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RESEARCH ARTICLE

The long-chain fatty acid receptors FFA1 and FFA4 are involved in food intake regulation in fish brain

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ABSTRACT

We hypothesized that the free fatty acid receptors FFA1 and FFA4 might be involved in the anorectic response observed in fish after rising levels of long-chain fatty acids (LCFAs) such as oleate. In one experiment we demonstrated that intracerebroventricular (i.c.v.) treatment of rainbow trout with FFA1 and FFA4 agonists elicited an anorectic response 2, 6 and 24 h after treatment. In a second experiment, the same i.c.v. treatment resulted after 2 h in an enhancement in the mRNA abundance of anorexigenic neuropeptides *pomca1* and *cartpt* and a decrease in the values of orexigenic peptides *npy* and *agrp1*. These changes occurred in parallel with those observed in the mRNA abundance and/or protein levels of the transcription factors Creb, Bsx and FoxO1, protein levels and phosphorylation status of Ampkα and Akt, and mRNA abundance of *plcb1* and *itrp3*. Finally, we assessed in a third experiment the response of all these parameters after 2 h of i.c.v. treatment with oleate (the endogenous ligand of both free fatty acid receptors) alone or in the presence of FFA1 and FFA4 antagonists. Most effects of oleate disappeared in the presence of FFA1 and FFA4 antagonists. The evidence obtained supports the involvement of FFA1 and FFA4 in fatty acid sensing in fish brain, and thus involvement in food intake regulation through mechanisms not exactly comparable (differential response of neuropeptides and cellular signalling) to those known in mammals.

KEY WORDS: FFA1, FFA4, Fish, Food intake, Hypothalamus, Oleate

INTRODUCTION

Free fatty acids (FFAs) act not only as energy sources, but also as natural ligands for a group of G protein-coupled receptors (GPCRs) named free fatty acid receptors (FFARs). These are widely expressed in various tissues, and contribute to important physiological processes that intertwine metabolism and immunity in multiple ways (Hara et al., 2014; Husted et al., 2017; Kimura et al., 2020), regulating energy homeostasis via modulation of cellular signal transduction pathways and ultimately cellular responses (Rohrer and Kobilka, 1998; Marinissen and Gutkind, 2001; Lagerström and Schiöth, 2008). Each FFAR can act as sensor with selectivity for a particular fatty acid carbon chain length (Kimura et al., 2020). Both FFA1 (formerly known as GPR40) and FFA4 (formerly known as GPR120) are known to be activated by long-chain fatty acids (LCFAs) (Husted et al., 2017). In mammals, several studies demonstrated that these FFARs are present in enteroendocrine cells of the gastrointestinal tract (GIT) where they relate the detection of changes in LCFA to the synthesis and release of gastrointestinal hormones (Lu et al., 2018). FFA1 and FFA4 are expressed not only in GIT, but also in a number of other tissues including liver, adipose tissue, taste buds and brain (Dragano et al., 2017; Kimura et al., 2020). In brain regions like the hypothalamus and hindbrain, the presence of these receptors has been related to their putative role as fatty acid sensors involved in the regulation of food intake and energy homeostasis (Hara et al., 2014; Husted et al., 2017; Kimura et al., 2020). However, most available information about fatty acid sensing in brain relates to other mechanisms, such as those based on carnitine palmitoyl transferase-1, fatty acid translocase, increased capacity of mitochondria to produce reactive oxygen species inhibiting ATP-dependent inward rectified potassium channels, and lipoprotein lipase activity (López et al., 2007; Magnan et al., 2015; Efeyan et al., 2015; Bruce et al., 2017). These mechanisms detect changes in LCFA to relate them to the modulation of food intake through changes in the expression of neuropeptides agouti-related protein (AgRP)/neuropeptide Y (NPY), and pro-opio melanocortin (POMC)/cocaine and amphetamine-related transcript (CART), ultimately leading to changes in food intake (Blouet and Schwartz, 2010). The number of available studies in mammals regarding the putative role of these FFARs as fatty acid sensors in brain is very limited compared with those of other fatty acid-sensing systems, and the role of these receptors in the brain remains unclear, as does the functional consequences of FFA1 and FFA4 activation. However, a relationship seems likely as treatment with agonists of FFA1 (Gorski et al., 2017) and FFA4 (Auguste et al., 2016) inhibits food intake. In vertebrates other than mammals, the available information is almost non-existent.

In fish, available studies support the presence of fatty acid-sensing mechanisms in rainbow trout (Librán-Pérez et al., 2012, 2013, 2014, 2015; Velasco et al., 2016), Senegalese sole (Conde-Sieira et al., 2015) and grass carp (Li et al., 2016; Gong et al., 2017). These mechanisms are comparable, in general, to those described in mammals (Blouet and Schwartz, 2010; Morton et al., 2014; Magnan et al., 2015) with the exception of the ability of fish systems for detecting not only changes in the levels of LCFA, but also medium-chain fatty acids including octanoate and polyunsaturated fatty acids like α-linolenate (Conde-Sieira and Soengas, 2017). However, there is no evidence for the presence and function in fish brain of FFARs and their possible involvement in food intake regulation. Based on the anorectic effects of raised levels of LCFA like oleate in rainbow trout (Librán-Pérez et al., 2012, 2014), we hypothesize that FFA1 and FFA4 might be involved in such a response. Therefore, we first examined whether intracerebroventricular (i.c.v.) treatment of rainbow trout with FFA1 and FFA4 agonists elicited an anorectic response. Then, in a second experiment, we assessed in hypothalamus and hindbrain the impact of the same treatment on mRNA abundance of neuropeptides involved in the metabolic

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control of food intake (*agrp1*, *npy*, *pomca1* and *cartpt*), mRNA abundance and protein phosphorylation status of the transcription factors brain homeobox transcription factor (Bsx), cAMP response element binding protein (Creb) and forkhead box protein O1 (FoxO1), phosphorylation status of AMP-activated protein kinase α (Ampkα) and protein kinase B (Akt), and mRNA abundance of phospholipase Cβ2 (*plcb*) and inositol 1,4,5-triphosphate receptor type 3 (*itpr3*), all of which are involved in the modulation of neuropeptide expression. Finally, we assessed in a third experiment the response of all these parameters in the presence of a representative LCFA such as oleate alone or in the presence of antagonists of FFA1 and FFA4 to elucidate the capacity of antagonists to revert the effects of oleate, thus providing further support to the involvement of FFARs in fatty acid sensing.

MATERIALS AND METHODS

Fish

Immature female rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)] of 97±2 g body mass and 21.7±0.44 cm body length were obtained from a local fish farm (Piscifactoria de la Calle, A. Estrada, Spain). Fish were maintained for 1 month in 100-liter tanks under laboratory conditions and 12 h:12 h light:dark photoperiod (lights on at 08:00 h, lights off at 20:00 h) in dechlorinated tap water at 15°C. 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.

Experimental design

Experiment 1: effects of i.c.v. administration of specific FFAR agonists on food intake

Following a 1-month acclimation period, fish were randomly assigned to 100-liter experimental tanks. On the day of the experiment, 10 fish per group were anesthetized with 2-phenoxyethanol (Sigma, St Louis, MO, USA; 0.02% v/v), and weighed to carry out i.c.v. administration as previously described (Polakof and Soengas, 2008). Briefly, fish were placed on a plexiglass board with Velcro straps adjusted to hold them in place. A 29½ gauge needle attached through a polyethylene cannula to a 10 µl Hamilton syringe was aligned with the sixth pre-orbital bone at the rear of the eye socket, and from this point the syringe was moved through the space in the frontal bone into the third ventricle. The plunger of the syringe was slowly depressed to dispense 1µl 100 g^{-1} body mass of DMSO–saline (1:3) alone (control), or containing 2 mmol l^{-1} of TUG424 (FFA1 agonist, Sigma), 1 mmol l^{-1} TUG891 (FFA4 agonist, Sigma) or 1 mmol l^{-1} GW9508 (FFA1+FFA4 agonist, Sigma); no effects were observed due to the vehicle alone (data not shown). The agonists and their doses were selected based on studies carried out in mammals (Darling et al., 2014; Dragano et al., 2017). Food intake was recorded for 7 days before treatment (to define basal line data) and then 2, 6 and 24 h after treatment. After feeding, the uneaten food remaining at the bottom (conical tanks) and feed waste were withdrawn, dried and weighed. The amount of food consumed by all fish in each tank was calculated (as previously described) as the difference from the feed offered (De Pedro et al., 1998; Polakof et al., 2008a; 2008b). The experiment was repeated three times, and therefore results are shown as means±s.e.m. of the data obtained in three different tanks per treatment.

Experiment 2: effects of i.c.v. administration of agonists of FFA1 and FFA4 on mechanisms involved in food intake control

Following acclimation, fish were randomly assigned to 100-liter experimental tanks (eight fish per tank) and fasted for 24 h before treatment to ensure that basal hormone and metabolite levels were achieved. On the day of the experiment, fish were anesthetized in their tanks with 2-phenoxyethanol (0.02% v/v), weighed and i.c.v. injected as described above with DMSO–saline (1:3) alone (control, *N*=8), or containing TUG424 (*N*=8), TUG891 (*N*=8) or GW9508 (*N*=8), specific agonists of FFA1, FFA4 and FFA1+FFA4, respectively, using the same concentrations as described above. After 2 h fish were anesthetized in tanks with 0.02% v/v 2-phenoxyethanol. 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. Fish were euthanized by decapitation and hypothalamus and hindbrain were taken, snap-frozen and stored at −80°C. Hypothalamus and hindbrain were used to assess changes in the levels of proteins of interest by western blot and for the assessment of mRNA abundance of transcripts by reverse transcription-quantitative PCR (RT-qPCR).

Experiment 3: i.c.v. administration of oleate alone or in the presence of an antagonist of FFA1 and FFA4

Following acclimation, fish were randomly assigned to 100-liter tanks (eight fish per tank) and were fasted for 24 h before treatment to ensure that basal hormone and metabolite levels were achieved. On the day of the experiment, fish were anesthetized in their tanks with 2-phenoxyethanol (0.02% v/v), weighed and i.c.v. injected as described above with vehicle alone (control, *N*=8), or containing 0.1 mmol l^{-1} DC260126 and 0.1 mmol l^{-1} AH7614 (FFA1 and FFA4 antagonists, Tocris; *N*=8), 1 µmol oleate (OL, Sigma; *N*=8), or OL+FFA1+FFA4 antagonist (*N*=8). The vehicle used was a mixture (1:3:3 in volume) of DMSO–saline–45% hydroxypropyl-beta-cyclodextrin (HPB). We used the saline–HPB fraction to a final concentration of 17 mmol l^{-1} HPB (Morgan et al., 2004) to safely deliver oleate, and DMSO to dissolve antagonists. No effects of HPB or DMSO alone occurred for any of the parameters assessed (data not shown). The dose of oleate treatment was selected based on studies carried out previously in our laboratory (Librán-Pérez et al., 2014; Velasco et al., 2016, 2017b). The antagonist and its dose were selected based on studies carried out in mammals (Sun et al., 2013; Quesada-López et al., 2016). After 2 h, fish were anesthetized, euthanized and sampled as described in Experiment 2.

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, triglyceride, fatty acid and total lipid levels, and from Fuji (Neuss, Germany) for fatty acids.

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). Tubes were kept on ice during the whole process to prevent protein denaturation, and

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were stored at −80°C at the end of the process. The concentration of protein in each sample was determined using Bradford assay with bovine serum albumin as standard. Hypothalamus and hindbrain protein lysates (20 µg) were subjected to western blotting using antibodies from Cell Signaling Technology (Leiden, The Netherlands): 1:1000 anti-phospho Akt (Ser473; reference no. 4060), 1:1000 anti-carboxyl terminal Akt (reference no. 9272), 1:250 anti-phospho AMPKα (Thr172, reference no. 2531), 1:250 anti-AMPKα (reference no. 2532), 1:500 anti-phospho Creb (Ser133, reference no. 9198), 1:500 anti-Creb (reference no. 9197), 1:250 anti-phospho-FoxO1 (Thr24, reference no. 9464), 1:250 anti-FoxO1 (reference no. 9454) and 1:1000 anti-β-tubulin reference (reference no. 2146); and Abcam (Cambridge, UK): 1:500 anti-Bsx (reference no. 56,092). 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 no. 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).

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). Total RNA (2 µg) was reverse transcribed using Superscript II reverse transcriptase (Promega) and random hexamers (Promega) to obtain 20 µl. Gene expression levels were determined by RT-qPCR using the iCycler iQ (Bio-Rad). Analyses were performed on 1 µl cDNA using Maxima SYBR Green qPCR Master Mix (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 (Panserat et al., 2000; Geurden et al., 2007; Kolditz et al., 2008; Lansard et al., 2009; Wacyk et al., 2012), with the exception of *plcb1*, *plcb3* and *plcb4*. For these transcripts, new primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3-.4.0/primer3/) from sequences available in GenBank (*plcb1*, XM_021611355.1; *plcb3*, XM_021577635.1; *plcb4*, XM_021600840.1). 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) in 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 each gene expression are shown in Table 1. Relative quantification of the target gene transcript was done using *actb* (β-actin) and *eef1a1* (elongation factor 1α) gene expressions as 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, 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 *actb* and *eef1a1* reference gene transcripts was made following the Pfaffl (2001) method.

Statistical analysis

In Experiment 1, comparisons between groups were carried out using two-way ANOVA with treatment and time as main factors. In Experiments 2 and 3, comparisons were carried out using one-way ANOVA. Shapiro–Wilk and Levene's 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 case of a significant effect ($P<0.05$), *post hoc* comparisons using a Student–Newman–Keuls (SNK) test were employed. Comparisons were carried out with the SigmaStat (Systat Software, San José, CA, USA) statistical package.

RESULTS

Food intake values in Experiment 1 (Fig. 1) are presented as daily values of the percentage of weight of eaten food with respect to the basal levels of each experimental tank. Central administration of FFA1 and FFA4 agonists resulted in a significant decrease of food intake post-treatment after 2 h (58.5 and 48.4%), 6 h (18 and 27.3%) and 24 h (32.5 and 71%), compared with the control group. A larger decrease was observed in fish treated with FFA1+FFA4 agonists after 2 h (84.6%) and 6 h (55%), while after 24 h (30.2%) the

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

	Forward primer	Reverse primer	Annealing temperature (°C)	Database	Accession number
actb	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59	GenBank	NM_ 001124235.1
agrp1	ACCAGCAGTCCTGTCTGGGTAA	AGTAGCAGATGGAGCCGAACA	60	GenBank	NM_001146677
bsx	CATCCAGAGTTACCCGGCAAG	TTTTCACCTGGGTTTCCGAGA	60	GenBank	MG310161
cartpt	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	60	GenBank	NM_001124627
creb1	CGGATACCAGTTGGAGGAGGA	AGCAGCAGCACTCGTTTAGGC	60	GenBank	MG310160
eefla1	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59	GenBank	AF498320
foxO1	AACTCCCACAGCCACAGCAAT	CGATGTCCTGTTCCAGGAAGG	60	GenBank	MG310159
itpr3	GCAGGGGACCTGGACTATCCT	TCATGGGGCACACTTTGAAGA	60	GenBank	XM_021616029.1
npy	CTCGTCTGGACCTTTATATGC	GTTCATCATATCTGGACTGTG	58	GenBank	NM_001124266
plcb1	GGAGTTGAAGCAGCAGAAGG	GGTGGTGTTTCCTGACCAAC	60	GenBank	XM_021611355.1
plcb2	GGATTGCTGGAAGGGAAAACC	CGGGGTACTGTGACGTCTTGA	60	GenBank	XM_021584705.1
plcb3	ATAGTGGACGGCATCGTAGC	TGTGTCAGCAGGAAGTCCAA	62	GenBank	XM_021577635.1
plcb4	ACCTCTCTGCCATGGTCAAC	CGACATGTTGTGGTGGATGT	60	GenBank	XM_021600840.1
pomca1	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	60	TIGR	TC86162

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 box O1; *itpr3*, inositol 1,4,5-triphosphate receptor type 3; *npy*, neuropeptide y; *plcb1*, phospholipase C β1; *plcb2*, phospholipase C β2; *plcb3*, phospholipase C β3; *plcb4*, phospholipase C β4; *pomca1*, pro-opio melanocortin a.

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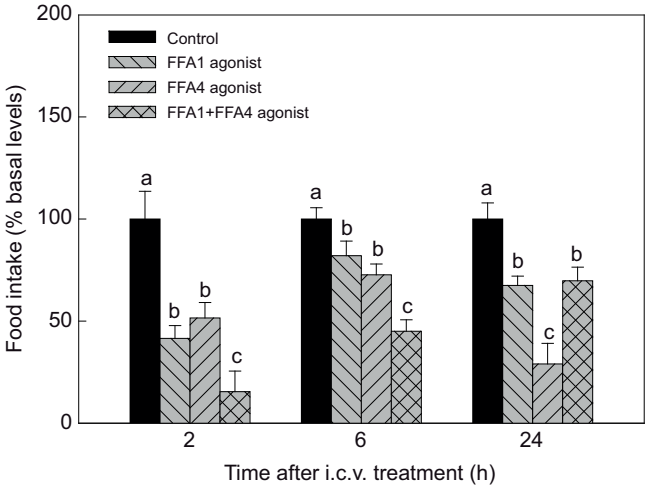

Fig. 1. Average food intake recorded in rainbow trout. Food intake was assessed in rainbow trout 2, 6 and 24 h after i.c.v. administration of 1 µl 100 g^{-1} body mass of DMSO–saline alone (control) or containing 2 mmol l^{-1} of FFA1 agonist (TUG424), or 1 mmol l^{-1} of FFA4 agonist (TUG891), or 1 mmol l^{-1} of FFA1+FFA4 agonist (GW9508). Food intake is displayed as the percentage of food ingested with respect to baseline levels (calculated as the average of food intake over the 7 days prior to experiment) and was normalized to control group (100%). The results 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. Different lower case letters indicate significant differences (P<0.05) from different treatments at the same time (two-way ANOVA, P<0.05; *post hoc* SNK test, P<0.05).

additive effect disappeared although remained below values of the control group.

In Experiments 2 and 3, levels of glucose (Fig. 2A,F), lactate (Fig. 2B,G), fatty acid (Fig. 2C,H), triglyceride (Fig. 2D,I) and total lipid (Fig. 2E,J) in plasma were not affected by treatments.

The mRNA abundance of neuropeptides involved in the metabolic regulation of food intake is shown in Fig. 3. *pomca1* mRNA abundance increased after 2 h treatment with FFA4 and FFA1+FFA4 agonists (Fig. 3A) and after 2 h treatment with oleate (Fig. 3E), both in the hypothalamus and hindbrain. *cartpt* mRNA abundance increased after 2 h treatment with FFA4 agonist in the hypothalamus and hindbrain (Fig. 3B) and after 2 h treatment with oleate in hindbrain (Fig. 3F). *npy* mRNA abundance decreased after 2 h treatment with FFA1 and FFA1+FFA4 agonists in the hypothalamus, after FFA4 and FFA1+FFA4 agonists in the hindbrain (Fig. 3C) and after 2 h treatment with oleate and oleate+FFA1+FFA4 antagonists in the hypothalamus and hindbrain (Fig. 3G). *agrp1* mRNA abundance decreased after 2 h treatment with FFA1 or FFA4 agonist in the hypothalamus and FFA4 and FFA1+FFA4 agonists in the hindbrain (Fig. 3D). Except in the case of *npy*, all effects observed by oleate treatment disappear in the presence of receptor antagonist (OL+FFA1+FFA4 antagonist).

The mRNA abundance of transcription factors is shown in Fig. 4. The value of *bsx* decreased 2 h after FFA1+FFA4 agonist treatment in the hypothalamus (Fig. 4A), as well as 2 h after oleate treatment in the hypothalamus and hindbrain (Fig. 4D). Intracerebroventricular treatments with specific agonists of FFA1 and FFA4 receptors did not alter the mRNA abundance of *creb1* (Fig. 4B), while oleate treatment decreased mRNA abundance of *creb1* (Fig. 4E). The value of *foxO1* increased 2 h after FFA1 agonist in the hypothalamus and hindbrain, after FFA4 agonist in the hypothalamus (Fig. 4C) and after oleate treatment in hindbrain (Fig. 4F). All effects observed in the values of transcription factors by oleate treatment disappeared in the presence of receptor antagonist (OL+FFA1+FFA4 antagonist).

Levels and phosphorylation status of transcription factors are shown in Fig. 5. Bsx protein levels decreased after FFA1 and FFA4 agonist treatment in the hypothalamus, and after FFA1, FFA4 and FFA1+FFA4 agonist treatments in hindbrain, compared with the control group (Fig. 5A). Bsx protein levels also decreased after oleate treatment in the hypothalamus, while no changes occurred after treatment with oleate or receptor antagonists (Fig. 5D). The phosphorylation status of Creb was not affected by any treatment, either in the hypothalamus or in the hindbrain (Fig. 5B,E). The phosphorylation status of FoxO1 in the hypothalamus decreased after FFA1 agonist treatment (Fig. 5C), but increased after oleate and oleate+FFA1+FFA4 antagonist treatments (Fig. 5F). No changes were observed in hindbrain.

The phosphorylation status of Akt in the hypothalamus increased after treatment with receptor agonists (Fig. 6A) and oleate (Fig. 6C) in comparison with the control group, whereas no significant changes occurred in hindbrain. The decreased value of Akt observed in the group treated with oleate disappeared in the group treated with oleate and receptors antagonist. The phosphorylation status of Ampkα decreased after treatment with receptors agonist in hypothalamus and hindbrain (Fig. 6C), and also decreased after oleate and oleate+FFA1+FFA4 antagonist treatment in the hypothalamus (Fig. 6D).

The mRNA abundance of parameters related to intracellular signaling is shown in Fig. 7. In the hypothalamus, the value of *plcb1* increased 2 h after treatment with FFA4 agonist (Fig. 7A) and oleate (Fig. 7F). The value of inositol 1,4,5-trisphosphate receptor type 3 (*itpr3*) increased 2 h after treatment with FFA1+FFA4 agonists (Fig. 7E) and oleate (Fig. 7J) in the hypothalamus, while no changes were observed in hindbrain. All effects observed by oleate treatment disappeared in the presence of the receptor antagonists (OL+FFA1+FFA4 antagonist). No significant changes occurred in the mRNA abundance of *plcb2*, *plcb3* and *plcb4*.

DISCUSSION

The present study evaluates, for the first time in fish, the role of FFA1 and FFA4 in the regulation of food intake through assessment of the effects of putative agonists and antagonists of those receptors on food intake, and in mechanisms involved in its control in hypothalamus and hindbrain. As the agonists used might also interact with FFAR other than FFA1 and FFA4 (Kimura et al., 2020), we cannot exclude the possibility that at least some of the changes observed might be attributed to the involvement of other FFARs. The absence of changes in plasma metabolite levels indicates that no major metabolic changes occurred in the periphery after central treatment with oleate and FFA1 and FFA4 agonists or antagonists. Therefore, in the present study, changes observed in parameters assessed in brain areas are due to the direct action of treatments, and are not the result of changes induced by altered levels of plasma metabolites.

Specific FFAR agonists decrease food intake in rainbow trout

Intracerebroventricular treatment with specific FFA1 and FFA4 agonists led to a significant reduction in food intake in rainbow trout, which was evident up to 24 h post-treatment. This has been assessed for the first time in fish in this study, but it is in agreement with available evidence in mammals after treatment with FFA1 or FFA4 agonists (Auguste et al., 2016; Dragano et al., 2017; Gorski et al., 2017). It is important to emphasize that the decrease was higher for the joint treatment of FFA1+FFA4 agonists, than for FFA1 and FFA4 agonist treatments separately, providing evidence for a synergistic effect 2 and 6 h post-treatment, in a way comparable

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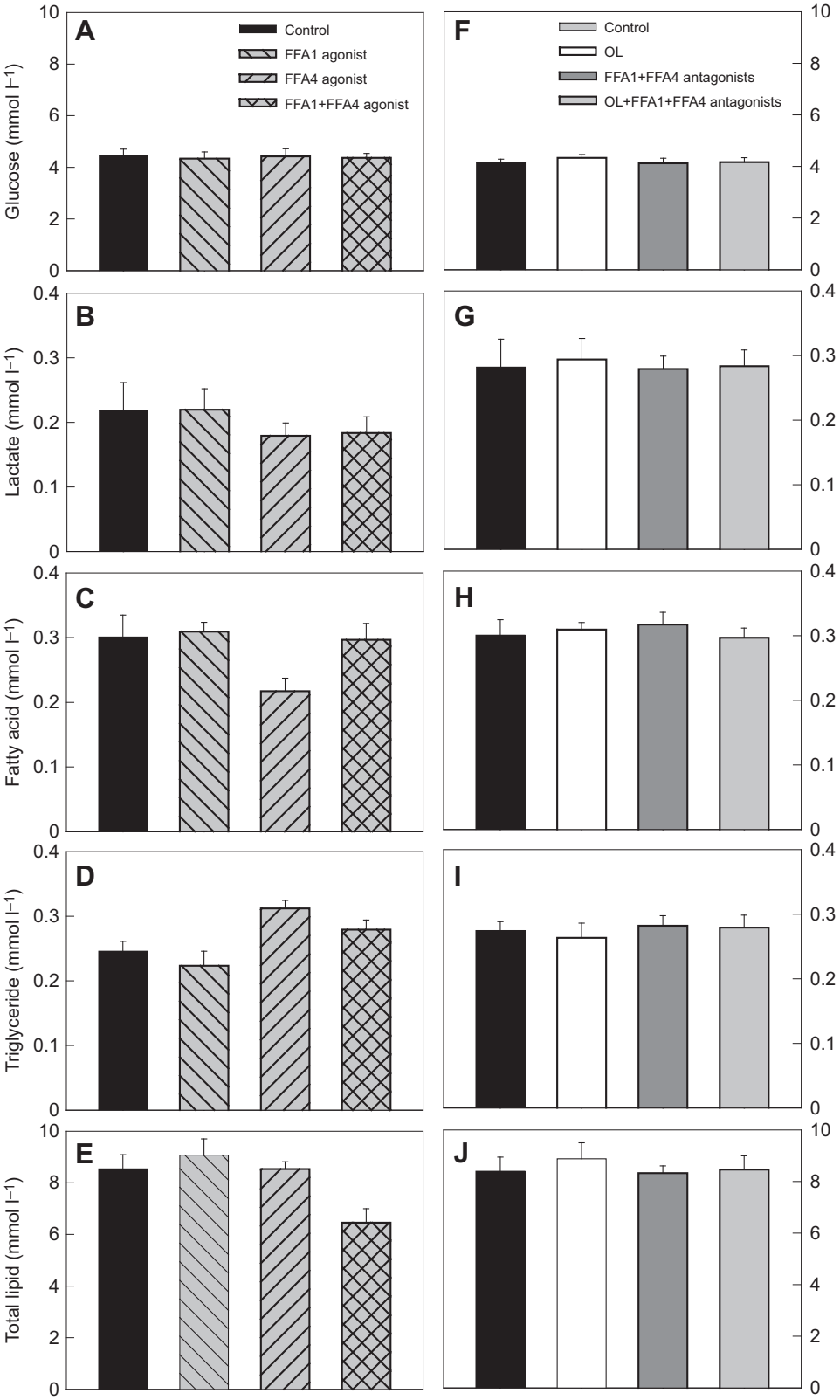

Fig. 2. Metabolite levels in the plasma of rainbow trout after i.c.v. treatments. Levels of glucose (A,F), lactate (B,G), fatty acid (C,H), triglyceride (D,I) and total lipid (E, J) in plasma of rainbow trout 2 h after i.c.v. administration of (left-hand panels) 1 µl 100 g^{-1} body mass of DMSO–saline alone (control), or containing 2 mmol l^{-1} of FFA1 agonist (TUG424), or 1 mmol l^{-1} of FFA4 agonist (TUG891), or 1 mmol l^{-1} of FFA1+FFA4 agonist (GW9508); and (right-hand panels) 1 µl 100 g^{-1} body mass of vehicle alone (control), or containing 1 µmol oleate (OL), or 0.1 mmol l^{-1} FFA1+FFA4 antagonists (C260126 and AH7614), or 1 µmol oleate+FFA1+FFA4 antagonists (OL+FFA1+FFA4 antagonists). Each value is the mean±s.e.m. of *N*=8 fish per treatment.

to that observed in mice (Dragano et al., 2017). The inhibition of food intake observed after i.c.v. treatment with any of the FFAR agonists was comparable to previous observations in the same species after i.c.v. treatment with oleate (Librán-Pérez et al., 2014; Velasco et al., 2016). Feeding regulatory systems seem to relate to behavioral control other than food intake such as swimming patterns, locomotor activity, etc. Although we did not visually observe behavioural changes, we cannot exclude the possibility of several of those behaviors being involved in at least some of the responses observed.

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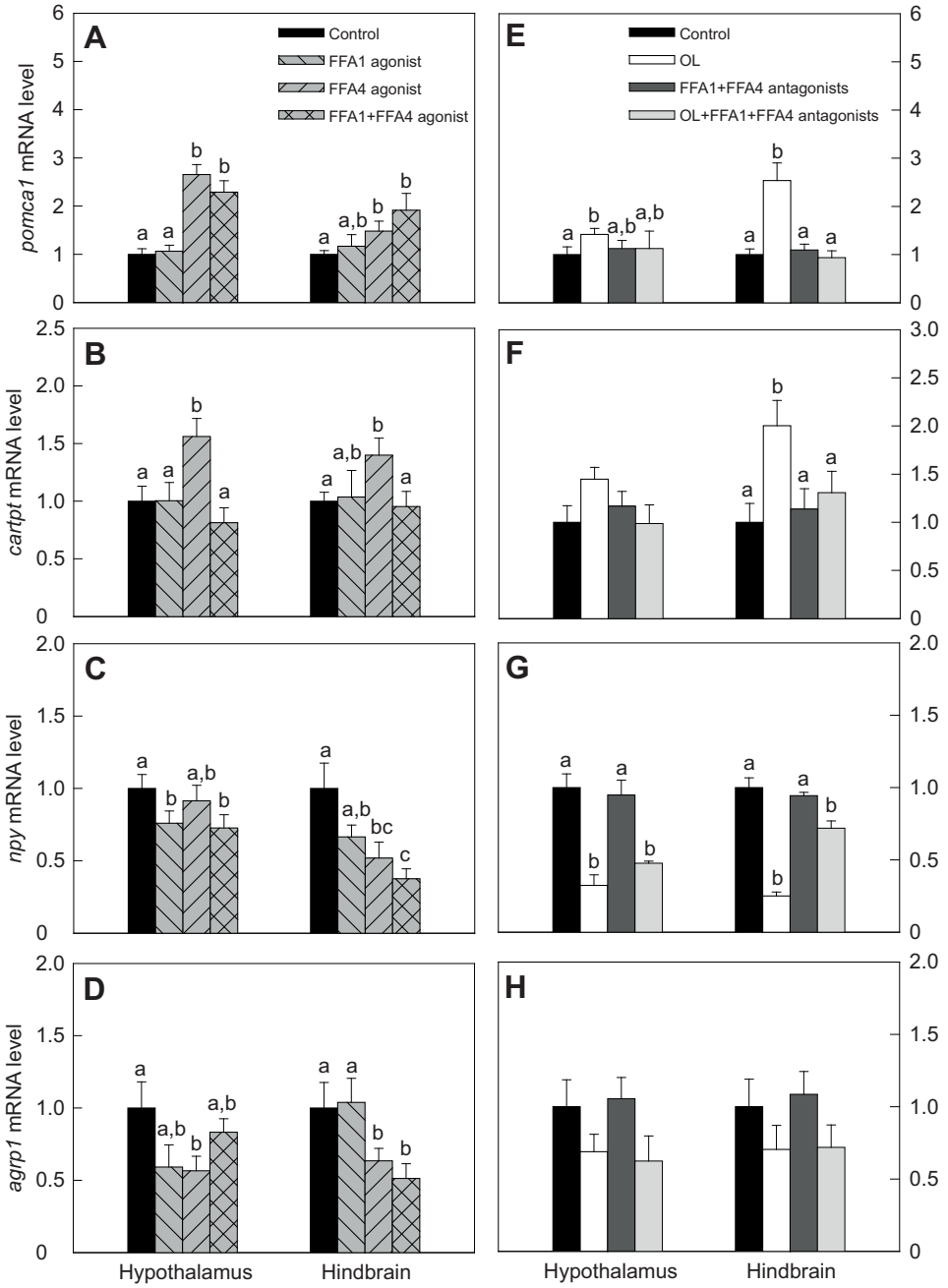

Fig. 3. Neuropeptide mRNA abundance. mRNA levels of *pomca1* (A,E), *cartpt* (B,F), *npy* (C,G) and *agrp1* (D,H) in the hypothalamus and hindbrain of rainbow trout 2 h after i.c.v. administration of (left-hand panels) 1 µl 100 g^{-1} body mass of DMSO–saline alone (control), or containing 2 mmol l^{-1} of FFA1 agonist (TUG424), or 1 mmol l^{-1} of FFA4 agonist (TUG891), or 1 mmol l^{-1} of FFA1+FFA4 agonist (GW9508); and (right-hand panels) 1 µl 100 g^{-1} body mass of vehicle alone (control), or containing 1 µmol oleate (OL), or 0.1 mmol l^{-1} FFA1+FFA4 antagonists (C260126 and AH7614), or 1 µmol oleate+FFA1+FFA4 antagonists (OL+FFA1+FFA4 antagonists). Each value is the mean±s.e.m. of N=8 fish per treatment. Gene expression results are given relative to the control group 2 h after treatment previously normalized by *actb* and *eef1a1* expression. Different lower case letters indicate significant differences (P<0.05) from different treatments (one-way ANOVA, P<0.05; *post hoc* SNK test, P<0.05).

Neuropeptide modulation by agonists and antagonists of FFA1 and FFA4

The anorectic effects of FFA1 and FFA4 agonists are consistent with changes observed in the mRNA abundance of some of the anorexigenic and orexigenic peptides involved in the metabolic regulation of food intake in mammals (Blouet and Schwartz, 2010) and fish (Soengas, 2014; Delgado et al., 2017; Soengas et al., 2018). In the hypothalamus, i.c.v. treatment with FFA4 agonist increased mRNA abundance of the anorexigenic peptides *pomca1* and *cartpt* and decreased *agrp1* mRNA abundance. Central treatment with FFA1 agonist decreased mRNA abundance of the orexigenic peptides *npy* and *agrp1*, while no changes occurred in the levels of the anorexigenic peptides *pomca1* and *cartpt*. These responses are different from those reported in mammals where FFA1 agonists increase the mRNA abundance of *pomc* without altering the expression of *npy*. This difference might relate to the relatively higher degree of co-localization between FFA1- and NPY-expressing neurons compared with the co-expression degree of FFA1 and POMC, as demonstrated in mammals (Dragano et al., 2017). A clearer difference between fish and mammals was observed regarding the impact of FFA4 agonist treatment that in fish decreased mRNA abundance of *agrp1* and increased that of *pomca1* and *cartpt*, whereas no changes occurred in mammals (Dragano et al., 2017). Altogether, these observations suggest that the mechanisms underlying the anorexigenic effects of FFA1 and FFA4 agonists are different in the hypothalamus of fish and mammals. Thus, FFA1 agonist seems to modulate different neuronal populations, i.e. POMC/CART neurons in mammals and NPY neurons in fish. However, FFA4 agonist seems to play a more important role in the modulation of these peptides in fish compared with mammals. Hindbrain also seems to be involved in modulating food intake through FFA4 as mRNA abundance of the anorexigenic

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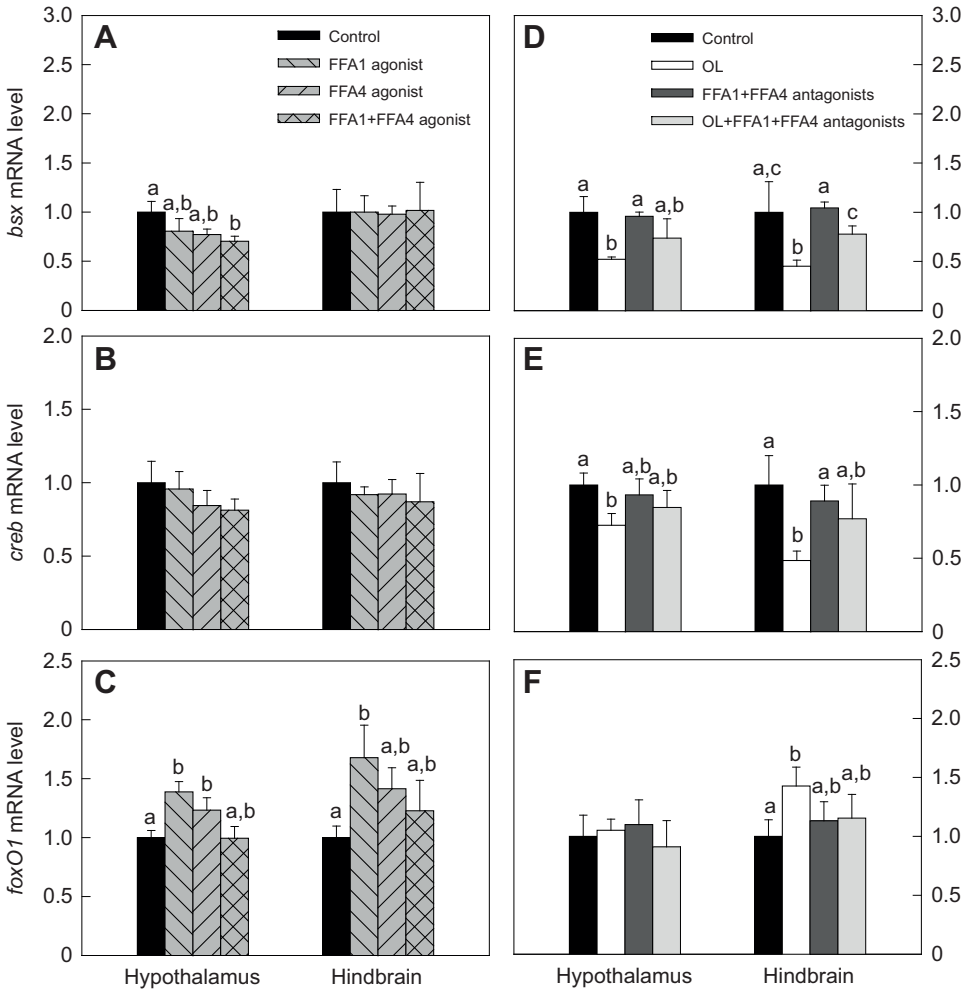

Fig. 4. mRNA abundance of transcription factors. mRNA abundance of *bsx* (A,D), *creb1* (B,E) and *foxO1* (C,F) in the hypothalamus and hindbrain of rainbow trout 2 h after i.c.v. administration of (left-hand panels) 1 µl 100 g^{-1} body mass of DMSO–saline alone (control), or containing 2 mmol l^{-1} of FFA1 agonist (TUG424), or 1 mmol l^{-1} of FFA4 agonist (TUG891), or 1 mmol l^{-1} of FFA1+FFA4 agonist (GW9508); and (right-hand panels) 1 µl 100 g^{-1} body mass of vehicle alone (control), or containing 1 µmol oleate (OL), or 0.1 mmol l^{-1} FFA1+FFA4 antagonists (C260126 and AH7614), or 1 µmol oleate+FFA1+FFA4 antagonists (OL+FFA1+FFA4 antagonists). Each value is the mean±s.e.m. of *N*=8 fish per treatment. Gene expression results are given relative to the control group 2 h after treatment previously normalized by *actb* and *eef1a1* expression. Different lower case letters indicate significant differences ($P<0.05$) from different treatments (one-way ANOVA, $P<0.05$; *post hoc* SNK test, $P<0.05$).

factors *pomca1* and *cartpt* increased after i.c.v. treatment with FFA4 agonist, and a decrease of *npy* and *agrp* levels was noted. The effects in hindbrain are not comparable to any study carried out in mammals. Changes in mRNA abundance of neuropeptides in both hypothalamus and hindbrain after agonist treatment are comparable to those observed after increased levels of oleate, as observed in Experiment 3 and in previous studies (Librán-Pérez et al., 2014; Velasco et al., 2016; 2017a). The involvement of FFA1 and FFA4 in the regulation of neuropeptide mRNA abundance is further supported by the finding that changes elicited by oleate generally disappeared in the presence of FFAR antagonists. However, this does not include orexigenic neuropeptides as decreased *npy* levels after oleate treatment remained in the presence of FFA1 and FFA4 antagonist. This is evidence that the action of oleate on orexigenic neuropeptides is also mediated by fatty acid sensor systems other than FFARs, as demonstrated in previous studies (Conde-Sieira and Soengas, 2017), and that fatty acid sensing in rainbow trout brain is only partially dependent on FFARs.

Role of FFA1 and FFA4 in the regulation of mechanisms involved in the control of food intake at the central level

The mechanisms linking the function of fatty acid-sensing systems with changes in the expression of neuropeptides, which ultimately regulate food intake, are partially known in mammals (Diéguez et al., 2011). They are apparently dependent on modulation of forkhead box O1 (FoxO1), cAMP response-element binding protein (Creb), and brain homeobox transcription factor (Bsx). Thus, decreased expression of Bsx and Creb and increased expression of FoxO1 occur in response to increased fatty acid levels (Nogueiras et al., 2008; Varela et al., 2011). Recent studies carried out in rainbow trout hypothalamus after oleate exposure (Conde-Sieira et al., 2018) displayed comparable responses. In the present study, we observed a similar response after oleate treatment, which was partially reverted in the presence of FFA1 and FFA4 antagonists. Considering the differences observed in the effects of different agonists, we suggest that only some of the effects of raised levels of oleate can be attributed to their detection by FFARs, with the remaining changes being attributable to other fatty acid-sensing systems. This is the first time, as far as we are aware, that changes in these transcription factors have been assessed in the brain of any animal after activation of FFARs. The changes observed suggest that the effects of FFARs on neuropeptide mRNA abundance might relate to changes observed in the transcription factors evaluated, with the possible exception of Creb.

Changes observed in transcription factors might relate to the activity of the different fatty acid-sensing systems, including FFARs, through different mechanisms (Diéguez et al., 2011; Gao et al., 2011; Morton et al., 2014) including modulation by AMP-activated protein kinase (AMPK) and protein kinase B (Akt). In the present study we observed that agonist treatment resulted in decreased phosphorylation status of Ampkα as well as increased phosphorylation status of Akt. The effect of FFA4 agonist on Akt phosphorylation is comparable to that observed in mammals (Im, 2018), whereas no other available studies assessed the impact of

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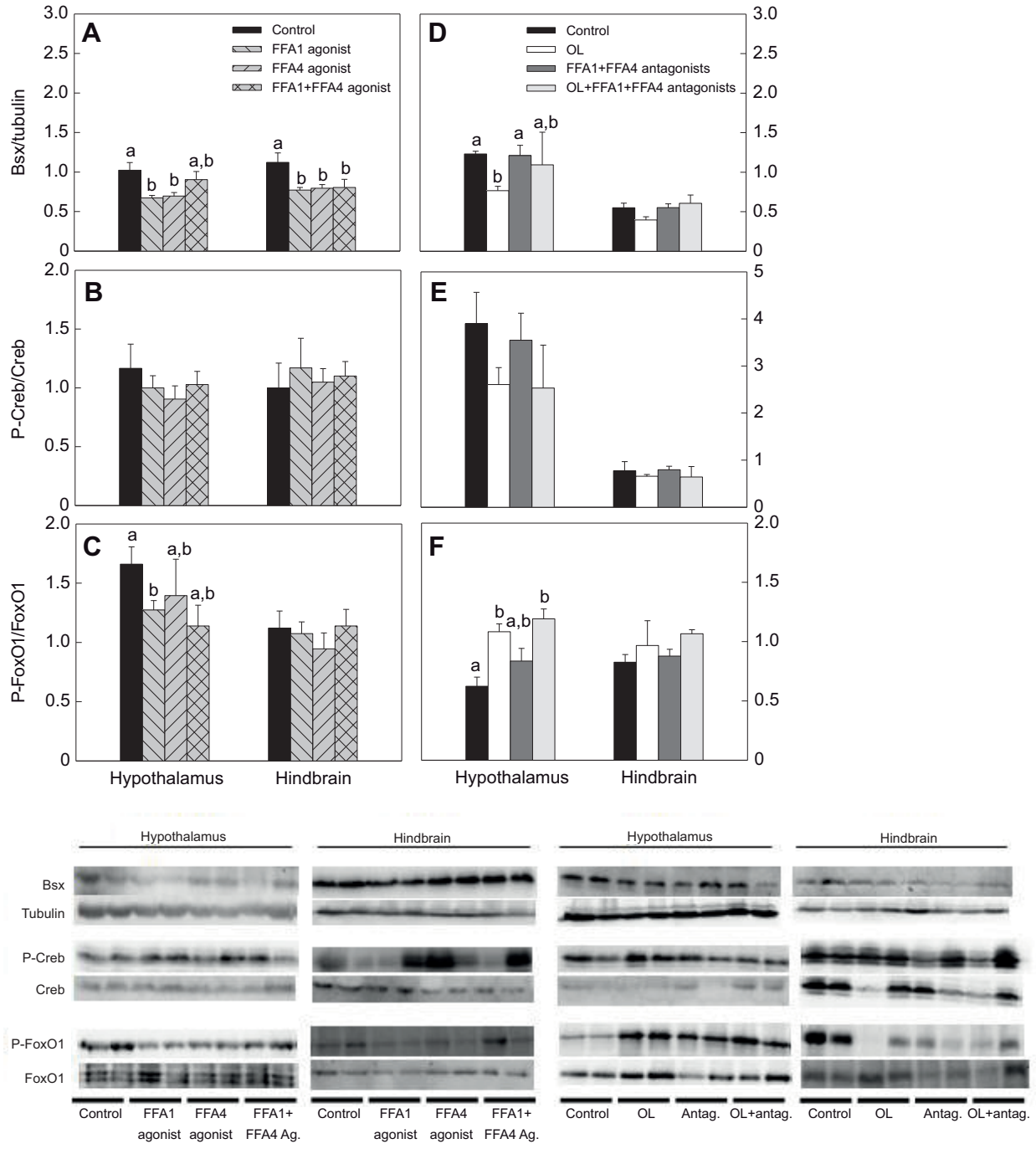

Fig. 5. Western blot analysis of transcription factors. Western blot analysis of Bsx (A,D), and phosphorylation status of Creb (B,E) and FoxO1 (C,F) in the hypothalamus and hindbrain of rainbow trout 2 h after i.c.v. administration of (left-hand panels) 1 µl 100 g^{-1} body mass of DMSO–saline alone (control), or containing 2 mmol l^{-1} of FFA1 agonist (TUG424), or 1 mmol l^{-1} of FFA4 agonist (TUG891), or 1 mmol l^{-1} of FFA1+FFA4 agonist (GW9508); and (right-hand panels) 1 µl 100 g^{-1} body mass of vehicle alone (control), or containing 1 µmol oleate (OL), or 0.1 mmol l^{-1} FFA1+FFA4 antagonists (C260126 and AH7614), or 1 µmol oleate+FFA1+FFA4 antagonists (OL+ FFA1+FFA4 antagonists). Total protein (20 µg) was loaded onto the gel per lane, and results were normalized by β-tubulin abundance. Western blots were performed on eight individual samples per treatment and two representative blots per treatment are shown here. Graphs of Creb and FoxO1 represent the ratio between the phosphorylated protein and the total amount of the target protein. Each value is the mean±s.e.m. of *N*=8 fish per treatment. Different lower case letters indicate significant differences (P<0.05) from different treatments (one-way ANOVA, P<0.05; *post hoc* SNK test, P<0.05).

FFA1 or FFA4 on AMPK function. However, the decreased phosphorylation of AMPKα is a typical response of hypothalamus after oleate exposure in both mammals (López et al., 2007) and fish (Velasco et al., 2017b). These changes were comparable to those elicited by oleate treatment alone, thus supporting that at least part of the signalling induced by the presence of oleate is elicited through these mechanisms. The partial reversal of effects of oleate in the presence of antagonist further supports this finding.

Changes in Ampkα and Akt should relate to changes in the signalling mechanisms activated by FFARs. Numerous studies have

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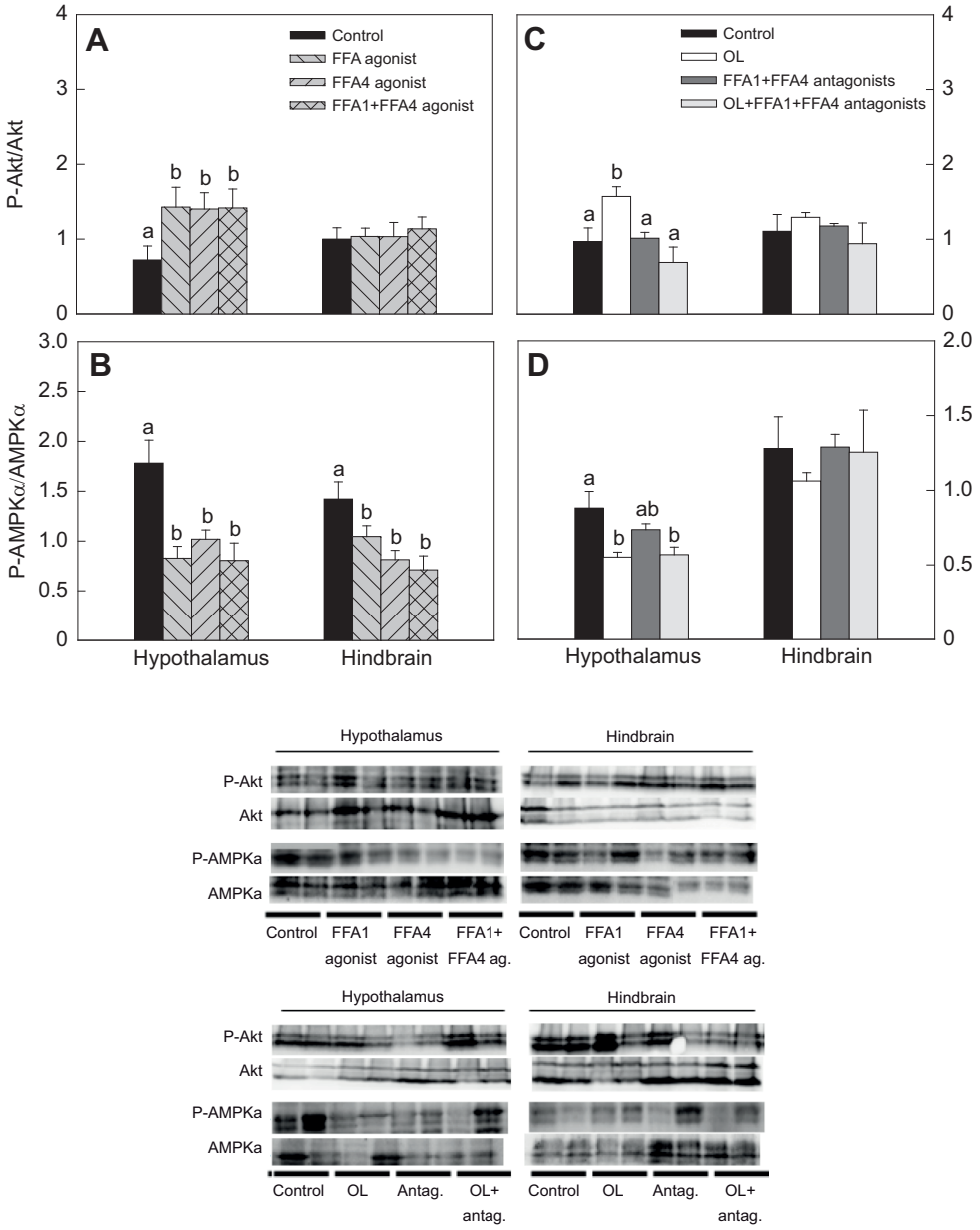

Fig. 6. Western blot analysis of Akt and AMPKα. Phosphorylation status of Akt (A, C) and AMPKα (B,D) in the hypothalamus and hindbrain of rainbow trout 2 h after i.c.v. administration of (left-hand panels) 1 µl 100 g^{-1} body mass of DMSO–saline alone (control), or containing 2 mmol l^{-1} of FFA1 agonist (TUG424), or 1 mmol l^{-1} of FFA4 agonist (TUG891), or 1 mmol l^{-1} of FFA1+FFA4 agonist (GW9508); and (right-hand panels) 1 µl 100 g^{-1} body mass of vehicle alone (control), or containing 1 µmol oleate (OL), or 0.1 mmol l^{-1} FFA1+FFA4 antagonists (C260126 and AH7614), or 1 µmol oleate+FFA1+FFA4 antagonists (OL+FFA1+FFA4 antagonists). Total protein (20 µg) was loaded onto the gel per lane, and results were normalized by β-tubulin abundance. Western blots were performed on eight 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. Each value is the mean±s.e.m. of *N*=8 fish per treatment. Different lower case letters indicate significant differences ($P<0.05$) from different treatments (one-way ANOVA, $P<0.05$; *post hoc* SNK test, $P<0.05$).

indicated that FFA1 and FFA4 are coupled with $G_{\alpha}q$ protein that activates phospholipase C-β (PLCβ), resulting in increased intracellular Ca^{2+} levels by 1,4,5-inositol triphosphate (IP_3) or diacylglycerol induced phosphorylation of protein kinase C (Kimura et al., 2020). Activation of extracellular signal regulated kinases (ERK1/2) has been confirmed as one of the downstream signalling cascades of FFAR1- and FFA4-$G_{\alpha}q$ protein signalling (Itoh et al., 2003; Katsuma et al., 2005). To our knowledge, there are no studies that assess the impact on these pathways of activation of these FFARs in brain. We have assessed some parameters related to these pathways in the present study. First, we evaluated changes in mRNA abundance of different forms of Plcβ. Of the four different *plcb* mRNAs assessed, only *plcb1* displayed changes in response to treatments, suggesting that this is the form involved in mediating FFAR action in fish brain. This mRNA abundance increased in hypothalamus after treatment with oleate, and this effect was reversed by the presence of FFA1 and FFA4 antagonists, suggesting that this parameter is involved in signalling in fish hypothalamus. The effect is probably attributable to FFA4 as this was the only FFA whose agonist induced a comparable increase in hypothalamus. We have also observed changes in mRNA abundance of *itpr3* that changed in a way comparable to that of *plcb1*, which is not surprising considering that IP_3 is placed 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 the signalling of FFARs assessed, especially for FFA1. It is also important to note that the changes described occurred only in hypothalamus. Thus, changes observed in hindbrain attributable to FFA1 and FFA4 must depend on other signalling pathways.

Conclusions

In summary, in rainbow trout brain areas involved in the control of food intake, such as hypothalamus and hindbrain, we have obtained evidence for a fatty acid-sensing role for FFA1 and FFA4. 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 neuropeptides *npy*, *agrp1*, *pomca1* and *cartpt*. Changes in neuropeptides can also relate to changes observed in

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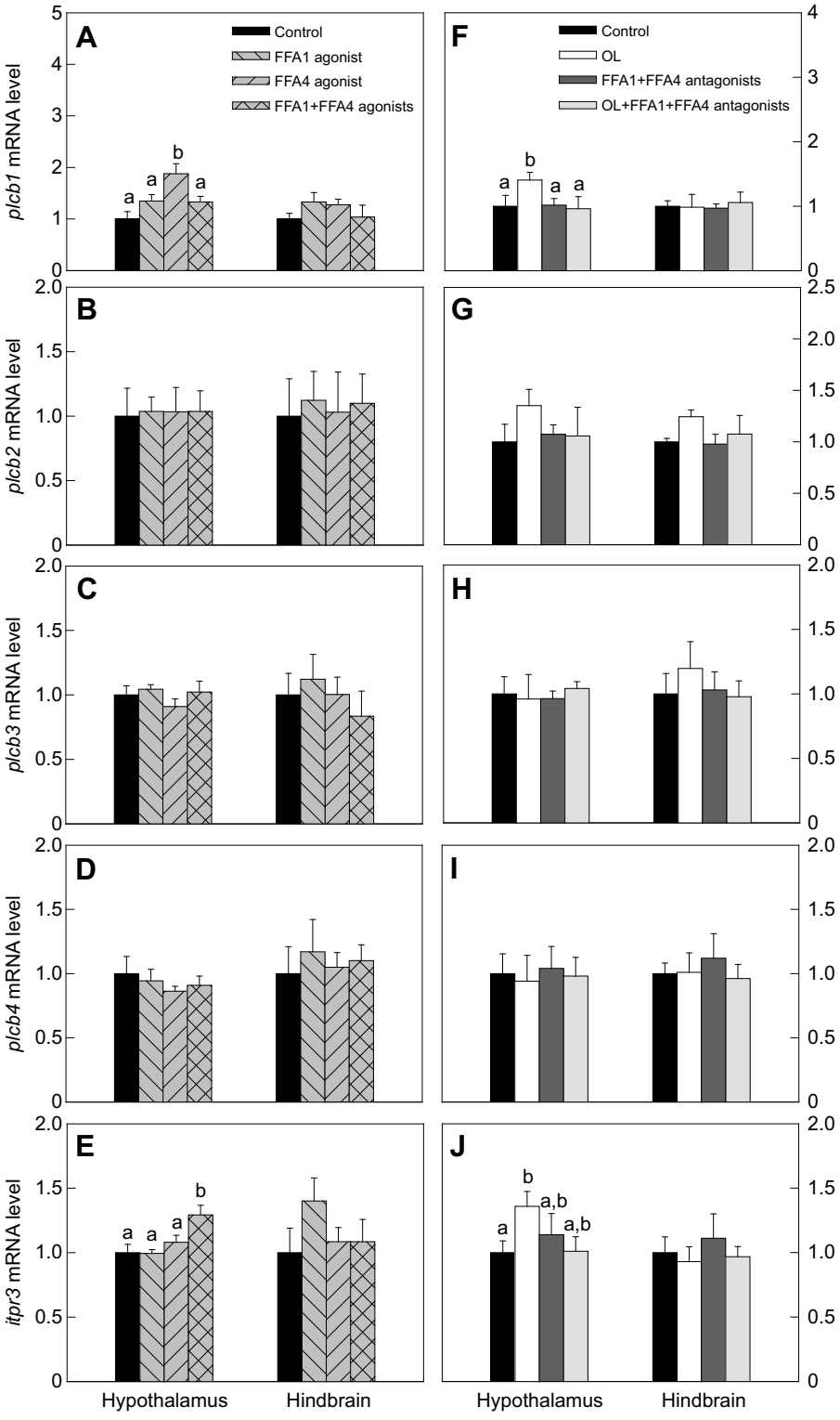

Fig. 7. mRNA abundance of downstream cascade components of FFAR1- and FFA4-$G_{\alpha}q$ protein. mRNA abundance of *plcb1* (A,F), *plcb2* (B, G), *plcb3* (C,H), *plcb4* (D,I) and *itpr3* (E,J) in the hypothalamus and hindbrain of rainbow trout 2 h after i.c.v. administration of (left-hand panels) 1 µl 100 g^{-1} body mass of DMSO–saline alone (control), or containing 2 mmol l^{-1} of FFA1 agonist (TUG424), or 1 mmol l^{-1} of FFA4 agonist (TUG891), or 1 mmol l^{-1} of FFA1+FFA4 agonist (GW9508); and (right-hand panels) 1 µl 100 g^{-1} body mass of vehicle alone (control), or containing 1 µmol oleate (OL), or 0.1 mmol l^{-1} FFA1+FFA4 antagonists (C260126 and AH7614), or 1 µmol oleate+FFA1+FFA4 antagonists (OL+ FFA1+FFA4 antagonists). Each value is the mean±s.e.m. of N=8 fish per treatment. Gene expression results given relative to the control group 2 h after treatment previously normalized by *actb* and *eef1a1* expression. Different lower case letters indicate significant differences (P<0.05) from different treatments (one-way ANOVA, P<0.05; *post hoc* SNK test, P<0.05).

mRNA abundance and protein levels/phosphorylation status of the transcription factors Bsx, Creb and FoxO1. These changes occurred in parallel with changes in phosphorylation status of Ampkα and Akt, as well as in signalling pathways related to PLCβ and IP_3 that might be involved in the action of both FFARs. Further support for these effects was obtained when comparing the effect of raised levels of oleate (the endogenous ligand of both FFARs) alone or in the presence of FFA1+FFA4 antagonist as most effects disappeared in the presence of antagonist. These changes support the idea that at least part of the capacity of fish brain to sense LCFA such as oleate depends on the function of these FFARs, and this is reflected in the control of food intake. Comparable studies in mammalian brain only evaluated changes in food intake, neuropeptide mRNA abundance and Akt levels after treatment with FFA1 or FFA4 agonist. Despite this limitation, when comparing the present study with those in mammals, a different response is apparent, especially considering

the response observed in neuropeptide mRNA abundance or the cellular signalling pathways involved. Overall, we provided evidence, for the first time in fish, for a role of FFA1 and FFA4 in central sensing of LCFA likely being involved in the regulation of food intake.

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Competing interests
The authors declare no competing or financial interests.

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

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References

Auguste, S., Fisette, A., Fernandes, M. F., Hryhorczuk, C., Poitout, V., Alquier, T. and Fulton, S. (2016). Central agonism of GPR120 acutely inhibits food intake and food reward and chronically suppresses anxiety-like behavior in mice. *Int. J. Neuropsychoph.* **19**, pyw014. doi:10.1093/ijnp/pyw014

Blouet, C. and Schwartz, G. J. (2010). Hypothalamic nutrient sensing in the control of energy homeostasis. *Behav. Brain Res.* **209**, 1-12. doi:10.1016/j.bbr.2009.12.024

Bruce, K. D., Zsombok, A. and Eckel, R. H. (2017). Lipid processing in the brain: a key regulator of systemic metabolism. *Front. Endocrinol.* **8**, 60. doi:10.3389/fendo.2017.00060

Conde-Sieira, M. and Soengas, J. L. (2017). Nutrient sensing systems in fish: impact on food intake regulation and energy homeostasis. *Front. Neurosci.* **10**, 603. doi:10.3389/fnins.2016.00603

Conde-Sieira, M., Bonacic, K., Velasco, C., Valente, L. M. P., Morais, S. and Soengas, J. L. (2015). Hypothalamic fatty acid sensing in Senegalese sole (*Solea senegalensis*): response to long-chain saturated, monounsaturated, and polyunsaturated (n-3) fatty acids. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **309**, R1521-R1531. doi:10.1152/ajpregu.00386.2015

Conde-Sieira, M., Ceinos, R. M., Velasco, C., Comesaña, S., López-Patiño, M. A., Míguez, J. M. and Soengas, J. L. (2018). Response of rainbow trout's (*Oncorhynchus mykiss*) hypothalamus to glucose and oleate assessed through transcription factors BSX, ChREBP, CREB, and FoxO1. *J. Comp. Physiol. A* **204**, 893-904. doi:10.1007/s00359-018-1288-7

Darling, R. A., Zhao, H., Kinch, D., Li, A.-J., Simasko, S. M. and Ritter, S. (2014). Mercaptoacetate and fatty acids exert direct and antagonistic effects on nodose neurons via GPR40 fatty acid receptors. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **307**, R35-R43. doi:10.1152/ajpregu.00536.2013

Delgado, M. J., Cerdá-Reverter, J. M. and Soengas, J. L. (2017). Hypothalamic integration of metabolic, endocrine, and circadian signals in fish: involvement in the control of food intake. *Front. Neurosci.* **11**, 355. doi:10.3389/fnins.2017.00354

de Pedro, N., Pinillos, M. L., Valenciano, A. I., Alonso-Bedate, M. and Delgado, M. J. (1998). Inhibitory effect of serotonin on feeding behavior in goldfish: involvement of CRF. *Peptides* **19**, 505-511. doi:10.1016/S0196-9781(97)00469-5

Diéguez, C., Vazquez, M. J., Romero, A., López, M. and Nogueiras, R. (2011). Hypothalamic control of lipid metabolism: focus on leptin, ghrelin and melanocortins. *Neuroendocrinology* **94**, 1-11. doi:10.1159/000328122

Dragano, N. R. V., Solon, C., Ramalho, A. F., de Moura, R. F., Razolli, D. S., Christiansen, E., Azevedo, C., Ulven, T. and Velloso, A. L. (2017). Polyunsaturated fatty acid receptors, GPR40 and GPR120, are expressed in the hypothalamus and control energy homeostasis and inflammation. *J. Neuroinflammation* **14**, 91. doi:10.1186/s12974-017-0869-7

Efeyan, A., Comb, W. C. and Sabatini, D. M. (2015). Nutrient sensing mechanisms and pathways. *Nature* **517**, 302-310. doi:10.1038/nature14190

Gao, S., Zhu, G., Gao, X., Wu, D., Carrasco, P., Casals, N., Hegardt, F. G., Moran, T. H. and Lopaschuk, G. D. (2011). Important roles of brain-specific carnitine palmitoyltransferase and ceramide metabolism in leptin hypothalamic control of feeding. *Proc. Natl. Acad. Sci. USA* **108**, 9691-9696. doi:10.1073/pnas.1103267108

Geurden, I., Aramendi, M., Zambonino-Infante, J. and Panserat, S. (2007). Early feeding of carnivorous rainbow trout (*Oncorhynchus mykiss*) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292**, R2275-R2283. doi:10.1152/ajpregu.00444.2006

Gong, Y., Chen, W., Han, D., Zhu, X., Yang, Y., Jin, J., Liu, H. and Xie, S. (2017). Effects of food restriction on growth, body composition and gene expression related in regulation of lipid metabolism and food intake in grass carp. *Aquaculture* **469**, 28-35. doi:10.1016/j.aquaculture.2016.12.003

Gorski, J. N., Pachanski, M. J., Mane, J., Plummer, C. W., Souza, S., Thomas-Fowlkes, B. S., Ogawa, A. M., Weinglass, A. B., Salvo, J. D., Cheewatrakoolpong, B. et al. (2017). GPR40 reduces food intake and body weight through GLP-1. *Am. J. Physiol. Endocrinol. Metab.* **313**, E37-E47. doi:10.1152/ajpendo.00435.2016

Hara, T., Kashihara, D., Ichimura, A., Kimura, I., Tsujimoto, G. and Hirasawa, A. (2014). Role of free fatty acid receptors in the regulation of energy metabolism. *Biochim. Biophys. Acta. Mol. Cell Biol. Lipids.* **1841**, 1292-1300. doi:10.1016/j.bbalip.2014.06.002

Husted, A. S., Trauelsen, M., Rudenko, O., Hjorth, S. A. and Schwartz, T. W. (2017). GPCR-mediated signaling of metabolites. *Cell Metab.* **25**, 777-796. doi:10.1016/j.cmet.2017.03.008

Im, D.-S. (2018). FFA4 (GPR120) as a fatty acid sensor involved in appetite control, insulin sensitivity and inflammation regulation. *Mol. Aspects Med.* **64**, 92-108. doi:10.1016/j.mam.2017.09.001

Itoh, Y., Kawamata, Y., Harada, M., Kobayashi, M., Fujii, R., Fukusumi, S., Ogi, K., Hosoya, M., Tanaka, Y., Uejima, H. et al. (2003). Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. *Nature* **422**, 173-176. doi:10.1038/nature01478

Kamalam, B. S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S. and Panserat, S. (2012). Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. *J. Exp. Biol.* **215**, 2567-2578. doi:10.1242/jeb.070581

Katsuma, S., Hatae, N., Yano, T., Ruike, Y., Kimura, M., Hirasawa, A. and Tsujimoto, G. (2005). Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1. *J. Biol. Chem.* **280**, 19507-19515. doi:10.1074/jbc.M412385200

Kimura, I., Ichimura, A., Ohue-Kitano, R. and Igarashi, M. (2020). Free fatty acid receptors in health and disease. *Physiol. Rev.* **100**, 171-210. doi:10.1152/physrev.00041.2018

Kolditz, C., Borthaire, M., Richard, N., Corraze, G., Panserat, S., Vachot, C., Lefèvre, F. and Médale, F. (2008). Liver and muscle metabolic changes induced by dietary energy content and genetic selection in rainbow trout (*Oncorhynchus mykiss*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R1154-R1164. doi:10.1152/ajpregu.00766.2007

Lagerström, M. C. and Schiöth, H. B. (2008). Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat. Rev. Drug Discov.* **7**, 339-357. doi:10.1038/nrd2518

Lansard, M., Panserat, S., Seiliez, I., Polakof, S., Plagnes-Juan, E., Geurden, I., Médale, F., Kaushik, S., Corraze, G. and Skiba-Cassy, S. (2009). Hepatic protein kinase B (Akt)-target of rapamycin (TOR)-signalling pathways and intermediary metabolism in rainbow trout (*Oncorhynchus mykiss*) are not significantly affected by feeding plant-based diets. *Br. J. Nutr.* **102**, 1564-1573. doi:10.1017/S000711450999095X

Li, A., Yuan, X., Liang, X.-F., Liu, L., Li, J., Li, B., Fang, J., Li, J., He, S., Xue, M. et al. (2016). Adaptations of lipid metabolism and food intake in response to low and high fat diets in juvenile grass carp (*Ctenopharyngodon idellus*). *Aquaculture* **457**, 43-49. doi:10.1016/j.aquaculture.2016.01.014

Librán-Pérez, M., Geurden, I., Dias, K., Corraze, G., Panserat, S. and Soengas, J. L. (2015). Feeding rainbow trout with a lipid-enriched diet: effects on fatty acid sensing, regulation of food intake and cellular signaling pathways. *J. Exp. Biol.* **218**, 2610-2619. doi:10.1242/jeb.123802

Librán-Pérez, M., López-Patiño, M. A., Míguez, J. M. and Soengas, J. L. (2013). Oleic acid and octanoic acid sensing capacity in rainbow trout *Oncorhynchus mykiss* is direct in hypothalamus and Brockmann bodies. *PLoS ONE* **8**, e59507. doi:10.1371/journal.pone.0059507

Librán-Pérez, M., Otero-Rodiño, C., López-Patiño, M. A., Míguez, J. M. and Soengas, J. L. (2014). Central administration of oleate or octanoate activates hypothalamic fatty acid sensing and inhibits food intake in rainbow trout. *Physiol. Behav.* **129**, 272-279. doi:10.1016/j.physbeh.2014.02.061

Librán-Pérez, M., Polakof, S., López-Patiño, M. A., Míguez, J. M. and Soengas, J. L. (2012). Evidence of a metabolic fatty-acid sensing system in the hypothalamus and Brockmann bodies of rainbow trout: implications in food intake regulation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **302**, R1340-R1350. doi:10.1152/ajpregu.00070.2012

López, M., Lelliott, C. J. and Vidal-Puig, A. (2007). Hypothalamic fatty acid metabolism: a housekeeping pathway that regulates food intake. *BioEssays* **29**, 248-261. doi:10.1002/bies.20539

Lu, V. B., Gribble, F. M. and Reimann, F. (2018). Free fatty acid receptors in enteroendocrine cells. *Endocrinology* **159**, 2826-2835. doi:10.1210/en.2018-00261

Magnan, C., Levin, B. E. and Luquet, S. (2015). Brain lipid sensing and the neural control of energy balance. *Mol. Cell. Endocrinol.* **418**, 3-8. doi:10.1016/j.mce.2015.09.019

Marinissen, M. J. and Gutkind, J. S. (2001). G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol. Sci.* **22**, 368-376. doi:10.1016/S0165-6147(00)01678-3

Morgan, K., Obici, S. and Rossetti, L. (2004). Hypothalamic responses to long-chain fatty acids are nutritionally regulated. *J. Biol. Chem.* **279**, 31139-31148. doi:10.1074/jbc.M400458200

Morton, G. J., Meek, T. H. and Schwartz, M. W. (2014). Neurobiology of food intake in health and disease. *Nat. Rev. Neurosci.* **15**, 367-378. doi:10.1038/nrn3745

Nogueiras, R., López, M., Lage, R., Perez-Tilve, D., Pfluger, P., Mendieta-Zerón, H., Sakkou, M., Wiedmer, P., Benoit, S. C., Datta, R. et al. (2008). Bsx, a novel hypothalamic factor linking feeding with locomotor activity, is regulated by energy availability. *Endocrinology* **149**, 3009-3015. doi:10.1210/en.2007-1684

Panserat, S., Blin, C., Médale, F., Plagnes-Juan, E., Brèque, J., Krishnamoorthy, J. and Kaushik, S. (2000). Molecular cloning, tissue distribution and sequence analysis of complete glucokinase cDNAs from gilthead seabream (*Sparus aurata*), rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). *Biochim. Biophys. Acta* **1474**, 61-69. doi:10.1016/S0304-4165(99)00213-5

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45. doi:10.1093/nar/29.9.e45

Polakof, S. and Soengas, J. L. (2008). Involvement of lactate in glucose metabolism and glucosensing function in selected tissues of rainbow trout. *J. Exp. Biol.* **211**, 1075-1086. doi:10.1242/jeb.014050

Polakof, S., Panserat, S., Plagnes-Juan, E. and Soengas, J. L. (2008a). Altered dietary carbohydrates significantly affect gene expression of the major glucosensing components in Brockmannn bodies and hypothalamus of rainbow trout. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R1077-R1088. doi:10.1152/ajpregu.90476.2008

Polakof, S., Míguez, J. M. and Soengas, J. L. (2008b). Dietary carbohydrates induce changes in glucosensing capacity and food intake in rainbow trout. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R478-R489. doi:10.1152/ajpregu.00176.2008

Quesada-López, T., Cereijo, R., Turatsinze, J.-V., Planavila, A., Cairó, M., Gavaldà-Navarro, A., Peyrou, M., Moure, R., Iglesias, R., Giralt, M. et al. (2016). The lipid sensor GPR120 promotes brown fat activation and FGF21 release from adipocytes. *Nat. Commun.* **7**, 13479. doi:10.1038/ncomms13479

Rohrer, D. K. and Kobilka, B. K. (1998). G protein-coupled receptors: functional and mechanistic insights through altered gene expression. *Physiol. Rev.* **78**, 35-52. doi:10.1152/physrev.1998.78.1.35

Skiba-Cassy, S., Lansard, M., Panserat, S. and Médale, F. (2009). Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, R1421-R1429. doi:10.1152/ajpregu.00312.2009

Soengas, J. L. (2014). Contribution of glucose- and fatty acid sensing systems to the regulation of food intake in fish. A review. *Gen. Comp. Endocrinol.* **205**, 36-48. doi:10.1016/j.ygcen.2014.01.015

Soengas, J. L., Cerdá-Reverter, J. M. and Delgado, M. J. (2018). Central regulation of food intake in fish: an evolutionary perspective. *J. Mol. Endocrinol.* **60**, R171-R199. doi:10.1530/JME-17-0320

Sun, P., Wang, T., Zhou, Y., Liu, H., Jiang, H., Zhu, W. and Wang, H. (2013). DC260126: a small-molecule antagonist of GPR40 that protects against pancreatic β-cells dysfunction in db/db mice. *PLoS ONE* **8**, e66744. doi:10.1371/journal.pone.0066744

Varela, L., Vázquez, M. J., Cordido, F., Nogueiras, R., Vidal-Puig, A., Diéguez, C. and López, M. (2011). Ghrelin and lipid metabolism: key partners in energy balance. *J. Mol. Endocrinol.* **46**, R43-R63. doi:10.1677/JME-10-0068

Velasco, C., Librán-Pérez, M., Otero-Rodiño, C., López-Patiño, M. A., Míguez, J. M., Cerdá-Reverter, J. M. and Soengas, J. L. (2016). Ghrelin modulates hypothalamic fatty acid-sensing and control of food intake in rainbow trout. *J. Endocrinol.* **228**, 25-37. doi:10.1530/JOE-15-0391

Velasco, C., Moreiras, G., Conde-Sieira, M., Leao, J. M., Míguez, J. M. and Soengas, J. L. (2017a). Ceramide counteracts the effects of ghrelin on the metabolic control of food intake in rainbow trout. *J. Exp. Biol.* **220**, 2563-2576. doi:10.1242/jeb.159871

Velasco, C., Otero-Rodiño, C., Comesaña, S., Míguez, J. M. and Soengas, J. L. (2017b). Hypothalamic mechanisms linking fatty acid sensing and food intake regulation in rainbow trout. *J. Mol. Endocrinol.* **59**, 377-390. doi:10.1530/JME-17-0148

Wacyk, J., Powell, M., Rodnick, K. J., Overturf, K., Hill, R. A. and Hardy, R. (2012). Dietary protein source significantly alters growth performance, plasma variables and hepatic gene expression in rainbow trout (*Oncorhynchus mykiss*) fed amino acid balanced diets. *Aquaculture* **356-357**, 223-234. doi:10.1016/j.aquaculture.2012.05.013