Treatment with specific agonists of GPR84 and GPR119 receptors did not alter the mRNA abundance of protein kinase AMP-activated catalytic subunit alpha 1 (*prkaa1*) (Fig. 6A,D), whereas *prkaa2* mRNA levels decreased 2 h after treatment with both agonists in the hindbrain (Fig. 6E). *mtor* mRNA levels increased after GPR84 agonist treatment in both the hypothalamus (Fig. 6C) and hindbrain (Fig. 6F).

Fig. 7 shows levels and phosphorylation status of proteins involved in cellular signalling. The original western blots are shown in Fig. S2. The phosphorylation status of Akt in the hypothalamus increased after treatment with agonists of both receptors (Fig. 7A). The phosphorylation status of AMPKα decreased after treatment with GPR84 and GPR119 agonists in the hypothalamus (Fig. 7B) and hindbrain (Fig. 7D).

Finally, Fig. 8 depicts the mRNA abundance of parameters related to the intracellular transduction of extracellular signals.

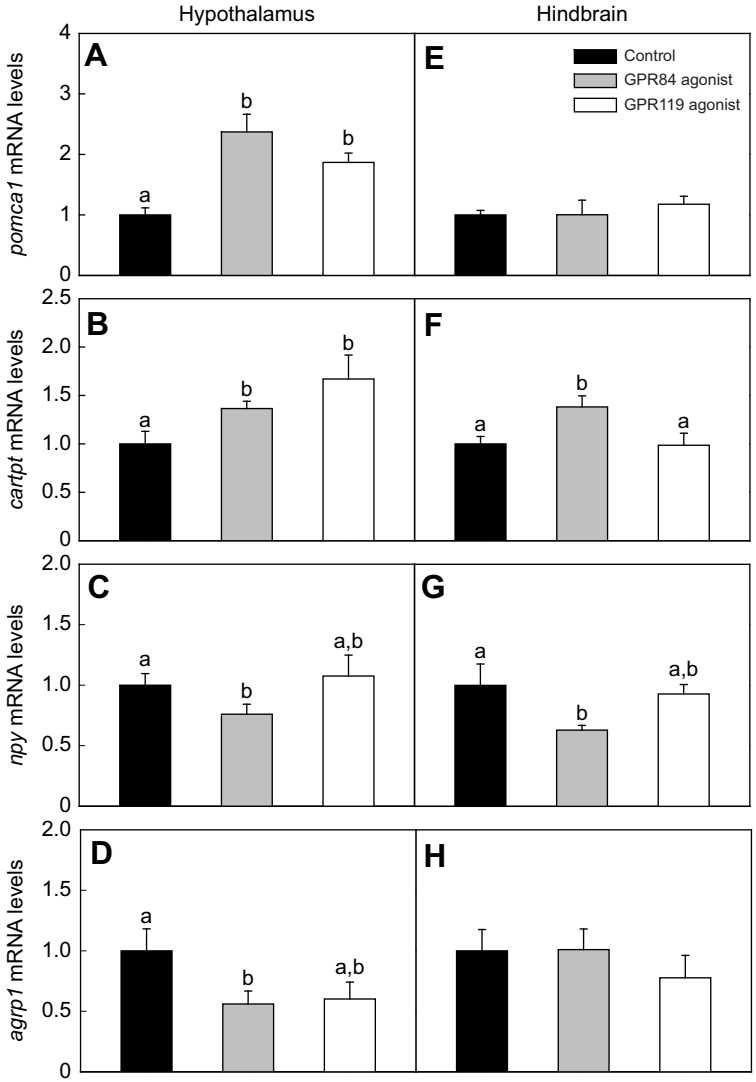

Fig. 3. mRNA abundance of neuropeptides after GPR84 and GPR119 agonist treatment. mRNA abundance of *pomca1* (A,E), *cartpt* (B,F), *npy* (C,G) and *agrp1* (D,H) in the hypothalamus (A–D) and hindbrain (E–H) of rainbow trout 2 h after i.c.v. administration of 1 µl 100 g^{-1} body mass of DMSO-saline alone (control) or containing 2 mmol l^{-1} 3,3′-diindolylmethane (GPR84 agonist) or 2 mmol l^{-1} AR231453 (GPR119 agonist). Data are means±s.e.m. of n=8 fish per treatment. Gene expression results are given with reference to control group levels and were previously normalized by *actb* and *eef1a1* expression. Different letters indicate significant differences (P<0.05) between treatments.

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1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-1 (*plcb1*) mRNA levels increased 2 h after treatment with the GPR84 agonist in the hypothalamus (Fig. 8A) and after GPR119 agonist treatment in the hypothalamus (Fig. 8A) and hindbrain (Fig. 8F). Inositol 1,4,5-trisphosphate receptor type 3 (*itpr3*) mRNA levels increased after treatment with both receptor agonists in the hypothalamus (Fig. 8E). No significant changes occurred in the mRNA abundance of *plcb2*, *plcb3* and *plcb4*.

DISCUSSION

The absolute plasma levels of glucose and lactate, and their lack of change in response to treatment indicate the absence a significant stress induced by treatments based on the role of these metabolites as markers of secondary stress responses in fish (Wendelaar Bonga, 1997). In contrast, unexpectedly, changes occurred in the level of both FA and triglyceride. Considering that GPR84 putatively responds to levels of MCFA and GPR119 to those of 2-MAGs derived from triglyceride, we cannot discard that at least some of the changes observed in the hypothalamus and hindbrain are not the result of the direct action of treatments but were induced indirectly by altered levels of these metabolites in plasma. Moreover, 3,3′-diindolylmethane is reported to have an oestrogenic effect in rainbow trout (Shilling et al., 2001). As oestrogens have been suggested to be involved in the regulation of food intake in mammals (Frank et al., 2014) and fish (Leal et al., 2009), we cannot exclude the possibility that at least some of the effects elicited by treatment with this agonist may result from a putative oestrogenic effect on the parameters assessed.

Specific GPR84 and GPR119 agonists decrease food intake of rainbow trout

The inhibition of food intake elicited by the GPR84 agonist was comparable to previous observations in the same species after i.c.v. treatment with the MCFA octanoate (Librán-Pérez et al., 2014), which makes sense assuming GPR84 is involved in MCFA detection in fish as in mammals (Luscombe et al., 2020). However, we must consider the different behaviour elicited by octanoate when comparing its impact on food intake regulation in mammals and fish. In mammals, despite octanoate not being obesogenic in comparison with LCFA (Du Toit et al., 2018), a recent study suggests that octanoate induces a rapid and transient anorexigenic effect, resulting in decreased food intake. However, in mammals, FFA4 (and not GPR84) appears to mediate this effect (Haynes et al., 2020). The distinct expression profile of GPR84 compared with that of FFARs in mammals does not support the hypothesis that its primary role is detecting MCFA centrally for regulation of energy metabolism including food intake (Luscombe et al., 2020). In contrast, in fish, octanoate effects are perfectly comparable to those of LCFA such oleate (Librán-Pérez et al., 2012, 2014; Velasco et al., 2017). We therefore suggest that a significant difference between the two groups of vertebrates exists. In fish, MCFA is involved in the regulation of food intake, probably through the action of GPR84, though this receptor would also probably be involved in immune function, as recently observed in zebrafish (Wang et al., 2019). In contrast, in mammals, GPR84 is probably involved in other processes not related to food intake, such as inflammatory and immune responses (Bouchard et al., 2007; Wei et al., 2017; Luscombe et al., 2020). This difference between mammals and fish might relate to the higher MCFA levels present in the diet of fish (Davis et al., 1999; Trushenski, 2009) versus that of mammals (Chen et al., 2020; Haynes et al., 2020). In this regard, it is interesting to mention that during milk suckling in newborn mammals, a situation in which high MCFA levels are present, high levels of GPR84 were observed in their gastric mucosa in comparison with that of weaned animals (Widmayer et al., 2017)

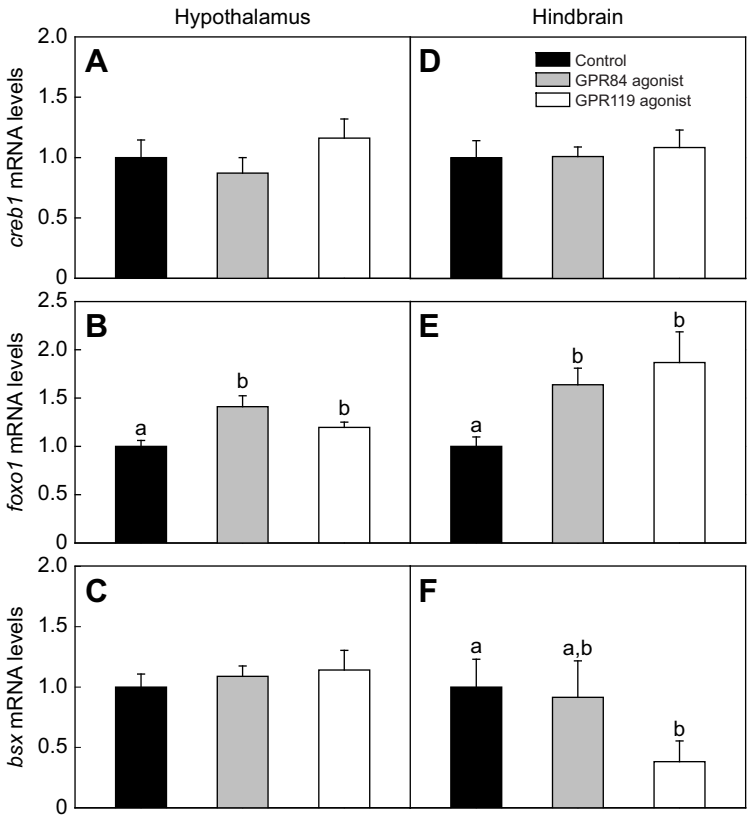

Fig. 4. mRNA abundance of transcription factors after GPR84 and GPR119 agonist treatment. mRNA abundance of *creb1* (A,D), *foxo1* (B,E) and *bsx* (C,F) in the hypothalamus (A–C) and hindbrain (D–F) of rainbow trout 2 h after i.c.v. administration of 1 µl 100 g^{-1} body mass of DMSO-saline alone (control) or containing 2 mmol l^{-1} of 3,3′-diindolylmethane (GPR84 agonist) or 2 mmol l^{-1} of AR231453 (GPR119 agonist). Data are means±s.e.m. of n=8 fish per treatment. Gene expression results are given with reference to control group levels and were previously normalized by *actb* and *eef1a1* expression. Different letters indicate significant differences (P<0.05) between treatments.

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The anorectic response elicited by treatment with the GPR119 agonist in the present study is similar to that characterized in other fish species after treatment with putative ligands of this receptor such as OEA (Gómez-Boronat et al., 2016) and PEA (Gómez-Boronat et al., 2020). In mammals, although GPR119 knockout did not cause changes in food intake when compared with wild-type animals (Higuchi et al., 2020), a reduction in food intake was also evident after treatment with OEA (Serrano et al., 2011; Azari et al., 2014; Romano et al., 2015) or PEA (Rodríguez de Fonseca et al., 2001; Mattace Raso et al., 2014).

Neuropeptide modulation by GPR84 and GPR119 agonists

The anorectic effects of GPR84 and GPR119 agonists are consistent with the changes in mRNA abundance of the two pairs of orexigenic and anorexigenic peptides involved in the metabolic regulation of food intake both in mammals (Blouet and Schwartz, 2010) and in fish (Soengas, 2014; Conde-Sieira and Soengas, 2017; Delgado et al., 2017; Soengas et al., 2018). Treatment with the GPR84 agonist increased the mRNA abundance in the hypothalamus of the anorexigenic peptides *pomca1* and *cartpt* and decreased *npy* and *agrp1* mRNA abundance while comparable changes occurred in the hindbrain for *cartpt* and *npy*. These changes are also comparable to those observed in the same species subjected to octanoate treatment (Librán-Pérez et al., 2012, 2014; Velasco et al., 2017). Indirect evidence supporting a role for GPR84 comes from zebrafish, where increased mRNA abundance occurred in the intestine during food deprivation (Huang et al., 2014). In comparison, treatment with the GPR119 agonist only affected anorexigenic neuropeptides, with increased mRNA abundance of *pomca1* and *cartpt*. This is the first time in any vertebrate species that changes in neuropeptide mRNA abundance have been assessed in response to agonists of these GPCRs. Finally, the changes described are comparable to those elicited by activation of FFA1 and FFA4 in the same species (Velasco et al., 2020), which also resulted in an anorectic response.

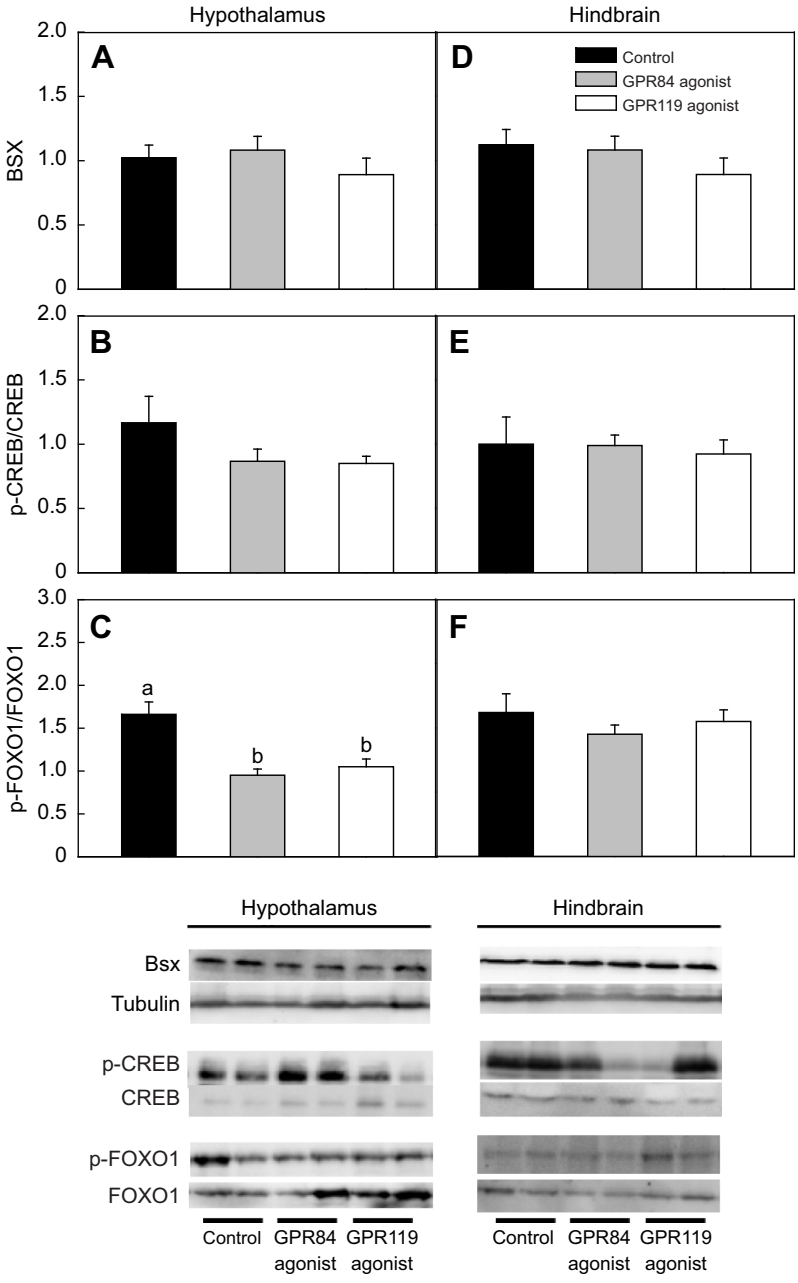

Fig. 5. Transcription factor levels and phosphorylation status after GPR84 and GPR119 agonist treatment. Western blot analysis of BSX (A,D) and phosphorylation status of CREB (B,E) and FOXO1 (C,F) in the hypothalamus (A–C) and hindbrain (D–F) of rainbow trout 2 h after i.c.v. administration of 1 µl 100 g^{-1} body mass of DMSO-saline alone (control) or containing 2 mmol l^{-1} 3,3′-diindolylmethane (GPR84 agonist) or 2 mmol l^{-1} AR231453 (GPR119 agonist). For analysis, 20 µg of total protein was loaded on the gel per lane, and results were normalized by β-tubulin abundance. Western blots were performed on 8 individual samples per treatment and two representative blots per treatment are shown here. Graphs of CREB and FOXO1 (B–F) represent the ratio between the phosphorylated protein and the total amount of the target protein. Data are means±s.e.m. of n=8 fish per treatment. Different letters indicate significant differences (P<0.05) between treatments.

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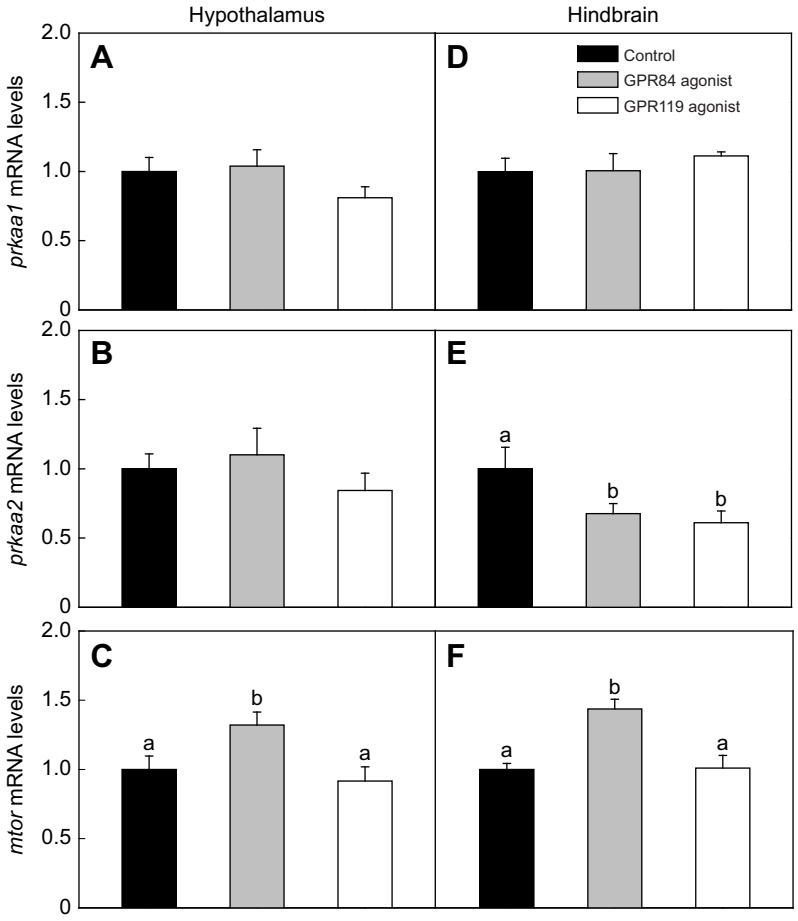

Fig. 6. mRNA abundance of cell signalling molecules after GPR84 and GPR119 agonist treatment. mRNA abundance of *prkaa1* (A,D), *prkaa2* (B,E) and *mtor* (C,F) in the hypothalamus (A–C) and hindbrain (D–F) of rainbow trout 2 h after i.c.v. administration of 1 µl 100 g^{-1} body mass of DMSO-saline alone (control) or containing 2 mmol l^{-1} 3,3′-diindolylmethane (GPR84 agonist) or 2 mmol l^{-1} AR231453 (GPR119 agonist). Data are means±s.e.m. of *n*=8 fish per treatment. Gene expression results are given with reference to control group levels and were previously normalized by *actb* and *eef1a1* expression. Different letters indicate significant differences (P<0.05) between treatments.

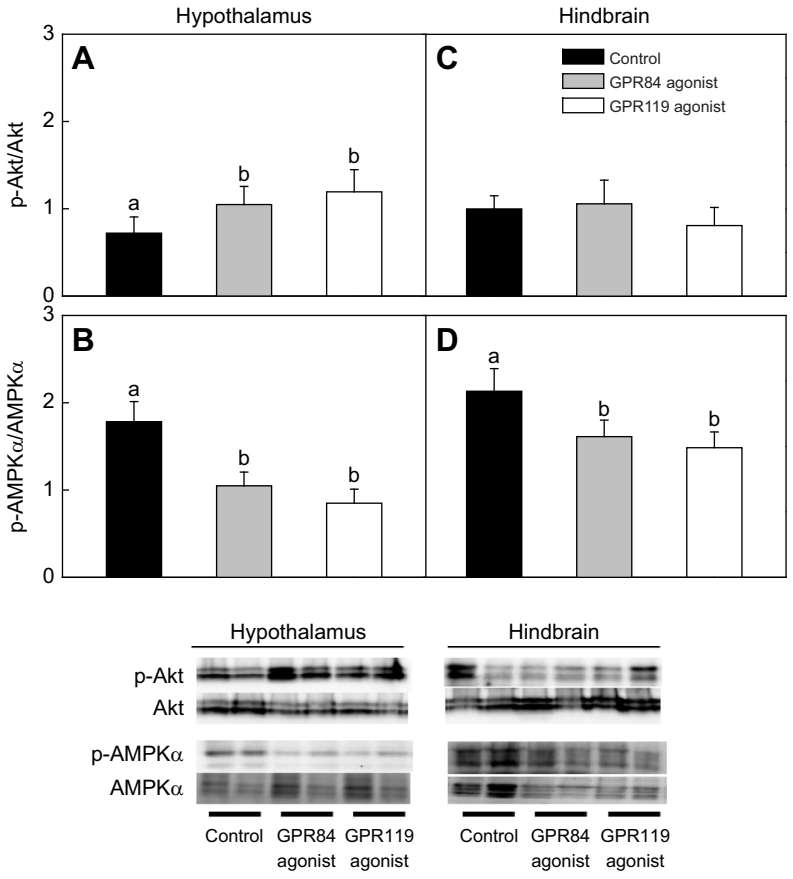

Fig. 7. AMPKα and Akt levels and phosphorylation status after GPR84 and GPR119 agonist treatment. Phosphorylation status of Akt (A,C) and AMPKα (B,D) in the hypothalamus (A,B) and hindbrain (C,D) of rainbow trout 2 h after i.c.v. administration of 1 µl 100 g^{-1} body mass of DMSO-saline alone (control) or containing 2 mmol l^{-1} 3,3′-diindolylmethane (GPR84 agonist) or 2 mmol l^{-1} AR231453 (GPR119 agonist). For analysis, 20 µg of total protein was loaded on the gel per lane, and results were normalized by β-tubulin abundance. Western blots were performed on 8 individual samples per treatment and two representative blots per treatment are shown here. Graphs represent the ratio between the phosphorylated protein and the total amount of the target protein. Data are means±s.e.m. of *n*=8 fish per treatment. Different letters indicate significant differences (P<0.05) between treatments.

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Role of GPR84 and GPR119 in the regulation of mechanisms involved in the control food intake at the central level

The mechanisms involved in the connection between FA-sensing systems and the expression of neuropeptides are partially known, both in mammals (Diéguez et al., 2011) and in fish (Soengas, 2021). They are apparently dependent on modulation of FOXO1, CREB and BSX. Thus, decreased expression of BSX and CREB and increased expression of FOXO1 occurred in response to increased FA levels (Nogueiras et al., 2008; Varela et al., 2011). These responses are also comparable to those observed in the hypothalamus of the same species after exposure to oleate (Conde-Sieira et al., 2018) or octanoate (Velasco et al., 2017). In the present study, we observed no relevant changes in CREB and BSX, whereas *foxo1* mRNA abundance increased after treatment with both agonists in the hypothalamus and hindbrain. However, this change did not parallel with phosphorylation status of FOXO1, which decreased in the hypothalamus after treatment with both agonists. As a whole, the changes observed suggest that the effects of both GPCRs on neuropeptide mRNA abundance might relate to changes observed in FOXO1 but not to the other transcription factors evaluated. This behaviour is different from that observed in these brain areas after exposure to oleate (Conde-Sieira et al., 2018; Velasco et al., 2017), octanoate (Velasco et al., 2017), or FFA1 and FFA4 agonists (Velasco et al., 2020) in which changes occurred in BSX and CREB. Therefore, the integrative mechanisms involved in the food intake response elicited by different GPCRs appear to be specific of the receptor involved.

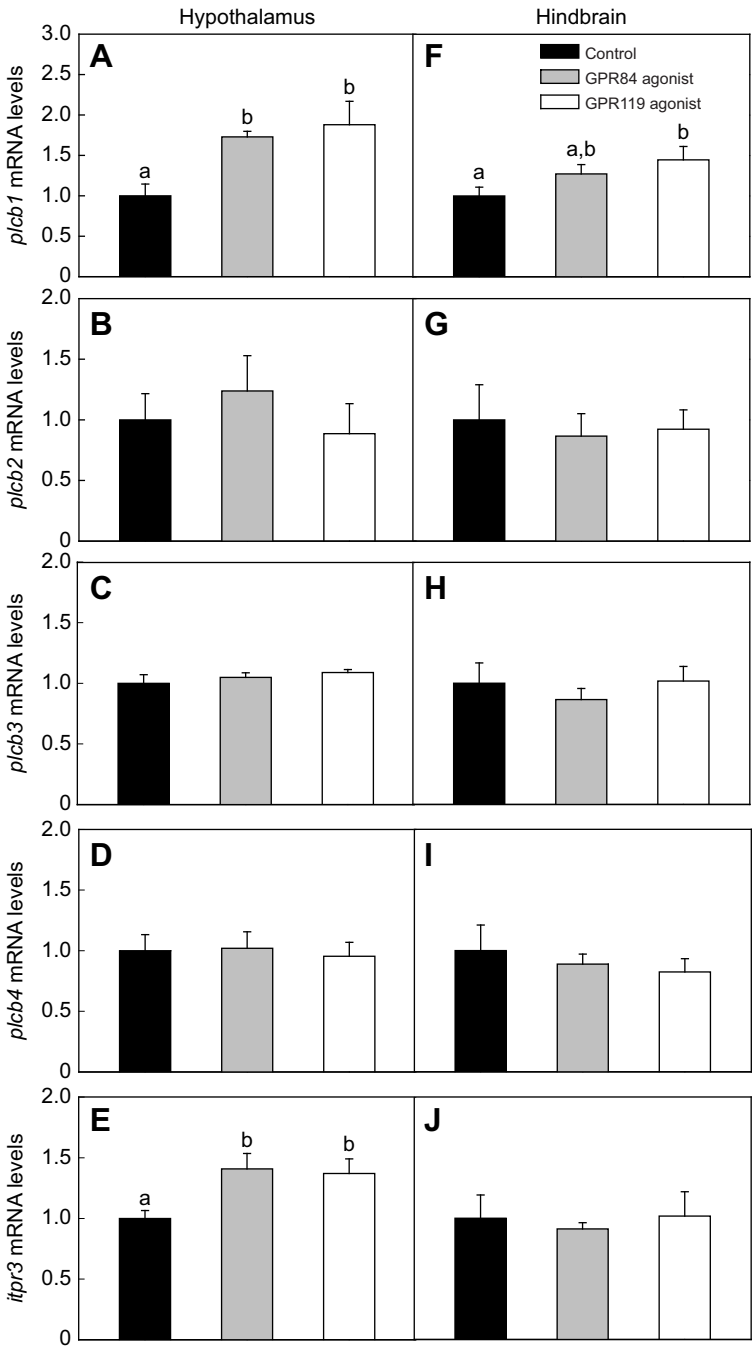

Fig. 8. mRNA abundance of downstream signal transduction cascade components after GPR84 and GPR119 agonist treatment. mRNA abundance of *plcb1* (A,F), *plcb2* (B,G), *plcb3* (C,H), *plcb4* (D,I) and *itpr3* (E,J) in the hypothalamus (A–E) and hindbrain (F–J) of rainbow trout 2 h after i.c.v. administration of 1 µl 100 g^{-1} body mass of DMSO-saline alone (control) or containing 2 mmol l^{-1} 3,3′-diindolylmethane (GPR84 agonist) or 2 mmol l^{-1} AR231453 (GPR119 agonist). Data are means±s.e.m. of n=8 fish per treatment. Gene expression results are given with reference to control group levels and were previously normalized by *actb* and *eef1a1* expression. Different letters indicate significant differences (P<0.05) between treatments.

Changes observed in transcription factors might relate to the activity of the different FA-sensing systems, including GPCRs, through different mechanisms including modulation by AMPK, mTOR and Akt (Diéguez et al., 2011; Morton et al., 2014). We observed in the present study that treatment with agonists of both receptors resulted in decreased phosphorylation of AMPKα as well as increased phosphorylation of Akt while only the GPR84 agonist increased mRNA abundance of *mtor*. These changes are similar to those described in mammalian nervous tissue for Akt after FFAR activation (Falomir-Lockhart et al., 2019) though no evidence is available regarding GPR84 and GPR119. The effects of the GPR84 agonist are comparable to those observed in the hypothalamus of the same species after treatment with octanoate (Velasco et al., 2017), further supporting the putative activation of this receptor by MCFA in fish. Furthermore, the effects of both agonists on Akt and AMPK are also comparable to the effects observed in rainbow trout hypothalamus after treatment with FFA1 and FFA4 agonists (Velasco et al., 2020), suggesting a comparable pathway of integrative mechanisms for all these GPCRs.

Changes in AMPKα, Akt and mTOR should relate to changes in the signalling mechanisms activated by GPCRs, and this might include 1,4,5-inositol triphosphate (IP3) or PLCβ (Kimura et al., 2020). No studies have assessed the impact on these pathways of activation of GPR84 and GPR119 in brain before. We evaluated changes in the mRNA abundance of four different forms of PLCβ. Only *plcb1* displayed changes in response to treatments, suggesting that this is the form involved in mediating GPR84 and GPR119 actions in fish brain. This pattern of response is the same as that observed previously in the same species after treatment with agonists of FFA1 and FFA4 (Velasco et al., 2020), suggesting similar extracellular mechanisms are involved in the response irrespective of the GPCR activated. We also observed changes in the mRNA abundance of *itpr3*, which changed in a way comparable to that of *plcb1*. This is not surprising considering that IP3 is downstream of PLCβ in the signalling cascade (Kimura et al., 2020). Changes in other signalling pathways not assessed in the present study might be involved in signalling in the hindbrain as no changes in *itpr3* mRNA abundance occurred in this area.

Conclusions

In summary, we obtained evidence in rainbow trout brain areas related to food intake control, such as the hypothalamus and hindbrain, for a lipid-sensing role for GPR84 and GPR119. The i.c.v. treatment with agonists of these receptors elicited an anorectic response in rainbow trout attributable to changes observed in the mRNA abundance of the four neuropeptides *npy*, *agrp1*, *pomca1* and *cartpt*. Changes in neuropeptides also relate to changes observed in mRNA abundance and phosphorylation status of the transcription factor FOXO1. These changes occurred in parallel with changes in the phosphorylation status of AMPKα and Akt, mRNA abundance of mTOR as well as in signalling pathways related to PLCβ and IP3 that might be involved in the action of both GPCRs. These results suggest that at least part of the capacity of fish brain to sense MCFA such as octanoate might relate to the function of GPR84, and this is reflected in decreased food intake. In a similar way, the changes observed also suggest the capacity of fish brain to sense NAE or triglyceride-derived molecules through binding of these ligands to GPR119, also eliciting an anorectic response. However, further studies assessing the effects of MCFA or NAE or triglyceride-derived molecules, and/or using other pharmacological tools (such as antagonists of these two receptors or knock-out models) are required to characterize the role of these GPCRs in the central regulation of food intake in fish.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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Data availability

Data are available from the Dryad digital repository: https://doi.org/10.5061/dryad.9kd51c5h8

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