Hypothalamic mechanisms linking fatty acid sensing and food intake regulation in rainbow trout

Cristina Velasco, Cristina Otero-Rodiño, Sara Comesaña, Jesús M Míguez and José L Soengas

Laboratorio de Fisiología Animal, Departamento de Biología Funcional e Ciencias da Saúde, Facultade de Biología and Centro Singular de Investigación Mariña-ECIMAT, Universidade de Vigo, Vigo, Spain

Correspondence should be addressed to J L Soengas
Email
jsoengas@uvigo.es

Abstract

We assessed in rainbow trout hypothalamus the effects of oleate and octanoate on levels and phosphorylation status of two transcription factors, FoxO1 and CREB, possibly involved in linking activation of fatty acid sensing with modulation of food intake through the expression of brain neuropeptides. Moreover, we assessed changes in the phosphorylation status of three proteins possibly involved in modulation of these transcription factors such as Akt, AMPK and mTOR. In a first experiment, we evaluated, in pools of hypothalamus incubated for 3 h and 6 h at 15°C in a modified Hanks' medium containing 100 or 500 µM oleate or octanoate, the response of fatty acid sensing, neuropeptide expression and phosphorylation status of proteins of interest. The activation of fatty acid sensing and enhanced anorectic potential occurred in parallel with the activation of Akt and mTOR, and the inhibition of AMPK. The changes in these proteins would relate to a neuropeptide expression through changes in the phosphorylation status of transcription factors under their control, such as CREB and FoxO1, which displayed inhibitory (CREB) or activatory (FoxO1) responses when tissues were incubated with oleate or octanoate. In a second experiment, we incubated hypothalamus for 6 h with 500 µM oleate or octanoate alone or in the presence of specific inhibitors of Akt, AMPK, mTOR, CREB or FoxO1. The presence of inhibitors counteracted the effects of oleate or octanoate on the phosphorylation status of the proteins of interest. The results support, for the first time in fish, the involvement of these proteins in the regulation of food intake by fatty acids.

Key Words

- ▶ trout
- ▶ hypothalamus
- ▶ fatty acid sensing
- ▶ AMPK
- ▶ mTOR

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Introduction

The detection of changes in nutrient levels in vertebrate brain is an essential process involved in the regulation of food intake and energy expenditure as demonstrated in mammals (Blouet & Schwartz 2010, Morton *et al.* 2014) and fish (Delgado *et al.* 2017). Accordingly, several mechanisms are present in brain areas, especially hypothalamus, detecting changes in the levels of glucose,

fatty acids and amino acids as demonstrated in mammals (Efeyan *et al.* 2015, Bruce *et al.* 2017) and fish (Soengas 2014, Conde-Sieira & Soengas 2017). In previous studies in fish, we demonstrated that in rainbow trout (Librán-Pérez *et al.* 2012, 2013, 2014, 2015, Velasco *et al.* 2016a) and Senegalese sole (Conde-Sieira *et al.* 2015), the hypothalamus detects changes in the levels of specific

long-chain fatty acids (LCFA) through fatty acid sensing mechanisms based on carnitine palmitoyl transferase-1 (CPT-1), fatty acid translocase (FAT/CD36), increased capacity of mitochondria to produce ROS inhibiting K^+_{ATP} and lipoprotein lipase (LPL) activity. There is evidence for these mechanisms in other fish species like grass carp (Li *et al.* 2016). These mechanisms are, in general, comparable to those described in mammals (Blouet & Schwartz 2010, Lipina *et al.* 2014, Morton *et al.* 2014, Magnan *et al.* 2015) with the notable exception of the ability of fish systems for detecting not only changes in the levels of LCFA but also medium-chain fatty acid like octanoate (rainbow trout) and polyunsaturated fatty acid like α -linolenate (Senegalese sole). The activation of these systems results in an increase in the anorexigenic potential (balance between mRNA abundance of anorexigenic and orexigenic neuropeptides) through increased production of the anorexigenic peptides' pro-opio melanocortin (POMC) and cocaine- and amphetamine-related transcript (CART), and decreased production of the orexigenic peptides' neuropeptide Y (NPY) and agouti-related peptide (AgRP) ultimately leading to decreased food intake (Librán-Pérez *et al.* 2012, 2014, Velasco *et al.* 2016a). Evidence of changes in the neuropeptide expression after treating fish with fatty acids or feeding fish with lipid-enriched diets has been also observed in other species (Tang *et al.* 2013, Li *et al.* 2016).

The mechanisms linking the function of fatty acid sensing systems with changes in the expression of neuropeptides, which ultimately regulate food intake, are mostly unknown even in mammals. Changes in the expression of neuropeptides might relate to modulation of forkhead boxO1 (FoxO1), cAMP response element binding protein (CREB) and/or brain homeobox transcription factor (BSX) (Diéguez *et al.* 2011). However, it is not clear how these transcription factors relate to the activity of the different nutrient sensing systems. Several possibilities have been suggested in mammals (Diéguez *et al.* 2011, Gao *et al.* 2013, Morton *et al.* 2014) including modulation by AMP-activated protein kinase (AMPK), mechanistic target of rapamycin (mTOR) and protein kinase B (Akt). There are very few studies addressing the effects in mammalian hypothalamus of raised levels of LCFA in the amount and/or phosphorylation status of these proteins (Mayer & Belsham 2010). The available studies in fish hypothalamus demonstrated a decrease in phosphorylation status of AMPK, and an increase in those of Akt, and mTOR in hypothalamus of rainbow trout fed a lipid-enriched diet (Librán-Pérez *et al.* 2015) or increased Akt protein levels in isolated rainbow trout hypothalamic cells incubated with leptin (Gong *et al.* 2016).

Therefore, we aimed to assess in rainbow trout hypothalamus the effects of raised levels of oleate or octanoate on the phosphorylation status of two of the transcription factors, CREB and FoxO1, possibly involved in linking the activation of fatty acid sensing systems with the modulation of food intake through the expression of brain neuropeptides. Moreover, we also assessed the changes in the phosphorylation status of three proteins, Akt, AMPK and mTOR possibly involved in the modulation of these transcription factors. Since we observed changes in these proteins, we assessed in a second experiment the specificity of the responses using appropriate inhibitors.

Materials and methods

Fish

Rainbow trout were obtained from a local fish farm (Javier de la Calle, A Estrada, Spain). Fish were maintained for 1 month in 100L tanks under laboratory conditions and 12L:12D photoperiod (lights on at 08:00h, lights off at 20:00h) in dechlorinated tap water at 15°C. Fish weight was 98 ± 2 g. Fish were fed once daily (10:00h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Segovia, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat and 11.5% ash; 20.2MJ/kg of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 55/2013) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

Experimental design

Experiment 1: in vitro incubation with increased concentrations of oleate or octanoate

Freshly obtained tissues were incubated as previously described (Polakof *et al.* 2007). Fish were fasted for 24h before treatment to ensure that basal hormone levels were achieved. Every morning of an experiment, fish were dipnetted from the tank, anaesthetized with 2-phenoxyethanol (Sigma; 0.2% v/v), killed by decapitation and weighed. The hypothalamus (approx. 20mg) was removed as described by Doyon *et al.* (2003), rinsed with a modified Hanks' medium (136.9mM NaCl; 5.4mM KCl, 5mM NaHCO_3 , 1.5mM CaCl_2 , 0.81mM MgSO_4 , 0.44mM KH_2PO_4 , 0.33mM Na_2HPO_4 , 2mM glucose, 10mM HEPES, 50U/mL penicillin and 50 μ g/mL streptomycin sulphate, pH 7.4; referred to a basal medium), sliced (approximately 300 μ m) on chilled Petri dishes and placed in a chilled Petri dish containing 100mL

of modified Hanks' medium/g tissue that was gassed with 0.5% CO₂/99.5% O₂. To ensure adequate mass, tissues were combined from different fish resulting in pools of 3–4 hypothalami. Tissues were incubated in 48-well culture plates at 15°C for 3–6 h containing per well 25 mg of tissue and 250 µL of modified Hanks' medium alone (control) or containing (final concentration) 100 or 500 µM oleate or octanoate directly dissolved into the medium. The wells were gassed with a 0.5% CO₂/99.5% O₂ mixture. After 3 or 6 h of incubation, tissues were quickly removed, rinsed, frozen in liquid nitrogen and stored at –80°C until assayed. Fatty acid concentrations were selected based on previous *in vitro* studies in rainbow trout (Sánchez-Gurmaches *et al.* 2010, Librán-Pérez *et al.* 2013). In each experiment, one set of 4 tissue pools was assessed for the assay of mRNA levels, and another set of 4 tissue pools was assessed for the assay by Western blot of changes in the levels of proteins. The number of independent experiments (one set of 4 tissue pools each) carried out was 6 (*N*=6).

Experiment 2: *in vitro* incubation with oleate or octanoate alone or in the presence of inhibitors of proteins of interest

Tissues were incubated for 6 h as described in experiment 1 in the modified Hanks' medium alone (C, control), or containing 500 µM oleate (OL), or containing 500 µM octanoate (OC), or containing 500 µM oleate and selected inhibitors of proteins of interest, or containing 500 µM octanoate and selected inhibitors of proteins of interest. The inhibitors used are 1 mM perifosine (Akt inhibitor; 1,4-[[hydroxy(octadecyloxy)phosphinyl]oxy]-1,1-dimethylpiperidinium inner salt), 10 µM compound C (AMPK inhibitor; 6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine), 100 nM rapamycin (mTOR inhibitor; 23,27-epoxy-3H-pyrido[2,1-c][1,4]oxazacyclohentacontine), 5 µM CBP–CREB interaction inhibitor (CREB inhibitor; N-(4-chlorophenyl)-3-hydroxy-2-naphthamide) and AS1842856 (FoxO1 inhibitor; 5-amino-7-(cyclohexylamino)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid). Inhibitors were purchased from Sigma (perifosine, compound C and rapamycin) and Calbiochem (CBP–CREB interaction inhibitor and AS1842856). The inhibitors were previously dissolved in DMSO; no effects were observed due to the vehicle alone (data not shown). The concentrations of inhibitors was selected based on previous studies in rainbow trout for rapamycin (Lansard *et al.* 2010) and compound C (Magnoni *et al.* 2012), and in mammals for perifosine (Li *et al.* 2010), CBP–CREB interaction

inhibitor (Corsini *et al.* 2014) and AS1842856 (Nagashima *et al.* 2010). Compound C besides inhibiting AMPK has some unspecific actions (Bain *et al.* 2007, López *et al.* 2008). Therefore, its inhibitory effects have to be taken with caution. After 6 h incubation, tissues were quickly removed, rinsed, frozen and stored at –80°C until assayed. In each experiment, one set of 4 tissue pools was assessed for the assay by Western blot of changes in the levels of proteins. The number of independent experiments (one set of 4 tissue pools each) carried out was 6 (*N*=6).

mRNA abundance analysis by RT-qPCR

Total RNA was extracted using Trizol reagent (Life Technologies) and subsequently treated with RQ1-DNase (Promega). Two micrograms of total RNA were reverse-transcribed using Superscript II reverse transcriptase (Promega) and random hexamers (Promega) to obtain approx. 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 Mastermix (Life Technologies), in a total PCR reaction volume of 15 µL, containing 50–500 nM of each primer. mRNA abundance of transcripts was determined as previously described in the same species by Craig & Moon (2013) (AMPKα1), Sánchez-Gurmaches *et al.* (2012) (FAT/CD36, PPARγ), Kolditz *et al.* (2008) (FAS, LPL, PPARα), Polakof *et al.* (2008) (Kir6.x-like; 2011a, CPT1c), Rolland *et al.* (2015) (mTOR), Figueiredo-Silva *et al.* (2012) (UCP2a), Leder & Silverstein (2006) (POMCa1), Conde-Sieira *et al.* (2010) (CART, NPY) and MacDonald *et al.* (2014) (AgRP). Sequences of the forward and reverse primers used for each gene expression are shown in Table 1. Relative quantification of the target gene transcript was done using β-actin gene expression as reference, which was stably expressed in this experiment. Thermal cycling was initiated with incubation at 95°C for 90 s using hot-start iTaq DNA polymerase activation followed by 35 cycles, each one consisting of heating at 95°C for 20 s, and specific annealing and extension temperatures for 20 s. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C/s from 55 to 94°C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without RNA were run for each reaction as negative controls. Relative quantification of the target gene transcript with the β-actin reference gene transcript was made following the Pfaffl method (Pfaffl 2001).

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

	Forward primer	Reverse primer	Annealing temperature (°C)	Data base	Accession number
β -actin	GATGGCCAGAAAGACAGCTA	TCGTCCAGTTGGTGACGAT	59	GenBank	NM_001124235.1
AgRP	ACCAGAGTCCTGTCTGGTAA	AGTAGCAGATGGAGCCGAACA	60	GenBank	CR376289
AMPK α 1	ATCTTCTTACGCCAGCTA	GGGAGCTCATCTTGAACCA	60	GenBank	HQ40367
CART	ACCATGGAGGCTCCAG	GGCAGTGTCTCCAA	60	GenBank	NM_001124627
CPT1c	CGCTTCAAGAATGGGTGTAT	CAACACCTGCTGTTCTCA	59	GenBank	AJ619768
FAS	GAGACCTAGTGAGGCTGTC	TCTTTGATGGTGACGTGT	59	Sigenae	tcab0001c.e.06 5.1.s.om.8
FAT/CD36	CAAGTCAGCGCAAAACCGA	ACTTGTAGCTCCACGGA	62	DFCI	AY606034.1
Kir6.x-like	TTGGCTCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	60	Sigenae	CA346261.1.s.om.8:1:773:1
LPL	TAATTGGCTGCAGAAAACAC	CGTCAGCAAACTCAAAAGT	59	GenBank	AJ224693
mTOR	ATGGTTCGATCACTGGTCATCA	TCCACTCTGCCACAGAGAC	60	GenBank	EU179853
NPY	CTCGCTGGACCTTTATATGC	GTTTCATCATCTGGACTGTG	58	GenBank	NM_001124266
POMCa1	CTCGCTGTCAGACCTCAACTCT	GAGTTGGTTGGAGATGGACCTC	60	Tigr	TC86162
PPAR α	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGACGACAGAT	55	GenBank	AY494835
PPAR γ	GACGGCGGTGCTAGTCTTA	ATGCTCTGGCGAACTCTGT	60	DFCI	CA345564
SIRT-1	GCTACTTGGGGACTGTGACG	CTCAAAGTCTCCGCCCAAC	60	GenBank	EZ774344.1
SREBP1c	GACAAAGTGGTCCAGTTGCT	CACAGTTAGTCCGCATCAC	60	GenBank	CA048941.1
UCP2a	TCCGGCTACAGATCCAGG	CTCTCCACAGACACGCA	57	GenBank	DQ295324

AgRP, agouti-related peptide; AMPK α 1, AMP-activated protein kinase subunit α 1; CART, cocaine- and amphetamine-related transcript; CPT1c, carnitine palmitoyl transferase type 1c; FAS, fatty acid synthase; FAT/CD36, fatty acid translocase; Kir6.x-like, inward rectifier K⁺ channel pore type 6.x-like; LPL, Lipoprotein lipase; mTOR, mechanistic target of rapamycin; NPY, neuropeptide Y; POMCa1, pro-opio melanocortin α 1; PPAR α , peroxisome proliferator-activated receptor type α ; PPAR γ , peroxisome proliferator-activated receptor type γ ; SIRT1, sirtuin 1; SREBP1c, sterol regulatory element-binding protein type 1c; UCP2a, mitochondrial uncoupling protein 2a.

Western blot analysis

Frozen samples (20 mg) were homogenized in 1 mL of buffer containing 150 mM NaCl, 10 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA (pH 7.4), 100 mM sodium fluoride, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1% Triton X-100, 0.5% NP40-IGEPAL and 1.02 mg/mL protease inhibitor cocktail (Sigma). Tubes were kept on ice during the whole process to prevent protein denaturation. Homogenates were centrifuged at 1000g for 15 min at 4°C, and supernatants were again centrifuged at 20,000g for 30 min. The resulting supernatants were recovered and stored at -80°C. The concentration of protein in each sample was determined using Bradford assay with bovine serum albumin as standard. Hypothalamus protein lysates (20 μ g) were Western blotted using appropriate antibodies from Cell Signaling Technology: anti-phospho Akt (Ser473), anti-carboxyl terminal Akt, anti-phospho AMPK α (Thr172), anti-AMPK α , anti-phospho CREB (Ser133), anti-CREB (48h2), anti-phospho-FoxO1 (Thr24), anti-FoxO1 (L27), anti-phospho-mTOR (Ser2448) and anti- β -tubulin. All these antibodies cross-react successfully with rainbow trout proteins of interest (Skiba-Cassy *et al.* 2009, Kamalam *et al.* 2012, Velasco *et al.* 2016b). After washing, membranes were incubated with an IgG-HRP secondary antibody (BIO-RAD) and spots were quantified by Image Lab software version 5.2.1 (BIO-RAD).

Statistics

In the first experiment, comparisons among groups were carried out using two-way ANOVA with fatty acid concentration (100 and 500 μ M) and time (3 and 6 h) as main factors. In the second experiment, comparisons among groups were carried out using one-way ANOVA. The normal distribution of variables and homoscedasticity were analysed by Kolmogorov-Smirnov and Levene tests, respectively. In those cases where a significant effect was observed for a factor, comparisons were carried out by a Student Newman-Keuls test. Differences were considered statistically significant at $P < 0.05$. Comparisons were carried out with the SigmaStat statistical package.

Results

Experiment 1

The mRNA abundance of parameters related to metabolic control of food intake after oleate treatment is shown in Table 2. Changes described represent the comparison of treated groups vs controls; the remaining comparisons

Table 2 Changes in mRNA abundance of selected transcripts in hypothalamus of rainbow trout incubated *in vitro* for 3 or 6 h at 15°C in the modified Hanks' medium alone (control) or containing 100 or 500 µM oleate.

	3 h incubation			6 h incubation		
	Control	100 µM oleate	500 µM oleate	Control	100 µM oleate	500 µM oleate
Fatty acid sensing						
CPT1c	1.00 ± 0.08 ^a	0.63 ± 0.12 ^b	0.65 ± 0.05 ^b	1.01 ± 0.12 ^a	0.68 ± 0.06 ^b	0.52 ± 0.10 ^b
FAS	1.00 ± 0.07 ^a	0.75 ± 0.12 ^{a,b}	0.70 ± 0.09 ^b	0.95 ± 0.11 ^a	0.56 ± 0.09 ^b	0.53 ± 0.07 ^b
FAT/CD36	1.00 ± 0.07 ^a	0.76 ± 0.02 ^b	0.79 ± 0.08 ^b	0.93 ± 0.12 ^a	0.74 ± 0.02 ^a	0.60 ± 0.10 ^b
Kir6.x-like	1.00 ± 0.12 ^a	0.55 ± 0.07 ^b	0.66 ± 0.09 ^b	1.00 ± 0.11 ^a	0.68 ± 0.09 ^b	0.88 ± 0.07 ^{a,b,#}
LPL	1.00 ± 0.19 ^a	0.35 ± 0.08 ^b	0.21 ± 0.04 ^b	0.79 ± 0.15 ^a	0.40 ± 0.04 ^b	0.51 ± 0.07 ^{a,b,#}
PPARα	1.00 ± 0.08 ^a	1.32 ± 0.18 ^{a,b}	1.41 ± 0.06 ^b	1.08 ± 0.06	1.27 ± 0.11	1.18 ± 0.05
PPARγ	1.00 ± 0.04 ^a	0.83 ± 0.11 ^{a,b}	0.57 ± 0.10 ^b	1.09 ± 0.11 ^a	0.47 ± 0.08 ^b	0.80 ± 0.01 ^{a,b,#}
SREBP1c	1.00 ± 0.06 ^a	1.46 ± 0.19 ^b	2.14 ± 0.14 ^c	1.17 ± 0.07 ^a	1.69 ± 0.01 ^b	1.89 ± 0.17 ^b
UCP2a	1.00 ± 0.09 ^a	0.46 ± 0.10 ^b	0.50 ± 0.10 ^b	1.16 ± 0.05 ^a	0.46 ± 0.15 ^b	0.42 ± 0.15 ^b
Sensors						
AMPKα1	1.00 ± 0.10	1.37 ± 0.10	1.00 ± 0.11	1.34 ± 0.17 ^a	1.88 ± 0.16 ^{b,#}	1.75 ± 0.18 ^{a,b,#}
mTOR	1.00 ± 0.10 ^a	1.53 ± 0.13 ^b	1.62 ± 0.06 ^b	1.13 ± 0.14 ^a	1.06 ± 0.06 ^{a,#}	1.41 ± 0.04 ^{b,#}
SIRT-1	1.00 ± 0.08 ^a	1.35 ± 0.06 ^b	1.03 ± 0.09 ^a	1.26 ± 0.15 ^a	2.01 ± 0.13 ^{b,#}	2.43 ± 0.19 ^{b,#}
Neuropeptides						
AgRP	1.00 ± 0.04 ^a	0.81 ± 0.16 ^{a,b}	0.58 ± 0.06 ^b	0.89 ± 0.12	0.81 ± 0.15	0.90 ± 0.13
CART	1.00 ± 0.18 ^a	1.34 ± 0.11 ^a	1.57 ± 0.16 ^b	1.02 ± 0.04 ^a	1.40 ± 0.13 ^{a,b}	1.68 ± 0.15 ^b
NPY	1.00 ± 0.11 ^a	0.57 ± 0.06 ^b	0.31 ± 0.09 ^c	0.93 ± 0.14 ^a	0.75 ± 0.13 ^{a,b}	0.34 ± 0.11 ^b
POMCa1	1.00 ± 0.03 ^a	1.58 ± 0.09 ^b	1.60 ± 0.21 ^b	1.23 ± 0.11 ^a	2.30 ± 0.18 ^b	2.16 ± 0.08 ^b

Data represent mean ± s.e.m. of 6 independent experiments carried out with pools of hypothalamus from 3 to 4 different fish. Data are referred to the control group incubated for 3 h (results were previously normalized by β-actin mRNA levels, which did not show changes among groups). Different letters indicate significant differences from different treatments at the same time (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$). #, significantly different ($P < 0.05$) from 3 h at the same treatment (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$).

are shown in the table. The values of CPT1c decreased 3 and 6 h after treatment with oleate at both concentrations assessed. The values of FAS decreased 3 h after treatment with 500 µM oleate and after 6 h with both concentrations. Values of FAT/C36 decreased 3 h after treatment with 100 and 500 µM oleate, and after 6 h with 500 µM. The values of Kir6.x-like decreased after treatment with oleate with both concentrations after 3 h, and after 6 h with 100 µM oleate. Values of LPL decreased 3 h after treatment with both concentrations of oleate and 6 h after treatment with 100 µM oleate. The values of PPARα increased after 3 h of treatment with 500 µM oleate. The values of PPARγ decreased after treatment for 3 h with 500 µM oleate or after 6 h for 100 µM oleate. Values of SREBP1c increased after oleate at both times assessed with the increase after 3 h being dose-dependent. The values of UCP2a decreased for oleate treatments after 3 and 6 h. The values of AMPKα1 increased 6 h after 100 µM oleate treatment. The values of mTOR increased for oleate for both concentrations after 3 h and for 500 µM after 6 h. Values of SIRT-1 increased 3 h after 100 µM oleate and for both concentrations after 6 h. Values of AgRP decreased after 3 h treatment with 500 µM oleate. CART values increased 3 or 6 h after treatment with 500 µM oleate. The values of NPY displayed in the oleate-treated group a dose-dependent decrease after 3 h and a decrease after 6 h for the 500 µM dose. POMCa1 values

increased after 3 or 6 h treatment with both concentrations of oleate.

The mRNA abundance of parameters related to metabolic control of food intake after octanoate treatment is shown in Table 3. Changes described represent the comparison of treated groups vs controls; the remaining comparisons are shown in the table. The values of CPT1c decreased 3 h after treatment with octanoate at both concentrations assessed, whereas after 6 h, a decrease occurred with 500 µM octanoate. The values of FAS decreased after treatment with octanoate for both concentrations after 3 h and 100 µM after 6 h. Values of FAT/C36 decreased 6 h after treatment with 500 µM octanoate. The values of Kir6.x-like decreased after treatment with both concentrations of octanoate after 3 h and after 6 h for 500 µM octanoate. Values of LPL decreased 3 and 6 h after treatment with both concentrations of octanoate. The values of PPARα displayed a dose-dependent increase after 3 h of treatment with octanoate. The values of PPARγ decreased after 3 h treatment with 100 µM octanoate, and after 6 h treatment at both concentrations. Values of SREBP1c increased 3 h after octanoate treatment with both concentrations, and 6 h after 500 µM octanoate treatment. The values of UCP2a decreased for both octanoate treatment after 3 and 6 h. The values of AMPKα1 increased after octanoate treatment for 3 h (500 µM) and

Table 3 Changes in mRNA abundance of selected transcripts in hypothalamus of rainbow trout incubated *in vitro* for 3 or 6 h at 15°C in the modified Hanks' medium alone (control) or containing 100 or 500 µM octanoate.

	3 h incubation			6 h incubation		
	Control	100 µM octanoate	500 µM octanoate	Control	100 µM octanoate	500 µM octanoate
Fatty acid sensing						
CPT1c	1.00 ± 0.08 ^a	0.54 ± 0.10 ^b	0.68 ± 0.06 ^b	1.01 ± 0.12 ^a	0.72 ± 0.13 ^{a,b}	0.66 ± 0.04 ^b
FAS	1.00 ± 0.07 ^a	0.65 ± 0.17 ^b	0.76 ± 0.11 ^b	0.95 ± 0.11 ^a	0.56 ± 0.06 ^b	0.64 ± 0.13 ^{a,b}
FAT/CD36	1.00 ± 0.07	0.69 ± 0.14	0.88 ± 0.10	0.93 ± 0.12 ^a	0.72 ± 0.07 ^a	0.58 ± 0.03 ^{b,#}
Kir6.x-like	1.00 ± 0.12 ^a	0.47 ± 0.08 ^b	0.78 ± 0.09 ^b	1.00 ± 0.11 ^a	0.80 ± 0.10 ^{a,b,#}	0.77 ± 0.04 ^b
LPL	1.00 ± 0.19 ^a	0.32 ± 0.06 ^b	0.24 ± 0.06 ^b	0.79 ± 0.15 ^a	0.44 ± 0.09 ^{b,#}	0.49 ± 0.05 ^{b,#}
PPAR α	1.00 ± 0.08 ^a	1.27 ± 0.06 ^b	2.24 ± 0.22 ^c	1.08 ± 0.06	1.19 ± 0.11	1.13 ± 0.10 [#]
PPAR γ	1.00 ± 0.04 ^a	0.59 ± 0.14 ^b	1.02 ± 0.08 ^a	1.09 ± 0.11 ^a	0.63 ± 0.11 ^b	0.69 ± 0.07 ^{b,#}
SREBP1c	1.00 ± 0.06 ^a	2.06 ± 0.10 ^c	1.74 ± 0.14 ^b	1.17 ± 0.07 ^a	1.53 ± 0.15 ^a	2.21 ± 0.17 ^{b,#}
UCP2a	1.00 ± 0.09 ^a	0.56 ± 0.05 ^b	0.71 ± 0.06 ^b	1.16 ± 0.05 ^a	0.28 ± 0.02 ^{b,#}	0.27 ± 0.04 ^{b,#}
Sensors						
AMPK α 1	1.00 ± 0.10 ^a	1.35 ± 0.14 ^{a,b}	1.41 ± 0.14 ^b	1.34 ± 0.17 ^a	2.22 ± 0.21 ^{b,#}	2.44 ± 0.21 ^{b,#}
Mtor	1.00 ± 0.10 ^a	1.43 ± 0.17 ^b	1.81 ± 0.22 ^b	1.13 ± 0.14 ^a	1.62 ± 0.20 ^b	1.99 ± 0.20 ^b
SIRT-1	1.00 ± 0.08 ^a	1.01 ± 0.13 ^a	1.48 ± 0.15 ^b	1.26 ± 0.15 ^a	1.41 ± 0.12 ^a	2.20 ± 0.07 ^{b,#}
Neuropeptides						
AgRP	1.00 ± 0.04 ^a	0.64 ± 0.13 ^b	0.89 ± 0.07 ^{a,b}	0.89 ± 0.12	0.95 ± 0.05 [#]	0.92 ± 0.04
CART	1.00 ± 0.18 ^a	1.67 ± 0.13 ^b	1.76 ± 0.12 ^b	1.02 ± 0.04 ^a	1.84 ± 0.19 ^b	1.96 ± 0.07 ^b
NPY	1.00 ± 0.11 ^a	0.72 ± 0.09 ^b	0.27 ± 0.01 ^c	0.93 ± 0.14 ^a	0.55 ± 0.10 ^b	0.71 ± 0.01 ^{b,#}
POMCa1	1.00 ± 0.03 ^a	1.51 ± 0.15 ^b	2.42 ± 0.26 ^c	1.23 ± 0.11 ^a	2.81 ± 0.26 ^{b,#}	2.44 ± 0.18 ^b

Data represent mean $\pm$ s.e.m. of 6 independent experiments carried out with pools of hypothalamus from 3 to 4 different fish. Data are referred to the control group incubated for 3 h (results were previously normalized by β -actin mRNA levels, which did not show changes among groups). Different letters indicate significant differences from different treatments at the same time (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$), #, significantly different ($P < 0.05$) from 3 h at the same treatment (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$).

6 h (both concentrations). The values of mTOR increased in treated groups at all concentrations and times. Values of SIRT-1 increased after 3 h and 6 h of 500 µM octanoate treatment. Values of AgRP decreased after 3 h treatment with 100 µM octanoate. CART values increased after 3 or 6 h treatment with all concentrations of octanoate. The values of NPY displayed a decrease after 3 or 6 h of treatment with octanoate with the decrease after 3 h being dose-dependent. POMCa1 values increased 3 or 6 h after

treatment with both concentrations of octanoate with the increase after 3 h being dose-dependent.

The phosphorylation status of Akt is shown in Fig. 1. The treatment with 500 µM oleate resulted in increased values after 6 h in comparison with the control group. 500 µM octanoate treatment increased values after 3 h and 6 h compared with control group.

The phosphorylation status of AMPK α is shown in Fig. 2. Values decreased after 6 h of 100 µM or 500 µM

Figure 1

Western blot analysis of Akt phosphorylation status in hypothalamus of rainbow trout incubated *in vitro* for 3 or 6 h at 15°C in modified Hanks' medium alone (control, C) or containing 100 or 500 µM oleate (OL) or octanoate (OC). 20 µg of total protein was loaded on the gel per lane. Western blots were performed on 6 individual samples per treatment and one representative blot per treatment is shown here. Graphs represent the ratio of phosphorylated protein to total amount of the target protein. Each value denotes the mean $\pm$ s.e.m. of 6 independent experiments carried out with pools of hypothalamus from 3 to 4 different fish. Different letters indicate significant differences from different treatments at the same time (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$), #, significantly different ($P < 0.05$) from 3 h at the same glucose concentration (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$).

Figure 2

Western blot analysis of AMPK α phosphorylation status in hypothalamus of rainbow trout incubated *in vitro* for 3 or 6 h at 15°C in the modified Hanks' medium alone (control, C) or containing 100 or 500 μ M oleate (OL) or octanoate (OC). 20 μ g of total protein was loaded on the gel per lane. Western blots were performed on 6 individual samples per treatment and one representative blot per treatment is shown here. Graphs represent the ratio of phosphorylated protein to total amount of the target protein. Each value denotes the mean $\pm$ S.E.M. of 6 independent experiments carried out with pools of hypothalamus from 3 to 4 different fish. Different letters indicate significant differences from different treatments at the same time (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$) #, significantly different ($P < 0.05$) from 3 h at the same glucose concentration (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$).

oleate treatment in comparison with the control group. The treatment with octanoate decreased values after 3 h (500 μ M) and 6 h (both 100 and 500 μ M) compared with controls.

The phosphorylation status of mTOR is shown in Fig. 3. The treatment with oleate increased values after 3 h (100 μ M) or 6 h (500 μ M) compared with controls. The treatment with 500 μ M octanoate raised values after 6 h of incubation.

The phosphorylation status of CREB is shown in Fig. 4. The treatment with 100 μ M oleate decreased values after 3 h in comparison with the control group. The treatment with octanoate (both 100 and 500 μ M) decreased values after 6 h compared with controls.

The phosphorylation status of FoxO1 is shown in Fig. 5. The treatment with 500 μ M oleate raised values compared with controls after 6 h of incubation.

The treatment with 500 μ M octanoate increased values in comparison with the control group both after 3 and 6 h.

Experiment 2

The response of phosphorylation status of proteins of interest to 6 h treatment with 500 μ M oleate alone or in combination with specific inhibitors is shown in Fig. 6. The increase in Akt values elicited by the presence of oleate was counteracted in the presence of perifosine (Fig. 6A). AMPK α values that decreased in the presence of oleate did not show significant differences when compared with the control group in the oleate-compound C treated group (Fig. 6B). The increased values of mTOR observed in the group treated with oleate disappeared in the group treated with oleate and rapamycin (Fig. 6C). The values of CREB that decreased in the presence of oleate alone did

Figure 3

Western blot analysis of mTOR phosphorylation status in hypothalamus of rainbow trout incubated *in vitro* for 3 or 6 h at 15°C in the modified Hanks' medium alone (control, C) or containing 100 or 500 μ M oleate (OL) or octanoate (OC). 20 μ g of total protein was loaded on the gel per lane. Western blots were performed on 6 individual samples per treatment and one representative blot per treatment is shown here. Graphs represent the ratio of phosphorylated protein to β -tubulin. Each value denotes the mean $\pm$ S.E.M. of 6 independent experiments carried out with pools of hypothalamus from 3 to 4 different fish. Different letters indicate significant differences from different treatments at the same time (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$) #, significantly different ($P < 0.05$) from 3 h at the same glucose concentration (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$).

Figure 4

Western blot analysis of CREB phosphorylation status in hypothalamus of rainbow trout incubated *in vitro* for 3 or 6 h at 15°C in the modified Hanks' medium alone (control, C) or containing 100 or 500 µM oleate (OL) or octanoate (OC). 20 µg of total protein was loaded on the gel per lane. Western blots were performed on 6 individual samples per treatment and one representative blot per treatment is shown here. Graphs represent the ratio of phosphorylated protein to total amount of the target protein. Each value denotes the mean + S.E.M. of 6 independent experiments carried out with pools of hypothalamus from 3 to 4 different fish. Different letters indicate significant differences from different treatments at the same time (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$) #, significantly different ($P < 0.05$) from 3 h at the same glucose concentration (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$).

not show differences with the control group after joint treatment of oleate and CBP-CREB interaction inhibitor (Fig. 6D). The values of FoxO1 increased in the presence of oleate and this effect was counteracted in the additional presence of AS1842856 (Fig. 6E).

The response of phosphorylation status of proteins of interest to 6 h treatment with 500 µM octanoate alone or in combination with specific inhibitors is shown in Fig. 7. The increase in Akt values elicited by the presence of octanoate was counteracted in the presence of perifosine (Fig. 7A). No significant changes were observed in AMPKα values (Fig. 7B). The increased values of mTOR observed in the group treated with octanoate disappeared in the group treated with octanoate and rapamycin (Fig. 7C). The values of CREB that decreased in the presence of octanoate alone did not show differences with the control group after joint treatment of octanoate and CBP-CREB interaction inhibitor (Fig. 7D). The values of

FoxO1 increased in the presence of octanoate and this effect was counteracted in the additional presence of AS1842856 (Fig. 7E).

Discussion

Oleate and octanoate treatments activate fatty acid sensing systems and modulate neuropeptide expression

The exposure to raised levels of oleate or octanoate induced changes in mRNA abundance of several parameters related to fatty acid sensing mechanisms present in rainbow trout hypothalamus (Conde-Sieira & Soengas 2017). These include the increase in mRNA levels of SREBP1c, SIRT-1 and PPARα, as well as the decrease in mRNA levels of FAT/CD36, CPT1c, FAS, Kir6.x-like, LPL, PPARγ and UCP2a. These changes were comparable to those previously observed in hypothalamus of the

Figure 5

Western blot analysis of FoxO1 phosphorylation status in hypothalamus of rainbow trout incubated *in vitro* for 3 or 6 h at 15°C in the modified Hanks' medium alone (control, C) or containing 100 or 500 µM oleate (OL) or octanoate (OC). 20 µg of total protein was loaded on the gel per lane. Western blots were performed on 6 individual samples per treatment and one representative blot per treatment is shown here. Graphs represent the ratio of phosphorylated protein to total amount of the target protein. Each value denotes the mean + S.E.M. of 6 independent experiments carried out with pools of hypothalamus from 3 to 4 different fish. Different letters indicate significant differences from different treatments at the same time (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$) #, significantly different ($P < 0.05$) from 3 h at the same glucose concentration (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$).

Figure 6

Western blot analysis of phosphorylation status of Akt (A), AMPK α (B), mTOR (C), CREB (D) and FoxO1 (E) in hypothalamus of rainbow trout incubated *in vitro* for 6 h at 15°C in the modified Hanks' medium alone (C, control) or containing 500 μ M oleate (OL) or containing 500 μ M oleate and selected inhibitors of proteins of interest. These included 1 mM perifosine (OL+P, Akt inhibitor), 10 μ M compound C (OL+C, AMPK inhibitor), 100 nM rapamycin (OL+R, mTOR inhibitor), 5 μ M CBP-CREB interaction inhibitor (OL+CB, CREB inhibitor) and AS1842856 (OL+A, FoxO1 inhibitor). 20 μ g of total protein was loaded on the gel per lane. Western blots were performed on 6 individual samples per treatment and one representative blot per treatment is shown here. Graphs represent the ratio of phosphorylated protein to total amount of the target protein (except mTOR for which β -tubulin was used as a reference). Each value denotes the mean $\pm$ S.E.M. of 6 independent experiments carried out with pools of hypothalamus from 3 to 4 different fish. Different letters indicate significant differences from different treatments at the same time (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$) #, significantly different ($P < 0.05$) from 3 h at the same glucose concentration (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$).

Figure 7

Western blot analysis of phosphorylation status of Akt (A), AMPK α (B), mTOR (C), CREB (D) and FoxO1 (E) in hypothalamus of rainbow trout incubated *in vitro* for 6 h at 15°C in the modified Hanks' medium alone (C, control) or containing 500 μ M octanoate (OC) or containing 500 μ M octanoate and selected inhibitors of proteins of interest. These included 1 mM perifosine (OC+P, Akt inhibitor), 10 μ M compound C (OC+C, AMPK inhibitor), 100 nM rapamycin (OC+R, mTOR inhibitor), 5 μ M CBP-CREB interaction inhibitor (OC+CB, CREB inhibitor) and AS1842856 (OC+A, FoxO1 inhibitor). 20 μ g of total protein was loaded on the gel per lane. Western blots were performed on 6 individual samples per treatment and one representative blot per treatment is shown here. Graphs represent the ratio of phosphorylated protein to total amount of the target protein (except mTOR for which β -tubulin was used as a reference). Each value denotes the mean $\pm$ S.E.M. of 6 independent experiments carried out with pools of hypothalamus from 3 to 4 different fish. Different letters indicate significant differences from different treatments at the same time (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$) #, significantly different ($P < 0.05$) from 3 h at the same glucose concentration (two-way ANOVA $P < 0.05$, *post hoc* SNK test $P < 0.05$).

same species under comparable experimental conditions (Librán-Pérez *et al.* 2013), thus validating the experimental design. It is important to remember that these results are comparable in general to those described in mammals for oleate (Morton *et al.* 2014, Magnan *et al.* 2015) but not for octanoate, whose responses appear to be specific of fish (Conde-Sieira & Soengas 2017, Delgado *et al.* 2017). As for neuropeptides involved in the control of food intake, a sharp increase occurred in the mRNA abundance of anorexigenic neuropeptides POMCa1 and CART, whereas a decrease occurred in the mRNA abundance of orexigenic neuropeptides AgRP and NPY with the increase of oleate or octanoate levels in the medium. These results agree with prior evidence obtained in rainbow trout hypothalamus exposed to oleate or octanoate (Librán-Pérez *et al.* 2012, 2013, 2014), again supporting the experimental design. The increased anorexigenic potential is also in agreement with decreased food intake observed *in vivo* in the same species when subjected to raised levels of oleate or octanoate (Librán-Pérez *et al.* 2012, 2014). Therefore, we aimed to evaluate if under those conditions of activation of fatty acid sensing systems (eliciting changes in the expression of neuropeptides) changes occurred in the phosphorylation status of several proteins that are putatively involved in linking those processes.

Oleate and octanoate treatments affect the phosphorylation status of the transcription factors CREB and FoxO1

CREB is one of the molecules hypothesized to be connecting changes in brain metabolism with the expression of neuropeptides. Thus, in mammals, decreased CREB levels resulted in decreased mRNA abundance of AgRP and NPY, thus favouring decreased food intake (Belgardt *et al.* 2009, Blanco de Morentín *et al.* 2011). Accordingly, in the present study, the phosphorylation status of CREB clearly decreased with the increase of oleate or octanoate in the medium. The specificity of the response is further supported by the findings of experiment 2 in which the response of CREB to the presence of fatty acids disappeared in the presence of CBP–CREB interaction inhibitor. This is the first time in which changes in CREB abundance have been assessed in fish hypothalamus after treatment with fatty acids. In other fish tissues, the presence of CREB had been characterized in liver of goldfish (Yan *et al.* 2016) while in zebrafish, liver (Craig & Moon 2011) food deprivation (situation opposed to the raised levels of nutrients of the present study) resulted in increased mRNA levels of CREB. Even in mammals, as far as we are aware, there are

no comparable studies available regarding the effects of fatty acids on the levels and/or phosphorylation status of this protein. However, the abundance of this protein is known to decrease under conditions in which food intake is inhibited resulting in decreased expression of NPY/AgRP and thus inducing anorexigenic potential, such as after leptin treatment (Blanco de Morentín *et al.* 2011, Diéguez *et al.* 2011, Kwon *et al.* 2016).

FoxO1 is another molecule hypothesized to be connecting changes in brain nutrient sensing with the expression of neuropeptides (Gross *et al.* 2009). Thus, increased FoxO1 resulted in increased mRNA abundance of POMC and CART, thus favouring decreased food intake (Belgardt *et al.* 2009, Blanco de Morentín *et al.* 2011). In the present study, the treatment with oleate or octanoate increased the phosphorylation status of FoxO1, and this effect disappeared in the presence of the inhibitor AS1842856, thus giving further support to the specificity of the response. The only available studies regarding this protein in fish hypothalamus demonstrated a decrease in its phosphorylation status in rainbow trout treated with the orexigenic hormone ghrelin (Velasco *et al.* 2017), i.e., an opposed response to the anorexigenic effects of oleate or octanoate observed in the present study. In other tissues like liver, a decrease in the abundance of phosphorylated FoxO1 also occurred under refeeding conditions (Dai *et al.* 2013). In other fish species such as grass carp, increased FoxO1 occurred in adipocytes during adipogenesis, i.e., a situation also reflecting lipid abundance (Sun *et al.* 2017). The increased FoxO1 phosphorylation would be in agreement with the increased phosphorylation status observed in mammalian hypothalamus under anorexigenic conditions eliciting an increased expression of POMC and CART, such as after leptin treatment (Diéguez *et al.* 2011, Kwon *et al.* 2016).

It is not clear, not even in mammals, how changes in these transcription factors relate to the activity of nutrient sensing systems. Of the different possibilities suggested in mammals (Diéguez *et al.* 2011, Gao *et al.* 2013, Morton *et al.* 2014), we have evaluated the possible roles of Akt, AMPK and mTOR.

The phosphorylation status of the cellular signalling proteins Akt, AMPK and mTOR changed after oleate or octanoate treatments

In a previous study, we demonstrated that feeding rainbow trout with a lipid-enriched diet resulted in increased phosphorylation status of Akt (Librán-Pérez *et al.* 2015). Now, we demonstrate the effects of raised levels of

oleate or octanoate on Akt phosphorylation status in fish hypothalamus, with the ratio displaying an increase in parallel with the increase of fatty acid concentration in the medium. The specificity of the response is supported by the fact that the increase disappeared in the presence of the Akt inhibitor perifosine. The increased phosphorylation status of Akt is comparable to that observed in hypothalamus under treatments resulting in raised POMC mRNA abundance and therefore decreased food intake, such as leptin as demonstrated both in mammals (Kwon *et al.* 2016) and in rainbow trout hypothalamic neurons (Gong *et al.* 2016). In this way, it is interesting that ICV treatment in rainbow trout with the orexigenic peptide ghrelin resulted in a decreased phosphorylation ratio of Akt (Velasco *et al.* 2017), i.e., the converse response to that herein observed under an anorexigenic situation. Furthermore, in other situations in which levels of nutrients increase, such as under refeeding conditions, increased phosphorylation status of Akt also occurred in peripheral tissues of fish as demonstrated in livers of rainbow trout (Lansard *et al.* 2009, Seiliez *et al.* 2011) and barramundi (Wade *et al.* 2014). The activation of Akt induces FoxO1 phosphorylation (Gross *et al.* 2009). Then, those situations, like the present study, in which Akt is activated could presumably result in increased FoxO1 phosphorylation (Belgardt *et al.* 2009), which, on the other hand, is known to promote expressions of POMC and CART (Diéguez *et al.* 2011, Kwon *et al.* 2016). Then, a possible relationship may exist between Akt and FoxO1 activations ultimately resulting in changes in the neuropeptide expression.

AMPK allows detection of lowered cell energy status with coupling to intrinsic cell mechanisms designed to restore energy balance (López 2017). In rainbow trout fed a lipid-enriched diet a decrease occurred in phosphorylation status of AMPK α (Librán-Pérez *et al.* 2015). In the present study, while mRNA abundance of AMPK α 1 increased in the presence of oleate or octanoate, a clear decrease in the phosphorylation status of AMPK α occurred in the presence of both fatty acids. In mammalian hypothalamus, no comparable experimental approaches have been carried out, though anorectic situations induced by leptin treatment also resulted in decreased levels of phosphorylated AMPK and phosphorylation status (López 2017). These results are also comparable to the effect of refeeding resulting in a decreased ratio in mammalian hypothalamus (Gao *et al.* 2013). In fish, these results are also comparable to those obtained in liver exposed to situations such as refeeding in rainbow trout (Polakof *et al.* 2011b), while

under food deprivation conditions, an increase in ratio is known to occur as demonstrated in zebrafish (Craig & Moon 2011). As a whole, the changes displayed in hypothalamus fit with the role of AMPK as an energy gauge, thus decreasing values under situations of enhanced energy availability, which usually result in decreased food intake (López 2017).

Hypothalamic mTOR responded with a rise to the presence of oleate or octanoate in the medium, and this was reflected by the changes in mRNA abundance and the phosphorylation status of the protein. These results are comparable to the rise in the mTOR phosphorylation status observed in hypothalamus of the same species after feeding a lipid-enriched diet (Librán-Pérez *et al.* 2015). The presence of rapamycin in the medium blocked the hypothalamic response to fatty acids, thus giving further support to the response since this molecule is known to inhibit mTOR in peripheral tissues of rainbow trout (Dai *et al.* 2013, 2014). Little is known on the mechanisms engaged by fatty acids to modulate mTOR (André & Cota 2012), though the phosphorylation status of mTOR is known to increase under anorectic conditions, like those induced by leptin treatment, and to increase under orexigenic conditions, like those induced by ghrelin treatment (Diéguez *et al.* 2011, André & Cota 2012). In other fish tissues like liver, increased mTOR phosphorylation status occurred after feeding fish with lipid-enriched diets (Librán-Pérez *et al.* 2015, Zeng *et al.* 2016) or after refeeding (Lansard *et al.* 2009, Wade *et al.* 2014), while a decrease occurred under food deprivation (Craig & Moon 2011).

In summary, we have demonstrated for the first time in fish that the activation of fatty acid sensing systems in hypothalamus by oleate or octanoate, resulting in increased anorectic potential, occurs in parallel with changes in the phosphorylation status of proteins that may be involved in linking both processes. Thus, the activation of fatty acid sensing systems induces changes in cellular metabolism presumably resulting in the activation of Akt and mTOR, and the inhibition of AMPK. The changes in these proteins would relate to a neuropeptide expression through changes in the phosphorylation status of transcription factors under their control such as CREB and FoxO1. Knowing the precise mechanisms involved in these mechanisms, as well as the role of other components not assessed yet such as BSX, still needs evaluation. However, these results provide for the first time in fish evidence for all the steps involved in the hypothalamic regulation of food intake by fatty acids, from the detection of the raised levels of

the metabolite to the changes in neuropeptide expression ultimately leading to the regulation of food intake. Main results are comparable to those suggested in mammals for oleate but not for octanoate, suggesting again that in fish, the latter fatty acid is particularly important in the metabolic regulation of food intake.

Declaration of interest

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

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