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Effects of CCK-8 and GLP-1 on fatty acid sensing and food intake regulation in trout

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Abstract

We hypothesize that cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) are involved in the modulation of metabolic regulation of food intake by fatty acids in fish. Therefore, we assessed in rainbow trout (*Oncorhynchus mykiss*) the effects of intracerebroventricular treatment with 1 ng/g of CCK-8 and with 2 ng/g of GLP-1 on food intake, expression of neuropeptides involved in food intake control and the activity of fatty acid-sensing systems in hypothalamus and hindbrain. Food intake decreased up to 24 h post-treatment to 49.8–72.3% and 3.1–17.8% for CCK-8 and GLP-1, respectively. These anorectic responses are associated with changes in fatty acid metabolism and an activation of fatty acid-sensing mechanisms in the hypothalamus and hindbrain. These changes occurred in parallel with those in the expression of anorexigenic and orexigenic peptides. Moreover, we observed that the activation of fatty acid sensing and the enhanced anorectic potential elicited by CCK-8 and GLP-1 treatments occurred in parallel with the activation of mTOR and FoxO1 and the inhibition of AMPK α , BSX and CREB. The results are discussed in the context of metabolic regulation of food intake in fish.

Key Words

- ▶ CCK-8
- ▶ GLP-1
- ▶ hypothalamus
- ▶ hindbrain
- ▶ fatty acid sensing
- ▶ fish

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Introduction

The regulation of food intake is a complex process in which changes in circulating levels of metabolites and hormones as well as nervous signals are integrated in specific brain regions as demonstrated in mammals (Blouet & Schwartz 2010, Efeyan *et al.* 2015, Bruce *et al.* 2017) and fish (Soengas 2014, Delgado *et al.* 2017, Soengas *et al.* 2018). In previous studies (see reviews by Soengas 2014, Conde-Sieira & Soengas 2016) we demonstrated that fish hypothalamus and hindbrain are able to detect changes in the levels of specific long-chain fatty acids (LCFA) through fatty acid-sensing mechanisms. These are based on carnitine palmitoyl transferase-1 (CPT-1), fatty acid translocase (FAT/CD36), increased capacity of mitochondria to produce reactive oxygen species

inhibiting ATP-dependent inward rectified potassium channel (K^+_{ATP}) and lipoprotein lipase (LPL) activity. These mechanisms are, in general, comparable to those described in mammals (Blouet & 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 acid like octanoate and poly unsaturated fatty acid like α -linolenate (reviewed in Delgado *et al.* 2017, Soengas *et al.* 2018). The activation of these systems in fish occur in parallel with decreased activity of the energy sensor 5'-AMP-activated protein kinase (AMPK) and increased activity of mechanistic target of rapamycin (mTOR) (Velasco *et al.* 2017). The activation of nutrient-sensing systems in fish generally

occurred in parallel with 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) (Conde-Sieira & Soengas 2016). These changes ultimately lead to decreased food intake (Soengas *et al.* 2018) in a way comparable to that observed in mammals (Belgardt *et al.* 2009, Blouet & Schwartz 2010, Morton *et al.* 2014). The mechanisms linking the function of nutrient-sensing systems to changes in the expression of neuropeptides are mostly unknown, even in mammals. Changes in the expression of neuropeptides might relate to the modulation of forkhead boxO1 (FoxO1), cAMP response element-binding protein (CREB) and brain homeobox transcription factor (BSX) (Belgardt *et al.* 2009, Diéguez *et al.* 2011, Gao *et al.* 2013, Morton *et al.* 2014). However, it is not clear how these transcription factors respond to the activity of the different nutrient-sensing systems.

The function of nutrient-sensing systems and their impact on food intake regulation can be modulated by changes in the levels of hormones like glucagon-like peptide 1 (GLP-1), cholecystokinin (CCK), leptin, insulin or ghrelin, as demonstrated in mammals (Blouet & Schwartz 2010, Morton *et al.* 2014). To date, the information available in fish regarding the endocrine modulation of central fatty acid-sensing systems is scarce and restricted to insulin (Librán-Pérez *et al.* 2015), ghrelin (Velasco *et al.* 2016a), PYY₁₋₃₆ (Velasco *et al.* 2018) and nesfatin-1 (Blanco *et al.* 2018) with no information available regarding other gastrointestinal tract (GIT) hormones like CCK or GLP-1.

CCK belongs to the CCK-gastrin family characterized by an evolutionary conserved C-terminal sequence required for its biological activity (Konturek *et al.* 2003). In mammals, this peptide once released by endocrine I cells in the GIT (in response to the presence of food) binds to CCKAR receptor in the gut and the signal is transmitted by the vagus nerve to the hindbrain, and then relayed to the hypothalamus (Cheung *et al.* 2009). The release of CCK is stimulated mainly by the presence of fat and small peptides from digestion (Moran & Kinzig 2004). In addition CCK is also produced directly by the brain acting as a neurohormone with CCK-8 being the most abundant form in this area (Strader & Woods 2005). In fish, CCK and its binding sites have been identified in the brain and GIT of a large number of species, including rainbow trout *Oncorhynchus mykiss* (Jensen *et al.* 2001). A differential distribution pattern of CCK receptor subtypes is present in fish, as described in goldfish *Carassius auratus* (Tinoco *et al.* 2015), with a high expression of CCK₁ subtype in

the intestine whereas the CCK₂ subtype predominantly expressed in the hypothalamus and vagal lobe. Similar to mammals (Chandra & Liddle 2007), CCK in fish elicits several effects such as decreasing gastric emptying, stimulating gall bladder contraction and gastrointestinal motility (Holmgren & Olsson 2009). CCK also acts as a satiety signal resulting in anorectic effects in several fish species (Delgado *et al.* 2017, Soengas *et al.* 2018) including rainbow trout (Gélineau & Boujard 2001, Jönsson *et al.* 2006).

GLP-1 belongs to the family of glucagon and, in mammals, is mainly secreted by intestinal L cells in response to food, playing an important role in glucose metabolism (Lockie 2013, Andersen *et al.* 2018). This peptide has been identified in several fish species where is produced by both pancreas and intestine (Silverstein *et al.* 2001) and receptors are present in brain regions including the hypothalamus and hindbrain (Yeung *et al.* 2002). The known actions of GLP-1 in fish and mammals for the regulation of glucose metabolism are remarkably different. Thus, in fish, GLP-1 stimulates gluconeogenesis and glycogenolysis (Mommensen *et al.* 1987, Mojsov 2000, Irwin & Mojsov 2018), thereby increasing glycaemia, whereas in mammals it stimulates insulin release (incretin) and slows gastric emptying reducing glucose availability (Andersen *et al.* 2018). GLP-1 is also produced by some neurons in brain areas like the hindbrain playing an important role in the regulation of feeding behaviour (Drucker 2002). Both intracerebroventricular (ICV) and peripheral injection of GLP-1 decrease food intake in mammals (Strader & Woods 2005) and in teleost fish species like channel catfish *Ictalurus punctatus* (Silverstein *et al.* 2001) and coho salmon *Oncorhynchus kisutch* (White *et al.* 2016).

CCK (Polakof *et al.* 2011a) or GLP-1 (Polakof *et al.* 2011b) treatments activate glucosensing capacity in the hypothalamus and hindbrain of rainbow trout, together with changes in mRNA abundance of several neuropeptides, suggesting that these peptides regulate not only food intake but also glucose homeostasis. Although the role of CCK and GLP-1 on fish appetite regulation has been relatively studied in fish (Delgado *et al.* 2017, Soengas *et al.* 2018), their involvement in the regulation of lipid metabolism has received little attention. Therefore, in this study, we aimed to assess in rainbow trout if CCK-8 and GLP-1 central treatment results in decreased food intake as demonstrated in other fish species. Once the anorectic role of these peptides were demonstrated, we evaluated under the same conditions changes in (i) brain expression of mRNAs encoding neuropeptides involved

in the metabolic control of food intake such as AgRP, NPY, POMCa1 and CART, (ii) parameters related to fatty acid-sensing systems and (iii) cellular signalling pathways putatively involved in linking changes in nutrient-sensing systems to neuropeptide expression, and ultimately food intake.

Materials and methods

Fish

Immature rainbow trout of both sexes were obtained from a local fish farm (A Estrada, Spain). These fish were maintained for 1 month in 100L tanks under laboratory conditions and 12L:12D photoperiod (lights on at 08:00, lights off at 20:00) in dechlorinated tap water at 15°C. Fish weight was 98 ± 2 g. Fish were fed once daily (10:00) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat and 11.5% ash; 20.2 MJ/kg of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 53/2013) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

Experimental design

In a first set of experiments, following 1 month acclimation period, fish were randomly assigned to 100L experimental tanks following ARRIVE guidelines. On the day of experiment, at 10:00, fish were anaesthetized with 2-phenoxyethanol (Sigma, 0.02% v/v), and weighed to carry out ICV administration as previously described (Polakof & Soengas 2008). Briefly, fish were placed on a plexiglass board with Velcro straps adjusted to hold them in place. A 29½ gauge needle attached through a polyethylene cannula to a 10 µL Hamilton syringe was aligned with the 6th preorbital bone at the rear of the eye socket, and from this point the syringe was moved through the space in the frontal bone into the third ventricle. The plunger of the syringe was slowly depressed to dispense $1 \mu\text{L} \times 100/\text{g}$ body mass of saline solution alone (control, $n=10$), or containing 1 ng/g of rainbow trout CCK-8 ($n=10$) or containing 2 ng/g rainbow trout GLP-1 ($n=10$). Both peptides were synthesized by Bachem (Bubendorf, Switzerland) according to rainbow trout sequences published by Jensen *et al.* (2001) and Irwin and Wong (1995), for CCK-8 and GLP-1, respectively. The doses of CCK-8 and GLP-1 were selected based on

studies carried out previously in our laboratory (Polakof *et al.* 2011a,b). Food intake was registered for 7 days before treatment (to define basal line data) and then 2, 6 and 24 h after treatment. After feeding, the remaining uneaten food at the bottom (conical tanks) and feed waste were withdrawn, dried and weighed. The amount of food consumed by all fish in each tank was calculated as previously described as the difference from the feed offered (De Pedro *et al.* 1998, Polakof *et al.* 2008a,b). The experiment was repeated three times, and therefore results are shown as the mean + s.e.m. of the data obtained in three different tanks per treatment.

In a second set of experiments, following 1 month acclimation period, fish were randomly assigned to 100L experimental tanks following ARRIVE guidelines. On the day of experiment, at 10:00, fish were anaesthetized with 2-phenoxyethanol (Sigma, 0.02% v/v), weighed and ICV injected, as described above, with saline alone (control, $n=21$ at 2 h, and $n=21$ at 6 h) or containing CCK-8 ($n=21$ at 2 h, and $n=21$ at 6 h) or containing GLP-1 ($n=21$ at 2 h, and $n=21$ at 6 h) using the same concentrations as described above. After 2 h or 6 h, fish were anaesthetized in tanks with 2-phenoxyethanol (Sigma, 0.02% v/v). Blood was collected by caudal puncture with ammonium-heparinized syringes, and plasma samples were obtained after blood centrifugation, deproteinized immediately (using 0.6 M perchloric acid) and neutralized (using 1 M potassium bicarbonate) before freezing and storage at -80°C until further assay. Fish were killed by decapitation and the hypothalamus and hindbrain were taken, snap-frozen and stored at -80°C . At each time, nine fish per group were used to assess enzyme activities and metabolite levels, six fish per group were used for the assessment of mRNA levels by qRT-PCR, whereas the remaining six fish per group were used to assess by Western blot changes in the levels and phosphorylation status of proteins of interest.

Assessment of metabolite levels and enzyme activities

Levels of fatty acid, total lipid, triglyceride, glucose and lactate in plasma were determined enzymatically using commercial kits (Wako Chemicals, for fatty acid; Spinreact, Barcelona, Spain, for total lipid, triglyceride, glucose and lactate) adapted to a microplate format.

Samples used to assess tissue metabolite levels were homogenized immediately by ultrasonic disruption in 7.5 vols of ice-cooled 0.6 M perchloric acid, and neutralized (using 1 M potassium bicarbonate). The homogenate was

centrifuged (10,000g), and the supernatant used to assay tissue metabolites. Fatty acid, total lipid and triglyceride levels were determined enzymatically using commercial kits as described above for plasma samples.

Samples for enzyme activities were homogenized by ultrasonic disruption with 9 vols of ice-cold buffer consisting of 50mM Tris (pH 7.6), 5mM EDTA, 2mM 1,4-dithiothreitol and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged (10,000g) and the supernatant used immediately for enzyme assays. Enzyme activities were determined using a microplate reader INFINITE 200 Pro (Tecan, Männedorf, Switzerland) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340nm or, in the case of carnitine palmitoyl transferase 1 (CPT-1) activity, of 5,5'-dithiobis(2-nitrobenzoic acid)-CoA complex at 412nm. The reactions were started by the addition of supernatant (15 µL) at a pre-established protein concentration, omitting the substrate in control wells (final volume 265–295 µL) and allowing the reactions to proceed at 20°C for pre-established times (3–10 min). Enzyme activities are expressed in terms of mg protein. Protein was assayed in triplicates in homogenates using microplates according to the bicinchoninic acid method with bovine serum albumin (Sigma) as standard. Initial rates of enzyme activities were assessed under optimal substrate concentrations (determined in preliminary tests). ATP-citrate lyase (ACLY, EC 4.1.3.8), CPT-1 (EC 2.3.1.21) and fatty acid synthase (FAS, EC 2.3.1.85) activities were determined following available methods (Alvarez *et al.* 2000, Polakof *et al.* 2011c, Ditlecadet & Driedzic 2013, respectively).

mRNA abundance analysis by RT-qPCR

Total RNA was extracted using Trizol reagent (Life Technologies) and subsequently treated with RQ1-DNase (Promega). Two µg 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 assay volume of 15 µL, containing 50–500 nM of each primer. mRNA abundance of transcripts was determined as previously described in the same species (Leder & Silverstein 2006, Ducasse-Cabanot *et al.* 2007, Kolditz *et al.* 2008, Conde-Sieira *et al.* 2010, 2018, Polakof *et al.* 2010, Figueiredo-Silva *et al.* 2012, Sanchez-Gurmaches *et al.* 2012, MacDonald *et al.*

2014). 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 90s using hot-start iTaq DNA polymerase activation followed by 35 cycles, each one consisting of heating at 95°C for 20s, and specific annealing and extension temperatures for 20s. 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 (2001).

Western blot

Frozen samples (20mg) were homogenized in 1mL of buffer containing 150mM NaCl, 10mM Tris-HCl, 1mM EGTA, 1mM EDTA (pH 7.4), 100mM sodium fluoride, 4mM sodium pyrophosphate, 2mM sodium orthovanadate, 1% Triton X-100, 0.5% NP40-IGEPAL and 1.02mg/mL protease inhibitor cocktail (Sigma). Tubes were kept on ice during the whole process to prevent protein denaturation. Homogenates were centrifuged at 1000g for 15min at 4°C, and supernatants were again centrifuged at 20,000g for 30min. 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 (10 µg) were used for Western blot with antibodies from (1) Cell Signaling Technology: anti-phospho AMPKα (Thr172) reference #2531, anti-AMPKα reference #2532, anti-phospho CREB (Ser133) reference #9198, anti-CREB reference #9197, anti-phospho-FoxO1 (Thr24) reference #9464, anti-FoxO1 reference #9454, anti-phospho mTOR (Ser2448) reference #5536 and anti-β-tubulin reference #2146; (2) Sigma: anti-mTOR reference #T2949; (3) Abcam: anti-BSX reference #56092. All these antibodies cross-react successfully with rainbow trout proteins of interest (Skiba-Cassy *et al.* 2009, Kamalam *et al.* 2012, Velasco *et al.* 2016a, Conde-Sieira *et al.* 2018). After washing, membranes were incubated with an IgG-HRP secondary antibody reference #2015718 (Abcam) and bands were quantified by Image Lab software version 5.2.1 (BIO-RAD) in a Chemidoc Touch imaging system (BIO-RAD).

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

	Forward primer	Reverse primer	Annealing temperature (°C)	Database	Accession number
β -Actin	GATGGGCCAGAAAGACAGCTA	TCGTCCAGTTGGTGACGAT	59	GenBank	NM_001124235.1
<i>acly</i>	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	60	GenBank	CA349411.1
<i>agrp1</i>	ACCAGCAGTCTGTCTGGGTAA	AGTAGCAGATGGAGCCGAACA	60	GenBank	NM_001146677
<i>bsx</i>	CATCCAGAGTTACCCGGCAAG	TTTTCACCTGGGTTCCGAGA	60	GenBank	MG310161
<i>cart</i>	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	60	GenBank	NM_001124627
<i>cpt1c</i>	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	59	GenBank	AJ619768
<i>creb</i>	CGGATACCAAGTTGGAGGAGGA	AGCAGCAGCACTCGTTTAGGC	60	GenBank	MG310160
<i>fasn</i>	GAGACCTAGTGGAGGCTGTC	TCTTGTGTAGTGGTGAGCTGT	59	Signae	tcab0001c.e.06 5.1.s.om.8
<i>cd36</i>	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCCTCCACAGGA	62	DFCI	AY606034.1
<i>foxo1</i>	AACTCCCACAGCCACAGCAAT	CGATGTCCTGTTCCAGGAAGG	60	GenBank	MG310159
<i>kir6.x-like</i>	TTGGCTCCTTTCGCCATGT	AAAGCCGATGGTCACTGGA	60	Signae	CA346261.1.s.om.8:1:773:1
<i>lpl</i>	TAATTGGCTGCAGAAAACAC	CGTCAGCAAACCTAAAGGT	59	GenBank	AJ224693
<i>npv</i>	CTCGTCTGGACCTTTATATGC	GTTTCATCATATCTGGACTGTG	58	GenBank	NM_001124266
<i>pomca1</i>	CTCGTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	60	Tigr	TC86162
<i>ppara</i>	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGACAGCAGAT	55	GenBank	AY494835
<i>pparg</i>	GACGGCGGGTCAGTACTTTA	ATGCTCTGGCGAACTCTGT	60	DFCI	CA345564
<i>srebp1c</i>	GACAAGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	60	GenBank	CA048941.1
<i>ucp2a</i>	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	57	GenBank	DQ295324

Statistics

Comparisons were carried out with the SigmaStat (v11) statistical package. Comparisons among groups were carried out using a two-way ANOVA with treatments (CCK-8 and GLP-1) and time (2 and 6 h). Where significant effects were obtained from the ANOVA, *post-hoc* comparisons were carried out using a Student–Newman–Keuls (SNK) test to assess the significance ($P < 0.05$) of differences among treatments and between sampling times.

Results

The statistical significance of the differences observed in the parameters assessed attributed to the main factors and its interaction in the two-way ANOVA are shown in Table 2 for parameters assessed in the hypothalamus and hindbrain. No significant differences resulted from the two-way ANOVA analysis of parameters assessed in plasma whereas food intake was significantly affected by treatment ($P < 0.001$) and not by time and treatment $\times$ time interaction. Treatment significantly affected food intake and most parameters assessed in the hypothalamus (except mRNA abundance of *agrp*, *cart*, *fat/cd36* and *ppara*, and levels of P-Mtor/ β -Tubulin) and hindbrain (except levels of fatty acid and total lipid, mRNA abundance of *acly*, *agrp*, *cpt1c*, *srebp1c* and *ucp2a*, and protein levels of Bsx/ β -Tubulin and P-Foxo1/Foxo1). Several time effects were observed in the hypothalamus for levels of metabolites (fatty acid), enzyme activities (FAS and ACLY), mRNA

abundance (*acly*, *bsx*, *creb*, *kir6.x-like*, *pomca1*, *ppara*) and protein levels (P-Ampk α /Ampk α and P-Mtor/ β -Tubulin). In the hindbrain, time effects were observed for metabolite levels (triglyceride) and mRNA abundance (*cart*, *creb*, *fat/cd36*, *foxo1*, *kir6.x-like*, *npv* and *srebp1c*). Finally, a few significant treatment $\times$ time interactions were observed in the hypothalamus (total lipid levels, mRNA abundance of *bsx*, *creb*, *fasn* and *ucp2a*, and protein levels of P-Ampk α /Ampk α) and hindbrain (ACLY activity, mRNA abundance of *cart* and protein levels of P-AmpkMPK α /Ampk α).

The significant differences resulting from post-hoc comparisons among treatments and between times are detailed in Figs 1, 2, 3, 4, 5, 6, 7 and Tables 3, 4. For simplicity, we will only describe in the next paragraphs the differences observed among treatments.

Figure 1 displays the food intake represented as percentage change versus basal levels in each group. Central administration of CCK-8 resulted in a significant decrease of food intake post-treatment after 2 h (55.2%), 6 h (72.3%) and 24 h (49.8%). A larger decrease was observed in fish treated with GLP-1 after 2 h (6.9%), 6 h (3.1%) and 24 h (17.8%).

Levels of plasma metabolites are shown in Fig. 2. Levels of fatty acid (Fig. 2A), triglyceride (Fig. 2B), total lipid (Fig. 2C), glucose (Fig. 2D) and lactate (Fig. 2E) were not affected by treatments.

Levels of metabolites in the hypothalamus and hindbrain are shown in Fig. 3. Levels of fatty acid (Fig. 3A) and triglyceride (Fig. 3B) in the hypothalamus increased after treatment with CCK-8 (6 h) and GLP-1 (2 and 6 h) compared with control group. Total lipid levels in the

Table 2 *P*-Values obtained after two-way analysis of variance of parameters assessed in the hypothalamus and hindbrain of rainbow trout.

Parameter	Hypothalamus			Hindbrain		
	Treatment	Time	Treatment × time	Treatment	Time	Treatment × time
Metabolite levels						
Fatty acid	0.035	0.031	–	–	–	–
Triglyceride	0.033	–	–	0.047	0.001	–
Total lipid	0.042	–	0.005	–	–	–
Enzyme activities						
CPT-1	0.041	–	–	0.026	–	–
FAS	<0.001	0.039	–	0.003	0.001	–
ACLY	0.046	0.042	–	0.047	–	0.002
mRNA abundance						
<i>acly</i>	0.015	0.002	–	–	–	–
<i>agrp</i>	–	–	–	–	–	–
<i>bsx</i>	0.031	0.032	0.027	0.048	–	–
<i>cart</i>	–	–	–	0.040	0.005	0.031
<i>cpt1c</i>	0.048	–	–	–	–	–
<i>creb</i>	<0.001	0.002	<0.001	0.041	0.002	–
<i>fasn</i>	0.021	–	0.044	0.039	–	–
<i>cd36</i>	–	–	–	0.008	<0.001	–
<i>foxo1</i>	0.007	–	–	0.031	<0.001	–
<i>kir6.x-like</i>	0.036	0.016	–	0.048	0.034	–
<i>lpl</i>	0.036	–	–	<0.001	–	–
<i>npv</i>	0.034	–	–	0.044	0.010	–
<i>pomca1</i>	0.029	<0.001	–	0.021	–	–
<i>ppara</i>	–	0.015	–	0.026	–	–
<i>pparg</i>	0.034	–	–	0.040	–	–
<i>srebp1c</i>	0.046	–	–	–	0.011	–
<i>ucp2a</i>	0.009	–	0.034	–	–	–
Levels or phosphorylation status of signalling proteins						
P-Ampkα/Ampkα	0.038	0.040	0.019	0.006	–	0.009
Bsx/β-Tubulin	0.043	–	–	–	–	–
P-Creb/Creb	0.039	–	–	0.013	–	–
P-Mtorβ-Ttubulin	–	<0.001	–	0.002	–	–
P-Foxo/Foxo1	<0.001	–	–	–	–	–

Treatment (control, CCK-8 and GLP-1) and time (2 h and 6 h) were the main factors, and treatment × time is the first-order interaction. All values are significantly different unless noted by a dash.

hypothalamus (Fig. 3C) decreased in fish treated with CCK-8 or GLP-1 (2h). In the hindbrain, no significant changes occurred in fatty acid (Fig. 3D) and total lipid (Fig. 3F) levels whereas triglyceride levels (Fig. 3E) were higher in fish treated with GLP-1 (2h) compared with control group.

Enzyme activities are shown in Fig. 4. In the hypothalamus CPT-1 activity (Fig. 4A) decreased after treatment with CCK-8 (2h) compared with control group. FAS activity in the hypothalamus (Fig. 4B) decreased in both CCK-8 and GLP-1 groups (6h). ACLY activity in the hypothalamus (Fig. 4C) decreased after GLP-1 treatment (6h) compared with control group. In the hindbrain, CPT-1 activity (Fig. 4D) decreased after treatment with CCK-8 or GLP-1 (2 and 6h) compared with control group. FAS activity in the hindbrain decreased after CCK-8 (6h) or GLP-1 (2 and 6h) treatments (Fig. 4E). ACLY activity in

the hindbrain (Fig. 4F) decreased 6h after CCK-8 or GLP-1 treatments.

The mRNA abundance of neuropeptides involved in the metabolic regulation of food intake is shown in Fig. 5. *npv* mRNA abundance decreased after 6h treatment with CCK-8 in the hypothalamus (Fig. 5A) and after GLP-1 treatment in the hindbrain (Fig. 5E). *pomca1* mRNA abundance increased after CCK-8 or GLP-1 treatments (2h) in the hypothalamus (Fig. 5C) and increased after treatment with CCK-8 (6h) or GLP-1 (2 and 6h) in the hindbrain (Fig. 5G) compared with control group. *cart* mRNA abundance in the hindbrain (Fig. 5H) increased 6h after CCK-8 treatment compared with control group. No significant differences were observed in *agrp1* mRNA abundance (Fig. 5B and F).

The mRNA abundance of parameters related to fatty acid sensing and transcription factors are shown in Table 3

Figure 1

Food intake in rainbow trout 2, 6 and 24 h after intracerebroventricular administration of 1 $\mu\text{L} \times 100/\text{g}$ body mass of saline alone (control, CTR) or containing 1 ng/g of rainbow trout CCK-8 or 2 ng/g rainbow trout GLP-1. Food intake is displayed as mean $\pm$ s.e.m. of the percentage of food ingested with respect to baseline levels (calculated as the average of food intake the 7 days prior to experiment). The results are shown as mean $\pm$ s.e.m. of the results obtained in three different experiments in which 10 fish were used per group in each tank. Different letters indicate significant differences ($P < 0.05$) from different treatment at the same time.

for hypothalamus, and in Table 4 for hindbrain. The value of ACLY decreased in the hypothalamus 6 h after treatment with CCK-8. The value of carnitine palmitoyl transferase type 1c (*cpt1c*) decreased 2 h after treatment with GLP-1 compared with control group in the hypothalamus, while no changes occurred in the hindbrain. *fasn* decreased in the hypothalamus and hindbrain after CCK-8 (6 h) treatment and in the hypothalamus after GLP-1 (2 and 6 h) treatment compared with control group. Fatty acid translocase (*cd36*) increased after CCK-8 (6 h) or GLP-1 (2 and 6 h) treatments compared with control groups in the hindbrain, while no changes occurred in the hypothalamus. Inward rectifier K^+ channel pore type 6.x-like (*kir6.x-like*) decreased in the hypothalamus after treatment with GLP-1 (6 h), whereas in the hindbrain mRNA abundance of *kir6.x-like* decreased after treatment with CCK-8 (2 h) or GLP-1 (2 and 6 h). *lpl* in the hypothalamus increased after treatment with CCK-8 (6 h) or GLP-1 (2 h), whereas in the hindbrain both treatments increased mRNA abundance of *lpl* after 2 and 6 h. Treatment with CCK-8 (2 and 6 h) or GLP-1 (2 h) resulted in a significant increase in values of peroxisome proliferator-activated receptor type α (*ppara*) compared with control group in the hindbrain, while no changes occurred in the hypothalamus. Peroxisome proliferator-activated receptor type γ (*pparg*) decreased after treatment with GLP-1 in the hypothalamus (2 and 6 h) or hindbrain

(2 h). Sterol regulatory element-binding protein type 1c (*srebp1c*) increased after treatment with CCK-8 (2 h) in the hypothalamus, while no changes occurred in the hindbrain. The value of mitochondrial uncoupling protein 2a (*ucp2a*) decreased 6 h after CCK-8 (hypothalamus and hindbrain) or GLP-1 treatments (hindbrain) compared with control group. The treatment with CCK-8 resulted in a significant decrease in mRNA abundance of *bsx* (hypothalamus) and *creb* (hypothalamus and hindbrain) after 6 h, and in a significant increase in mRNA abundance of *foxo1* after 2 (hypothalamus) and 6 h (hindbrain). The treatment with GLP-1 resulted in a significant decrease in mRNA abundance of *bsx* after 2 (hindbrain) and 6 h (hypothalamus) and *creb* after 6 h (hindbrain), and in a significant increase in mRNA abundance of *foxo1* after 2 (hypothalamus and hindbrain) and 6 h (hindbrain).

The phosphorylation status of Ampk α in the hypothalamus (Fig. 6A) decreased after CCK-8 (6 h) or GLP-1 (2 h) treatment compared with control group. In the hindbrain, the phosphorylation status of Ampk α (Fig. 6C) decreased after CCK-8 (2 and 6 h) or GLP-1 (2 h) treatments compared with control group. The phosphorylation status of Mtor in the hindbrain (Fig. 6D) increased after CCK-8

Figure 2

Levels of fatty acid (A), triglyceride (B), total lipid (C), glucose (D) and lactate (E) in plasma of rainbow trout 2 h or 6 h after intracerebroventricular administration of 1 $\mu\text{L} \times 100/\text{g}$ body mass of saline alone (control, CTR) or containing 1 ng/g of rainbow trout CCK-8 or 2 ng/g rainbow trout GLP-1. Each value is the mean $\pm$ s.e.m. of $n = 15$ fish per treatment.

Figure 3

Levels of fatty acid (A and D), triglyceride (B and E) and total lipid (C and F) in the hypothalamus (A, B and C) and hindbrain (D, E and F) of rainbow trout 2 and 6 h after intracerebroventricular administration of $1 \mu\text{L} \times 100/\text{g}$ body mass of saline alone (control, CTR) or containing 1 ng/g of rainbow trout CCK-8 or 2 ng/g rainbow trout GLP-1. Each value is the mean $\pm$ S.E.M. of $n = 9$ fish per treatment. Different letters indicate significant differences ($P < 0.05$) from different treatment at the same time. # indicates significantly different ($P < 0.05$) from 2 h at the same treatment.

or GLP-1 treatment (2 and 6 h), whereas no significant changes occurred in the hypothalamus.

Levels and phosphorylation status of transcription factors are shown in Fig. 7. In the hypothalamus, Bsx protein levels (Fig. 7A) decreased after GLP-1 treatment (2 h), whereas no significant changes occurred in the hindbrain (Fig. 7D). The phosphorylation status of Creb in the hypothalamus (Fig. 7B) decreased after CCK-8 or GLP-1 treatments (2 h) whereas in the hindbrain (Fig. 7E) decreased after CCK-8 (2 and 6 h) or GLP-1 treatments (6 h). The phosphorylation status of Foxo1 (Fig. 7C) increased in the hypothalamus after CCK-8 or GLP-1 treatments (6 h) and no significant changes occurred in the hindbrain (Fig. 7F).

Discussion

The absence of changes in plasma metabolite levels indicates that no major metabolic changes occurred in the periphery after central treatment with CCK-8 and GLP-1. Therefore, in the present study changes observed

in parameters assessed in brain areas are due to CCK-8 and GLP-1 direct action, and are not the result of changes induced by altered levels of plasma metabolites.

The few interactions observed from the two-way ANOVA allow us to consider that most effects of treatments were comparable between the two sampling times assessed.

CCK-8 and GLP-1 ICV treatments inhibit food intake in rainbow trout

The central administration of CCK-8 elicited a clear anorectic effect in rainbow trout, which was evident up to 24 h post-treatment in agreement with available evidence in other fish species such as goldfish (Himick & Peter 1994, Kang *et al.* 2010) and channel catfish (Silverstein & Plisetskaya 2000). In the same way, GLP-1 central treatment resulted in an even more clear inhibition of food intake in agreement with evidence obtained in channel catfish (Silverstein *et al.* 2001) and coho salmon

Figure 4

Activities of CPT-1 (A and D), FAS (B and E) and ACLY (C and F) in the hypothalamus (A, B and C) and hindbrain (D, E and F) of rainbow trout 2 and 6 h after intracerebroventricular administration of $1 \mu\text{L} \times 100/\text{g}$ body mass of saline alone (control, CTR) or containing 1 ng/g of rainbow trout CCK-8 or 2 ng/g rainbow trout GLP-1. Each value is the mean $\pm$ S.E.M. of $n = 9$ fish per treatment. Different letters indicate significant differences ($P < 0.05$) from different treatment at the same time. # indicates significantly different ($P < 0.05$) from 2 h at the same treatment.

Figure 5

mRNA abundance of *npy* (A and E), *agRP* (B and F), *pomc* (C and G) and *cart* (D and H) in the hypothalamus (A, B, C and D) and hindbrain (E, F, G and H) of rainbow trout 2 and 6 h after intracerebroventricular administration of 1 μ L $\times$ 100/g body mass of saline alone (control, CTR) or containing 1 ng/g of rainbow trout CCK-8 or 2 ng/g rainbow trout GLP-1. Each value is the mean $\pm$ S.E.M. of $n = 6$ fish per treatment. Gene expression results are referred to control group 2 h after treatment previously normalized by β -actin expression. Different letters indicate significant differences ($P < 0.05$) from different treatment at the same time. # indicates significantly different ($P < 0.05$) from 2 h at the same treatment.

(White *et al.* 2016). The effect of GLP-1 was impressive and strongly repetitive as supported by the reduced SEM displayed. The anorectic response after CCK-8 or GLP-1 treatments is also in agreement with that observed in mammals, in which increased levels of both peptides (or agonists) are associated with decreased food intake (Strader & Woods 2005, Burmeister *et al.* 2017). The mechanisms underlying the anorectic effects of CCK-8 and GLP-1 peptides are still unknown. The anorectic action of these peptides has been suggested to relate to the ileal brake mechanism (the slowing of gastric emptying causes the

sensation of satiety) in mammals (Mojsov 2000, Strader & Woods 2005) and fish (Tinoco *et al.* 2015), but might also be attributed to a modulatory action as a central satiety signal (Strader & Woods 2005, Nogueiras *et al.* 2010).

In the present study, the anorectic effects of CCK-8 and GLP-1 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 & Schwartz 2010) and fish (Soengas 2014, Soengas *et al.* 2018). Thus, the increase in mRNA abundance of the anorexigen *cart* and *pomc* and the decrease in mRNA abundance of the orexigen *npy* after CCK-8 and GLP-1 treatments agree with prior evidence obtained in rainbow trout hypothalamus and hindbrain (Polakof *et al.* 2011a,b) occurring in parallel with the activation of glucosensing systems. Nevertheless, the effect of GLP-1 is different to that known in mammals where GLP-1 induced in the hypothalamus an increase in mRNA abundance of *pomc/cart* without changing mRNA abundance of *npy/agRP* (Martínez de Morentin *et al.* 2011, Lockie 2013).

CCK-8 and GLP-1 activate fatty acid sensing in the hypothalamus and hindbrain of rainbow trout

The treatment with CCK-8 or GLP-1 induced in general a raise in the levels of fatty acid and triglyceride in the hypothalamus and hindbrain. This raise is comparable to that observed in the same areas of the same species after ICV treatment with oleate or octanoate (Librán-Pérez *et al.* 2014) also resulting in an anorectic response.

The mechanism related to binding of fatty acid to FAT/CD36 and subsequent modulation of transcription factors was apparently activated in the hindbrain by central administration of CCK-8 and GLP-1, as evidenced by increased mRNA abundance of *cd36*, *ppara* and *srebp1c*, although not significant in the latter case. These results are comparable to those observed at central level after treatment with PYY₁₋₃₆, another gastrointestinal hormone that similarly plays an anorectic role in fish, including rainbow trout (Velasco *et al.* 2018). The results are also comparable to those observed in the same species after treatment with enhanced levels of oleate or octanoate (Librán-Pérez *et al.* 2012, 2013, 2014). Further support to these results come from the fact that central administration of ghrelin, a GIT hormone known for its orexigenic effect both in mammals (López *et al.* 2008) and fish species like goldfish (Tinoco *et al.* 2014) and rainbow trout (Velasco *et al.* 2016b) elicited opposed responses in parameters. Despite few changes occurred at hypothalamic level, the

Figure 6

Phosphorylation status of Ampkα (A and C) and Mtor (B and D) in the hypothalamus (A and B) and hindbrain (C and D) of rainbow trout 2 and 6 h after intracerebroventricular administration of 1 μ L $\times$ 100/g body mass of saline alone (control, CTR) or containing 1 ng/g of rainbow trout CCK-8 or 2 ng/g rainbow trout GLP-1. 20 μ g of total protein was loaded on the gel per lane, and results were normalized by β -tubulin abundance. Western blots were performed on six individual samples per treatment and two representative blots per time and 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 $\pm$ S.E.M. of $n = 6$ fish per treatment. Different letters indicate significant differences ($P < 0.05$) from different treatment at the same time. # indicates significantly different ($P < 0.05$) from 2 h at the same treatment.

increase observed in mRNA abundance of *srebp1c* after CCK-8 treatment and the decrease in *pparg* mRNA levels after GLP-1 treatment evidence that these peptides also affect this fatty acid mechanism at hypothalamic level.

Another mechanism of fatty acid sensing relates fatty acid abundance to decreased lipogenic potential resulting in CPT-1 inhibition (Soengas 2014). In the present study, many parameters displayed in general changes comparable with those observed when this system is activated by oleate or octanoate in the same species (Librán-Pérez *et al.* 2012, 2013, 2014), as demonstrated by enhanced levels of fatty acid and triglyceride and inhibition of CPT-1 activity after treatment with both hormones at central level. Thus, treatment with CCK-8 decreased FAS (also mRNA) and ACLY activities in both the hypothalamus and hindbrain; and treatment with GLP-1 decreased ACLY (hindbrain) and FAS (hindbrain and hypothalamus) activity, and mRNA abundance of *fasn* and *cpt1c* in the hypothalamus. These changes are similar to those observed in the same groups after central treatment with oleate or octanoate (Librán-Pérez *et al.* 2014). However, mRNA abundance of a pro-lipogenic factor such as *lpl* increased in the hypothalamus and hindbrain after treatment with both

peptides, which is in contrast with the decrease observed after oleate treatment (Velasco *et al.* 2016a). Altogether, it seems that fatty acid metabolism is activated by treatment with CCK-8 and GLP-1 in both the hypothalamus and hindbrain of rainbow trout.

The central detection of fatty acid is also related in fish to mitochondrial activity through enhanced production of reactive oxygen species, resulting in an inhibition of K^+_{ATP} (Soengas 2014). CCK-8 treatment elicited changes in the parameters related to this system including decreased expression of *kir6.x-like* and *ucp2a* both in the hypothalamus and hindbrain whereas GLP-1 treatment decreased expression of *kir6.x-like* in the hypothalamus and hindbrain, and *ucp2a* in the hypothalamus. These results are, in general, in agreement with those observed when this system is activated in the same species by the presence of oleate or octanoate (Librán-Pérez *et al.* 2012, 2013, 2014), and are also comparable with the response observed after central treatment with the anorectic peptide PYY₁₋₃₆ (Velasco *et al.* 2018).

As a whole the treatment with CCK-8 or GLP-1 activated fatty acid-sensing systems in the hypothalamus and hindbrain of rainbow trout in a way comparable to

Figure 7

Western blot analysis of Bsx (A and D), and phosphorylation status of Creb (B and E) and Foxo1 (C and F) in the hypothalamus (A, B and C) and hindbrain (D, E and F) of rainbow trout 2 and 6 h after intracerebroventricular administration of 1 μ L $\times$ 100/g body mass of saline alone (control, CTR) or containing 1 ng/g of rainbow trout CCK-8 or 2 ng/g rainbow trout GLP-1. 20 μ g of total protein was loaded on the gel per lane, and results were normalized by β -tubulin abundance. Western blots were performed on six individual samples per treatment and two representative blots per time and 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 $\pm$ S.E.M. of $n = 6$ fish per treatment. Different letters indicate significant differences ($P < 0.05$) from different treatment at the same time.

the effects elicited by the treatment with specific fatty acids in the same species. The anorectic effects of these peptides appear to modulate food intake directly but also indirectly through changes in nutrient-sensing systems.

CCK-8 and GLP-1 treatment elicit changes in integrative pathways and transcription factors

The knowledge regarding central integration of metabolic and endocrine information eliciting changes in neuropeptide expression ultimately regulating food

intake is scarce in fish (Delgado *et al.* 2017, Soengas *et al.* 2018). Available studies in fish (Dai *et al.* 2014, Otero-Rodiño *et al.* 2017, Velasco *et al.* 2017, Conde-Sieira *et al.* 2018) pointed to a network comparable to that known in mammals (Diéguez *et al.* 2011, Martínez de Morentin *et al.* 2011, Gao *et al.* 2013). In this network, mTOR and AMPK would operate as mediators between the activation of nutrient-sensing systems and the modulation of the expression of neuropeptides via modulation of the transcription factors FoxO1, CREB and BSX. In this study, we observed in the hypothalamus and hindbrain

Table 3 Changes in mRNA abundance of selected transcripts in hypothalamus of rainbow trout 2 and 6 h after intracerebroventricular administration of 1 $\mu\text{L} \times 100/\text{g}$ body mass of saline alone (control, CTR) or containing 1 ng/g of rainbow trout CCK-8 or 2 ng/g rainbow trout GLP-1.

	2 h post treatment			6 h post treatment		
	CTR	CCK-8	GLP-1	CTR	CCK-8	GLP-1
Fatty acid sensing						
<i>acly</i>	1.00 $\pm$ 0.13	0.87 $\pm$ 0.07	1.14 $\pm$ 0.18	1.83 $\pm$ 0.22 ^{a#}	1.02 $\pm$ 0.13 ^b	1.52 $\pm$ 0.14 ^a
<i>cpt1c</i>	1.00 $\pm$ 0.08 ^a	1.00 $\pm$ 0.07 ^a	0.73 $\pm$ 0.10 ^b	0.91 $\pm$ 0.10	0.97 $\pm$ 0.09	0.79 $\pm$ 0.13
<i>fasn</i>	1.00 $\pm$ 0.10 ^a	1.05 $\pm$ 0.08 ^a	0.75 $\pm$ 0.07 ^b	1.08 $\pm$ 0.11 ^a	0.62 $\pm$ 0.14 ^{b#}	0.74 $\pm$ 0.11 ^b
<i>cd36</i>	1.00 $\pm$ 0.11	1.29 $\pm$ 0.16	1.00 $\pm$ 0.14	1.09 $\pm$ 0.10	1.07 $\pm$ 0.10	1.02 $\pm$ 0.04
<i>kir6.x-like</i>	1.00 $\pm$ 0.10	0.98 $\pm$ 0.21	1.07 $\pm$ 0.13	1.70 $\pm$ 0.03 ^{a#}	1.35 $\pm$ 0.27 ^{ab#}	1.02 $\pm$ 0.12 ^b
<i>lpl</i>	1.00 $\pm$ 0.12 ^a	1.29 $\pm$ 0.21 ^{ab}	1.44 $\pm$ 0.21 ^b	1.19 $\pm$ 0.17 ^a	1.67 $\pm$ 0.22 ^b	1.37 $\pm$ 0.23 ^{ab}
<i>ppara</i>	1.00 $\pm$ 0.07	0.93 $\pm$ 0.06	0.83 $\pm$ 0.15	1.34 $\pm$ 0.11 [#]	1.12 $\pm$ 0.12	1.20 $\pm$ 0.06
<i>pparg</i>	1.00 $\pm$ 0.13 ^a	0.87 $\pm$ 0.05 ^{ab}	0.69 $\pm$ 0.04 ^b	1.38 $\pm$ 0.05 ^a	1.26 $\pm$ 0.20 ^{ab}	0.97 $\pm$ 0.17 ^b
<i>srebp1c</i>	1.00 $\pm$ 0.06 ^a	1.29 $\pm$ 0.13 ^b	1.00 $\pm$ 0.14 ^a	1.21 $\pm$ 0.22	0.83 $\pm$ 0.12	0.90 $\pm$ 0.16
<i>ucp2a</i>	1.00 $\pm$ 0.08	1.10 $\pm$ 0.08	0.89 $\pm$ 0.07	1.45 $\pm$ 0.06 ^a	0.75 $\pm$ 0.18 ^b	1.04 $\pm$ 0.11 ^b
Transcription factors						
<i>bsx</i>	1.00 $\pm$ 0.15	1.11 $\pm$ 0.13	0.84 $\pm$ 0.10	1.08 $\pm$ 0.18 ^a	0.45 $\pm$ 0.13 ^{b#}	0.70 $\pm$ 0.07 ^b
<i>creb</i>	1.00 $\pm$ 0.14	1.06 $\pm$ 0.10	0.82 $\pm$ 0.05	1.86 $\pm$ 0.08 ^{a#}	0.92 $\pm$ 0.12 ^b	1.14 $\pm$ 0.13 ^{b#}
<i>foxo1</i>	1.00 $\pm$ 0.15 ^a	2.09 $\pm$ 0.22 ^b	1.61 $\pm$ 0.15 ^b	1.44 $\pm$ 0.20	1.71 $\pm$ 0.24	1.84 $\pm$ 0.19

Data represent mean $\pm$ S.E.M. of $n = 6$ fish per treatment. Gene expression results are referred to control group 2 h after treatment previously normalized by β -actin expression. Different letters indicate significant differences ($P < 0.05$) from different treatment at the same time.

#Significantly different ($P < 0.05$) from 2 h at the same treatment.

a significant decrease in the phosphorylation status of Ampk α after treatment with CCK-8 or GLP-1 though the results observed were not exactly the same after 2 h or 6 h of treatment. This central AMPK inhibition is consistent with responses observed after activation of fatty acid-sensing systems in fish (Velasco *et al.* 2017). Also in mammals, the decrease in AMPK α phosphorylation status

in the hypothalamus also inhibits CPT-1 activity (Chari *et al.* 2010) and leads to decreased fatty acid oxidation and to increased lipogenic potential (Martínez de Morentin *et al.* 2011), as in the present study. Furthermore, in mammals, the activation of GLP-1 receptors decreased food intake via inhibition of AMPK and activation of mTOR (Burmeister *et al.* 2017). Mtor signalling at central

Table 4 Changes in mRNA abundance of selected transcripts in the hindbrain of rainbow trout 2 and 6 h after intracerebroventricular administration of 1 $\mu\text{L} \times 100/\text{g}$ body mass of saline alone (control, CTR) or containing 1 ng/g of rainbow trout CCK-8 or 2 ng/g rainbow trout GLP-1.

	2 h post treatment			6 h post treatment		
	CTR	CCK-8	GLP-1	CTR	CCK-8	GLP-1
Fatty acid sensing						
<i>acly</i>	1.00 $\pm$ 0.18	0.89 $\pm$ 0.21	0.96 $\pm$ 0.17	1.01 $\pm$ 0.27	1.10 $\pm$ 0.03	1.06 $\pm$ 0.14
<i>cpt1c</i>	1.00 $\pm$ 0.22	0.62 $\pm$ 0.18	0.82 $\pm$ 0.07	0.96 $\pm$ 0.11	0.94 $\pm$ 0.06	0.82 $\pm$ 0.11
<i>fasn</i>	1.00 $\pm$ 0.24	0.92 $\pm$ 0.19	0.89 $\pm$ 0.10	0.93 $\pm$ 0.10 ^a	1.38 $\pm$ 0.14 ^b	1.02 $\pm$ 0.15 ^a
<i>cd36</i>	1.00 $\pm$ 0.14 ^a	1.23 $\pm$ 0.23 ^{ab}	1.55 $\pm$ 0.17 ^b	1.45 $\pm$ 0.20 ^a	2.36 $\pm$ 0.21 ^{b#}	2.20 $\pm$ 0.13 ^{b#}
<i>kir6.x-like</i>	1.00 $\pm$ 0.03 ^a	0.73 $\pm$ 0.16 ^b	0.80 $\pm$ 0.05 ^b	1.21 $\pm$ 0.11 ^a	1.17 $\pm$ 0.13 ^{ab#}	0.84 $\pm$ 0.09 ^b
<i>lpl</i>	1.00 $\pm$ 0.19 ^a	2.49 $\pm$ 0.20 ^b	1.81 $\pm$ 0.17 ^c	1.33 $\pm$ 0.10 ^a	1.93 $\pm$ 0.24 ^b	2.10 $\pm$ 0.15 ^b
<i>ppara</i>	1.00 $\pm$ 0.15 ^a	1.79 $\pm$ 0.21 ^b	2.07 $\pm$ 0.33 ^b	1.54 $\pm$ 0.23 ^a	2.75 $\pm$ 0.60 ^b	1.31 $\pm$ 0.08 ^a
<i>pparg</i>	1.00 $\pm$ 0.19 ^a	0.79 $\pm$ 0.09 ^a	1.53 $\pm$ 0.13 ^b	1.05 $\pm$ 0.17	1.39 $\pm$ 0.19	1.31 $\pm$ 0.19
<i>srebp1c</i>	1.00 $\pm$ 0.16	0.76 $\pm$ 0.14	0.80 $\pm$ 0.16	1.00 $\pm$ 0.22	1.39 $\pm$ 0.13 [#]	1.15 $\pm$ 0.22
<i>ucp2a</i>	1.00 $\pm$ 0.21	0.76 $\pm$ 0.16	1.05 $\pm$ 0.13	1.09 $\pm$ 0.03 ^a	1.37 $\pm$ 0.09 ^b	0.97 $\pm$ 0.11 ^a
Transcription factors						
<i>bsx</i>	1.00 $\pm$ 0.16 ^a	0.96 $\pm$ 0.12 ^a	0.53 $\pm$ 0.03 ^b	0.69 $\pm$ 0.14	0.64 $\pm$ 0.14	0.71 $\pm$ 0.09
<i>creb</i>	1.00 $\pm$ 0.15	0.81 $\pm$ 0.14	0.87 $\pm$ 0.11	1.49 $\pm$ 0.13 ^{a#}	1.19 $\pm$ 0.10 ^b	1.13 $\pm$ 0.12 ^b
<i>foxo1</i>	1.00 $\pm$ 0.13 ^a	1.19 $\pm$ 0.18 ^{ab}	1.39 $\pm$ 0.05 ^b	1.62 $\pm$ 0.21 ^{a#}	2.23 $\pm$ 0.19 ^{b#}	2.10 $\pm$ 0.19 ^{b#}

Data represent mean $\pm$ S.E.M. of $n = 6$ fish per treatment. Gene expression results are referred to control group 2 h after treatment previously normalized by β -actin expression. Different letters indicate significant differences ($P < 0.05$) from different treatment at the same time.

#Significantly different ($P < 0.05$) from 2 h at the same treatment.

level plays a main role in modulating energy homeostasis by responding to nutrient availability and hormonal signals in mammals (Blouet & Schwartz 2010). In this study, Mtor phosphorylation status increased significantly in the hindbrain after treatment with both peptides. The changes observed in the hindbrain are consistent with those observed in mammals after central administration of hormones (i.e. leptin) or metabolites (i.e. leucine, α -lipoic acid), which regulate feeding through modulation of mTOR (Martins *et al.* 2012), in a way that short-term central administration of anorectic factors activate central mTOR signalling (Cota *et al.* 2008). Moreover, in rainbow trout liver, the activation of Mtor occurs in parallel with increased mRNA abundance of *srebp1c* (Seilliez *et al.* 2011), as in the present study. These results allow us to suggest that in rainbow trout CCK-8 and GLP-1 treatment modulates cellular signalling pathways related to Ampk α in the hypothalamus and hindbrain, as well as Mtor in the hindbrain.

Regarding transcription factors, BSX regulates hypothalamic NPY/AgRP expression in mammals in a way that when BSX mRNA levels increase a parallel increase occur in mRNA abundance of both peptides (Nogueiras *et al.* 2008, Varela *et al.* 2011). In the present study, mRNA abundance and protein levels of hypothalamic BSX decreased after treatment with CCK-8 and GLP-1 in parallel with decreased *npv* mRNA and food intake thus supporting a comparable role to that of mammals. This situation is comparable to that observed in the same species after oleate treatment (Conde-Sieira *et al.* 2018), which makes sense considering that oleate treatment resulted in decreased food intake in rainbow trout (Conde-Sieira & Soengas 2016, Soengas *et al.* 2018). Moreover, in mammals activation of CPT-1 occurred in parallel with increased values of BSX mRNA in the hypothalamus (Mera *et al.* 2014), as in the present study. In mammals, although both AgRP and NPY share BSX as a common transcriptional factor, BSX needs to interact with FoxO1 to increase *agrp* mRNA abundance, and with CREB to increase *npv* mRNA abundance (Varela *et al.* 2011). Accordingly, in rainbow trout the phosphorylation status and mRNA abundance of CREB decreased in the hypothalamus and hindbrain after treatment with CCK-8 and GLP-1. This situation is comparable to that observed in mammals under food deprivation (Ren *et al.* 2013) and in fish under anorexigenic conditions such as those elicited by oleate ICV treatment (Conde-Sieira *et al.* 2018). Nevertheless, the phosphorylation status (hypothalamus) and mRNA abundance of FoxO1 (hypothalamus and

hindbrain) increased after treatment with CCK-8 and GLP-1. These changes are comparable to those observed in mammals after treatment with other anorectic hormones such as leptin or insulin (Diéguez *et al.* 2011, Varela *et al.* 2011), and also comparable to that observed in fish under anorexigenic conditions (Velasco *et al.* 2017). These changes suggest that, in rainbow trout, an inhibition of CREB and an activation of FoxO1 occurred in parallel with decreased *npv* and increased *pomc* and *cart* mRNA abundance.

In summary, we have demonstrated, for the first time in fish, that the anorectic effects of central treatment with CCK and GLP-1 in rainbow trout are associated with changes in fatty acid metabolism and activation of fatty acid-sensing systems in the hypothalamus and hindbrain. Central activation of fatty acid-sensing systems by these peptides occurred in parallel with the inhibition of AMPK α and the activation of mTOR. These changes would relate to neuropeptide expression through changes in the phosphorylation status of transcription factors under their control such as BSX, CREB and FoxO1 finally resulting in changes in neuropeptide mRNA abundance leading to increased anorexigenic potential and a subsequent decrease in food intake. In general, the effects of both peptides on fatty acid metabolism are comparable to those known in mammals. In the case of GLP-1 this is surprising considering that the effects of this peptide in fish are completely different than those in mammals concerning glucose metabolism (Polakof *et al.* 2011b, Irwin & Mojssov 2018). It seems that in terms of regulation of food intake, the modulatory effects associated with fatty acid are more preserved throughout phylogeny than those related to glucose. This might relate to the reduced importance of glucose metabolism in many teleost fish species (Polakof *et al.* 2012) and clearly deserves further research.

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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Author contribution statement

C V, J M M and J L S conceived and designed the research; C V, S C and M C-S performed the experiments; C V, S C and M C-S analysed the data; all authors interpreted results of the experiments; C V and J L S prepared figures; all authors drafted, edited and revised manuscript; J L S approved the final version of manuscript.

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