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Differential Role of Hypothalamic AMPKα Isoforms in Fish: an Evolutive Perspective

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Abstract

In mammals, hypothalamic AMP-activated protein kinase (AMPK) α1 and α2 isoforms mainly relate to regulation of thermogenesis/liver metabolism and food intake, respectively. Since both isoforms are present in fish, which do not thermoregulate, we assessed their role(s) in hypothalamus regarding control of food intake and energy homeostasis. Since many fish species are carnivorous and mostly mammals are omnivorous, assessing if the role of hypothalamic AMPK is different is also an open question. Using the rainbow trout as a fish model, we first observed that food deprivation for 5 days did not significantly increase phosphorylation status of AMPKα in hypothalamus. Then, we administered adenoviral vectors that express dominant negative (DN) AMPKα1 or AMPKα2 isoforms. The inhibition of AMPKα2 (but not AMPKα1) led to decreased food intake. The central inhibition of AMPKα2 resulted in liver with decreased capacity of use and synthesis of glucose, lipids, and amino acids suggesting that a signal of nutrient abundance flows from hypothalamus to the liver, thus suggesting a role for central AMPKα2 in the regulation of peripheral metabolism in fishes. The central inhibition of AMPKα1 induced comparable changes in liver metabolism though at a lower extent. From an evolutionary point of view, it is of interest that the function of central AMPKα2 remained similar throughout the vertebrate lineage. In contrast, the function of central AMPKα1 in fish relates to modulation of liver metabolism whereas in mammals modulates not only liver metabolism but also brown adipose tissue and thermogenesis.

Keywords Trout · Hypothalamus · AMPK isoforms · Food intake · Hepatic metabolism

Introduction

AMP-activated protein kinase (AMPK) is the energy sensor primarily activated by the deficiency of energy in the cell (increased AMP/ATP ratio) and is associated with cellular mechanisms responsible for the restoration of energy balance by shutting down anabolic pathways and stimulating catabolic pathways [1, 2].

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AMPK is a ubiquitous protein but its function at central level has special relevance in the control of whole body energy homeostasis [2, 3]. Thus, in mammals, energy-related functions such as food intake [4–6], thermogenesis/browning in adipose tissue [7–9], hepatic and muscular metabolism [10–13], or glucose homeostasis [14–16] are regulated by hypothalamic AMPK. The role of hypothalamic AMPK as an appetite regulator relies on its capacity to integrate peripheral signals like levels of nutrients (glucose, fatty acid, or amino acids) and hormones (leptin, insulin, ghrelin, and GLP-1, among others) and alter subsequently the function of the neuropeptide-expressing AgRP/NPY and POMC neurons [1, 3]. Moreover, changes in hypothalamic AMPK alter energy expenditure through changes in peripheral tissues like liver or muscle by activation of sympathetic and vagal outflow [3, 8, 13, 17, 18].

In fishes, the available knowledge regarding AMPK function is more limited. In peripheral tissues (mainly liver and skeletal muscle), AMPK is a modulator of the response of energy metabolism to different energetic challenges. These

include food deprivation [19–21], nutritional conditions [22–27], exercise [28–30], low oxygen conditions [31, 32], temperature or salinity fluctuation [33, 34], stress by subordination [35], and toxic exposure [36]. At central level, in rainbow trout (*Oncorhynchus mykiss*) hypothalamus, the phosphorylation status of AMPKα decreased when fish were fed a lipid-enriched diet [37] or in the presence of oleate/octanoate [38] or glucose [39]. These variations in AMPKα occurred in parallel with the activation of gluco- and fatty acid-sensing mechanisms and changes in mRNA abundance of NPY, AgRP, POMC, and CART [38, 39] suggesting that in fish hypothalamus AMPK is an energy gauge as in mammals [1, 2]. However, there is no evidence in fish regarding the impact of changes in central AMPK activity on metabolic changes in peripheral tissues.

AMPK is a heterotrimeric protein formed by α (α1 or α2), β (β1 or β2), and γ (γ1, γ2, or γ3) subunits [40]. This structure is well conserved throughout animal phylogeny with the α subunit being the holder of the catalytic domain and the main site of protein phosphorylation [41]. Vertebrates α1 and α2 show different cellular distribution and specific physiological functions [11, 41]. Available studies revealed that in mammalian hypothalamus, the AMPKα2 isoform is involved in appetite and body weight regulation [11, 42, 43]. In contrast, the hypothalamic AMPKα1 isoform is mainly involved in the regulation of peripheral metabolism by controlling the thermogenic capacity of brown adipose tissue (BAT) and liver lipid metabolism [8, 13]. Both paralogues, α1 and α2, are present in fish displaying a high degree of homology with other vertebrates [11, 21, 36, 41, 44] with some functional evidence available in peripheral tissues [24, 26, 35, 44]. These paralogues have been also described in the whole fish brain [31, 34, 36, 44] though their specific functions have not been addressed in brain areas like the hypothalamus. Metabolically, fish display important differences when comparing with known mammalian models [45–47]. These include the fact that fishes do not expend energy to maintain a constant body temperature and require less energy for the excretion of waste nitrogenous products resulting in lower metabolic rates [48]. Since AMPKα1 and α2 isoforms are present in the fish hypothalamus, and fish do not thermoregulate, an interesting question arises regarding the possible role(s) of both central isoforms regarding the control of energy balance and also its possible evolutionary aspects. Moreover, since many fish species are carnivorous, to what extent the role of hypothalamic AMPK is different in these species compared with the mostly omnivore mammalian models assessed so far is also an open question. Therefore, the objective of the present study was to elucidate the possible role of hypothalamic AMPKα1 and α2 isoforms in the regulation of energy homeostasis in fish, using the rainbow trout as a model.

Materials and Methods

Fish

Immature rainbow trout were obtained from a local fish farm (A Estrada, Spain). Fish were maintained for 1 month in 100-l tanks (15 fish per tank) 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 at the beginning of the experiment was 67 ± 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 55/2013) for the use of animals in research and were approved by the Ethics Committee of the Universidade de Vigo.

Fasting/Feeding Trial

After 1 month of acclimation, two groups of fish ($N = 6$) were fasted for 5 days or were normally fed and then anesthetized in their holding tanks with 2-phenoxyethanol (0.02% *v*/*v*) (Sigma, St. Louis, MO, USA). Blood was collected by caudal puncture with ammonium-heparinized syringes and plasma samples were obtained after blood centrifugation, deproteinized immediately (using 0.6 mol.l^{-1} perchloric acid), and neutralized (using 1 mol l^{-1} potassium bicarbonate) before freezing on dry ice and storage at − 80 °C until further assay. Fish were killed by decapitation and hypothalamus was dissected, snap-frozen, and stored at − 80 °C to be used later for the assay by Western blot of changes in the levels of pAMPKα and AMPKα.

Intracerebroventricular Injection of Adenoviral Vectors

Following acclimation, fish were fasted for 24 h before treatment to ensure basal hormone and metabolite levels were achieved. On the day of experiment, fish were anesthesized in their tanks with 2-phenoxyethanol (0.02% *v*/*v*) and weighed. Then, intracerebroventricular (icv) administration was performed as described previously [49]. Briefly, fish ($N = 14$ per group) were placed on a Plexiglass board with Velcro straps adjusted to hold them in place. A 29½-gauge needle attached through a polyethylene cannula to a 10-μl Hamilton syringe was aligned with the sixth 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 injecting 0.5 μl of adenoviral vectors (*Viraquest*; North Liberty, IA, USA) containing green fluorescence protein (GFP) alone (control) or linked to a constitutive inactive

isoform of AMPKα1 or AMPKα2 (AMPKα1-DN and AMPKα2-DN, respectively) (wild-type, at 1.1×10^{12} pfu/ml) as previously shown [6, 8, 13, 50–56]. Immediately after injection, fish returned to their experimental tanks.

After 24 h of icv injection, fish were fed and food intake (FI) was assessed as follows: food was supplied in batches of approx. 5 g every 2 min until satiation. After feeding, the food uneaten remaining at the bottom (conical tanks) was withdrawn, dried, and weighed. The amount of food consumed by fish was calculated as previously described [57] as the difference from the feed offered. FI values registered after treatment are referred to those of basal values (assessed in each tank throughout 7 days prior to icv injection). During the following 12 days after icv injection, fish were food supplied once a day and FI levels were recorded as described. On day 13 after icv, fish were anesthetized in their holding tanks, and samples of plasma, liver, and hypothalamus were obtained from each experimental group ($N = 10$) as described above. Hypothalamus was used for the assay by Western blot of changes in the levels of pACCα and ACCα, and livers were used to assess enzyme activities, metabolite levels, and mRNA levels by quantitative real-time PCR (qRT-PCR). Additionally, the entire brain of four fish from each experimental group was used for immunohistochemical analyses.

Immunohistochemical Analyses

Sampled brains ($N = 4$ per group) were immediately fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4 for 24 h at 4 °C. Pieces were then dehydrated in a graded series of alcohols and embedded in paraffin. Serial transverse sections ranging from 7 to 10 μm were obtained and processed by immunofluorescence. Alternate sections were stained using the hematoxylin-eosin (H-E) to study the overall structure. The primary antibody used in this study was polyclonal rabbit anti-GFP (Abcam, Ab 290) whose specificity was previously tested by Western blotting. Sections were processed as follows: (1) preincubation with 0.1% bovine serum albumin (BSA) in PBS containing 0.25% Triton X-100 (PBS-T) for 1 h, to inhibit non-specific reactivity; (2) incubation with anti-GFP (1:100; Abcam) antibody diluted 1:100 in PBS-T overnight at room temperature in a humid chamber; (3) incubation with secondary specific antibody (Alexa Fluor 488 GAR-conjugated, Invitrogen) diluted 1:400 in PBS-T. After incubation, sections were washed in PBS-T and coverslipped using Prolong Gold with DAPI (Molecular Probes) to delay fluorescence fading. Immunostaining was not observed when primary antiserum was omitted from the protocol. In addition, an untreated trout was processed in parallel to confirm the specificity of immunostaining and no immunoreactivity was observed (results not shown). Slides were observed and photographed under an Olympus photomicroscope (BX51) equipped with digital camera (Olympus DP71). The plates were composed and labeled with CorelDRAW Software (Corel Corporation).

Assessment of Metabolite Levels and Enzyme Activities

Plasma glucose, lactate, triglyceride, and fatty acid levels were determined enzymatically using commercial kits (Spinreact, Barcelona, Spain, for glucose, lactate, and triglyceride and Wako Chemicals, Neuss, Germany, for fatty acid) adapted to a microplate format. Total α-amino acid levels were assessed using the ninhydrin method [58], with alanine as standard.

Samples used to assess metabolite levels in liver were homogenized immediately by ultrasonic disruption in 7.5 volumes of ice-cooled 0.6 mol l^{-1} perchloric acid and neutralized (using 1 mol l^{-1} potassium bicarbonate). The homogenate was centrifuged (10,000×*g*) and the supernatant used to assay tissue metabolites. Glycogen levels were assessed using the method of Keppler and Decker [59]. Glucose obtained after glycogen breakdown (after subtracting free glucose levels), lactate, triglyceride, and fatty acid levels was determined with commercial kits, as described above for plasma samples. Tissue total α-amino acid levels were determined colorimetrically as described above for plasma samples.

Samples of liver for determination of enzyme activities were homogenized by ultrasonic disruption with 9 vols of ice-cold stopping buffer containing 50 mmol l^{-1} imidazole-HCl (pH 7.6), 15 mmol l^{-1} 2-mercaptoethanol, 100 mmol l^{-1} KF, 5 mmol l^{-1} EDTA, 5 mmol l^{-1} EGTA, and a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA). The homogenate was centrifuged and the supernatant used immediately for enzyme assays. Enzyme activities were determined using a microplate reader INFINITE 200 Pro (Tecan, Männedorf, Switzerland). Reaction rates of enzymes were determined by the decrease in absorbance of NADH at 340 nm. 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 time periods. Enzyme activities are expressed per protein level, which was assayed with the bicinchoninic acid method with bovine serum albumin (Sigma) as standard. Enzyme activities were assessed at maximum rates determined by preliminary tests to determine optimal substrate concentrations. Hexokinase (low Km HK, *EC* 2.7.1.1), glucokinase (GK, *EC* 2.7.1.2), glycogen synthase (GSase, *EC* 1.1.1.35), glycogen phosphorylase (GPase, *EC* 2.4.1.1), glucose 6-phosphatase (G6Pase, *EC* 3.1.3.9), fructose 1,6-bisphosphatase (FBPase, *EC* 3.1.3.11.), carnitine palmitoyl transferase 1 (CPT-1, *EC* 2.3.1.21), fatty acid synthase (FAS, *EC* 2.3.1.85), glutamate dehydrogenase (GDH, *EC* 1.4.1.4), and glutamate pyruvate transaminase (GPT, *EC* 2.6.1.15)

activities were determined as described previously [60–62]. PFK activity was determined at low (0.1 mmol l^{-1}) and high (5 mmol l^{-1}) fructose 6-phosphate concentrations (omitted for controls), and an activity ratio was calculated as the activity at low fructose 6-phosphate/high fructose 6-phosphate concentrations. GPase a activity was measured with 10 mmol l^{-1} caffeine present, and total GPase activities were estimated without caffeine; the ratio of GPase activities with and without caffeine multiplied by 100 represents the percentage of total GPase (a + b) in the active form (% GPase a). Total GSase activities were measured with 5 mmol l^{-1} glucose 6-phosphate (G6P) present, and GSase a activities were estimated lacking G6P; the ratio of GSase activities without and with G6P multiplied by 100 represents the percentage of total GSase (a + b) in the active form (% GSase a).

Western Blot Analysis

Frozen samples (20 mg) were homogenized in 1 ml of buffer containing 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA (pH 7.4), 100 mmol l^{-1} sodium fluoride, 4 mmol l^{-1} sodium pyrophosphate, 2 mmol l^{-1} sodium orthovanadate, 1% Triton X-100, 0.5% NP40-IGEPAL, and 1.02 mg ml^{-1} protease inhibitor cocktail (Sigma). Tubes were kept on ice during the whole process to prevent protein denaturation. Homogenates were centrifuged at 1000×*g* for 15 min at 4 °C, and supernatants were again centrifuged at 20,000×*g* for 30 min. The resulting supernatants were recovered and stored at − 80 °C. The concentration of protein in each sample was determined using Bradford assay with bovine serum albumin as standard. Hypothalamic protein lysates (10 μg) were Western blotted using appropriate antibodies (dilution 1:1000) from Cell Signaling Technology (Leiden, The Netherlands): anti-phospho acetyl-CoA carboxylase α (ACCα) reference no. 3661, anti-ACCα reference no. 3662, anti-phospho AMPKα (Thr172) reference no. 2535, anti-AMPKα reference no. 2532, and anti-β-tubulin reference no. 2146. Anti-pACCα and anti-ACCα were validated for use in rainbow trout hypothalamus as shown in Fig. 2 while the remaining antibodies cross-react successfully with rainbow trout proteins of interest [22, 37, 63]. After washing, membranes were incubated with an IgG-HRP secondary antibody from DAKO (Glostrup, Denmark) and bands were quantified by Image Lab software version 5.2.1 (Bio-Rad) in a Chemidoc Touch imaging system (Bio-Rad).

mRNA Abundance Analysis by RT-qPCR

Total RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA) and subsequently treated with RQ1-DNAse (Promega, Madison, WI, USA). Two micrograms of total RNA was reverse transcribed using Superscript II reverse transcriptase (Promega) and random hexamers (Promega) to obtain 20 μl. Gene expression levels were determined by RT-qPCR using the iCycler iQ (Bio-Rad, Hercules, CA, USA). Analyses were performed on 1 μl cDNA using MAXIMA SYBR Green qPCR Mastermix (Life Technologies), in a total PCR reaction volume of 15 μl, containing 50–500 nM of each primer. mRNA abundance of transcripts was determined as previously described in the same species [64–69]. 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 and elongation factor 1α (EF1α) gene expressions as reference, which were 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 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 and EF1α reference gene transcripts was made following the Pfaffl method [70].

Statistical Analysis

Comparisons among groups were carried out using a Student *t* test in the fasting/food deprivation trial. In the icv treatment, comparisons among groups were carried out using two-way ANOVA. The normal distribution of variables and homoscedasticity were analyzed by Kolmogorov-Smirnov tests. Post hoc comparisons were carried out by a Sidac test. Comparisons were carried out with the GraphPad statistical package and differences were considered statistically significant at $p < 0.05$.

Results

The Phosphorylation Status of Hypothalamic AMPKα Did Not Change with Food Deprivation in Rainbow Trout

In hypothalamus, food deprivation for 5 days did not affect levels of pAMPKα and AMPKα (Fig. 1).

The Administration of Adenoviral Vectors Expressing AMPKα-DN Isoforms Was Appropriate to Assess the Physiological Role of Both AMPKα Isoforms

The photomicrographs of hypothalamic sections (Fig. 2) showed GFP immunoreactivity (GFP-ir) in control,

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	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59	GenBank	NM_ 001124235.1
CPT1a	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	55	GenBank	AF327058
EF1α	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59	GenBank	AF498320
FAS	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	59	Sigenae	tcab0001c.e.06 5.1.s.om.8
FBPase	GCTGGACCCTTCCATCGG	CGACATAACGCCCACCATAGG	59	GenBank	AF333188
G6Pase	CTCAGTGGCGACAGAAAGG	TACACAGCAGCATCCAGAGC	55	Sigenae	cay0019b.d.18_ 3.1.s.om.8.1-1693
GDH	TGCTGACACCTATGCCAACAC	CCTGGCTGATGGGCTTACC	58	GenBank	AJ556997
GK	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCCTTTGGTCCAG	60	GenBank	AF053331
GPT	CTGGGCTGTTCGTCACTTCA	AATGACTGCGACAGGACAATGT	58	GenBank	BT045760
GSase	CGTGGTGAGAGGAAGGAACTGAGC	CCGTTGAGACCGTGGAGACA	59	GenBank	BT073381.1
PFK	GGTGGAGATGCACAAGGAAT	CTTGATGTTGTCCCCTCCAT	59	Sigenae	tcbk0069c.k.05_s.1

CPT1a carnitine palmitoyl transferase type 1a, *EF1α* elongation factor 1α, *FAS* fatty acid synthase, *FBPase* fructose 1,6-bisphosphatase, *GDH* glutamate dehydrogenase, *GK* glucokinase, *GPT* glutamate-pyruvate transaminase, *GSase* glycogen synthase, *PFK* 6-phosphofucto 1-kinase

AMPKα1-DN, and AMPKα2-DN groups. GFP-ir was observed in the hypothalamic III ventricle (V) in cells that present morphological features in common with cerebrospinal fluid-contacting (CSF-C) cells. These cells exhibited a round or pear-shaped perikarya and a long thin apical dendrite that ended in a ventricular bulb and a small soma located away from the ventricle (b, d, f).

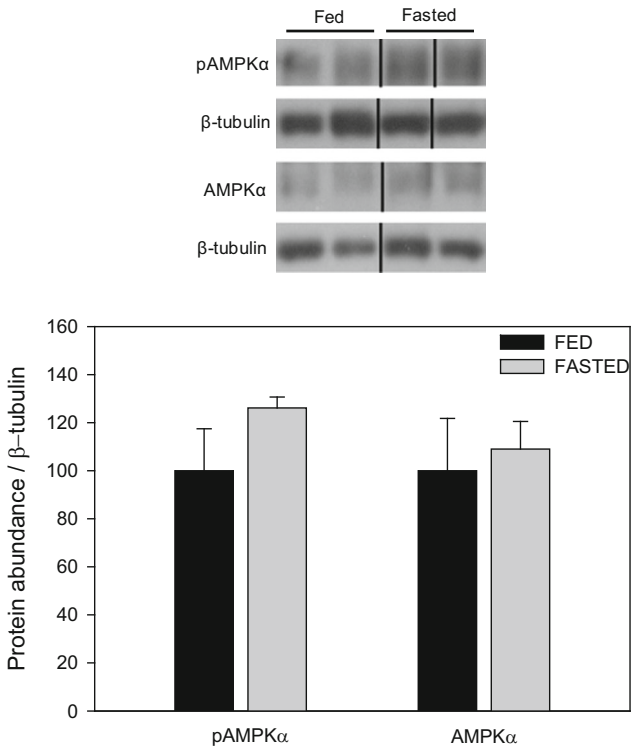

Fig. 1 AMPKα is not affected by short-term food deprivation in fish hypothalamus. Western blot analysis of pAMPKα and AMPKα protein levels in the hypothalamus of rainbow trout fed or fasted for 5 days. Ten micrograms of total protein was loaded on the gel per lane. Western blots were performed on six individual samples per treatment, and two representative blots are shown. Graphs represent the ratio between the protein of interest and β-tubulin. Each value is the mean ± SEM of $n = 6$ fish per treatment

To further support and control the effect of both adenoviral treatments, we also assessed protein levels of pACCα and ACCα under the different icv treatments as presented in Fig. 3. The icv injection of AMPKα2-DN decreased values of pACCα compared to controls, whereas no significant changes, but a trend to decrease, were found for AMPKα1-DN. ACCα levels were not affected by any of the treatments.

Average Daily Food Intake Decreased in AMPKα2-DN Group

The values of food intake (Fig. 4) are presented as daily values of the percentage of weight of eaten food with respect to the basal levels of each group ($n = 14$). The average was calculated from day 4 (time needed for food intake to stabilize) until day 13 after icv treatment. A lower FI was observed in AMPKα2-DN group with respect to control and AMPKα1-DN group.

Plasma and Liver Metabolite Levels Are Affected by Both AMPKα1-DN and AMPKα2-DN Treatments

In plasma, glucose levels (Fig. 5a) decreased in AMPKα1-DN and AMPKα2-DN groups compared with control, whereas lactate values (Fig. 5b) did not display any significant variation. In the case of fatty acid levels (Fig. 5c), these were lower in AMPKα1-DN group in contrast with the higher values for AMPKα2-DN compared with controls. Triglyceride levels in plasma (Fig. 5d) presented lower values in both AMPKα-DN groups in relation with controls. In the case of amino acids (Fig. 5e), no significant changes were detected for any experimental group.

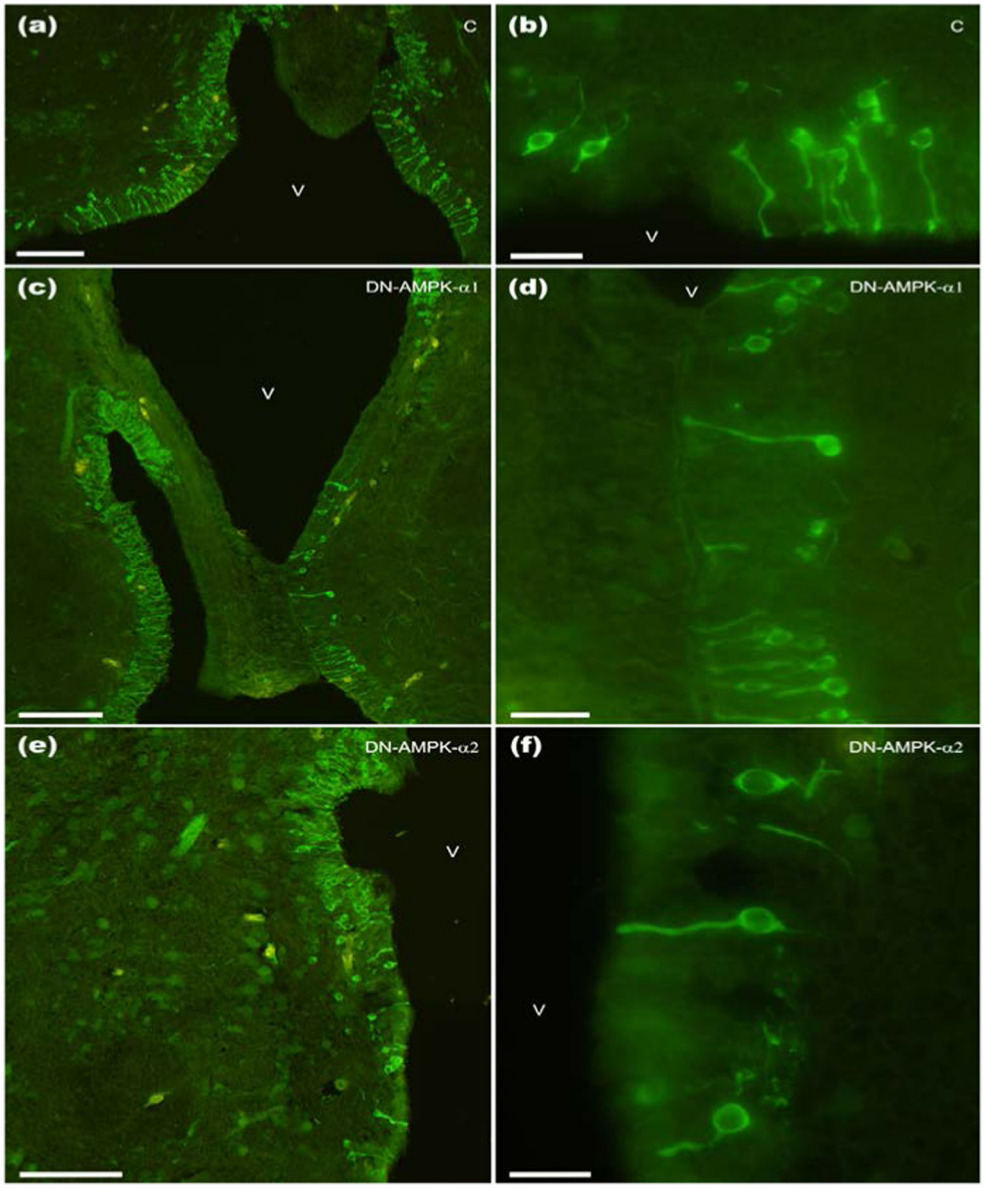

Fig. 2 Histochemical validation of treatment with adenoviral vectors expressing AMPKα-DN isoforms. Photomicrographs of transverse sections through the III ventricle (V) of rainbow trout brain showing GFP immunoreactivity (GFP-ir) 13 days after intracerebroventricular administration of adenovirus tagged with GFP (Control) or adenovirus tagged with GFP expressing a dominant negative AMPKα1 isoform (AMPKα1-DN) or expressing a dominant negative AMPKα2 isoform (AMPKα2-DN). At this level, most of those GFP-ir cells had morphological features in common with cerebrospinal fluid-contacting (CSF-C) cells, a peculiar cell population with a bipolar morphology, and a long and thin process that ended in the ventricle (**b**, **d**, **f**). Scale bar, 100 μm in **a**, **c**, and **e** and 25 μm in **b**, **d**, and **f**

In the liver, glucose levels (Fig. 6a) did not display any significant changes, whereas glycogen values (Fig. 6b) are lower in AMPKα2-DN-treated group than in the control group. Lactate levels (Fig. 6c) decreased in groups injected with AMPKα-DN compared to controls. Triglyceride (Fig. 6d) and fatty acid (Fig. 6e) levels in liver displayed significantly decreased values for AMPKα2-DN-treated group than controls, whereas amino acids were higher than controls in this experimental group (Fig. 6f).

Hepatic Glucose, Lipid, and Amino Acid Metabolism Are Affected by AMPKα1-DN and AMPKα2-DN Treatments

HK (Fig. 7a) and GK (Fig. 7b) activities were lower in AMPKα2-DN group than in controls. mRNA levels of GK (Fig. 7c) displayed lower values in both AMPKα-DN groups (α1 and α2) compared with controls. PFK activity ratio (Fig. 7d) displayed decreased levels in AMPKα2-DN group in relation with control, whereas PFK activity (Fig. 7e) and mRNA abundance (Fig. 7f) did not display any significant changes. GPase (Fig. 7k) and GSase (Fig. 7h) activities increased in the liver of fish expressing AMPKα-DN while the percentage of active form is lower for GPase (Fig. 7j) and higher for GSase (Fig. 7g) in AMPKα2-DN when compared with control and with α1 in the case of GPase. FBPase activity decreased in AMPKα1-DN with respect to controls (Fig. 8a) and G6Pase activity (Fig. 8c) was lower than control group for both AMPKα-DN groups, whereas no differences occurred for the mRNA abundance of these enzymes. CPT1 showed decreased levels for both AMPKα-DN groups either for the activity (Fig. 8e) and the mRNA abundance of CPT1a (Fig. 8f). Regarding FAS, it

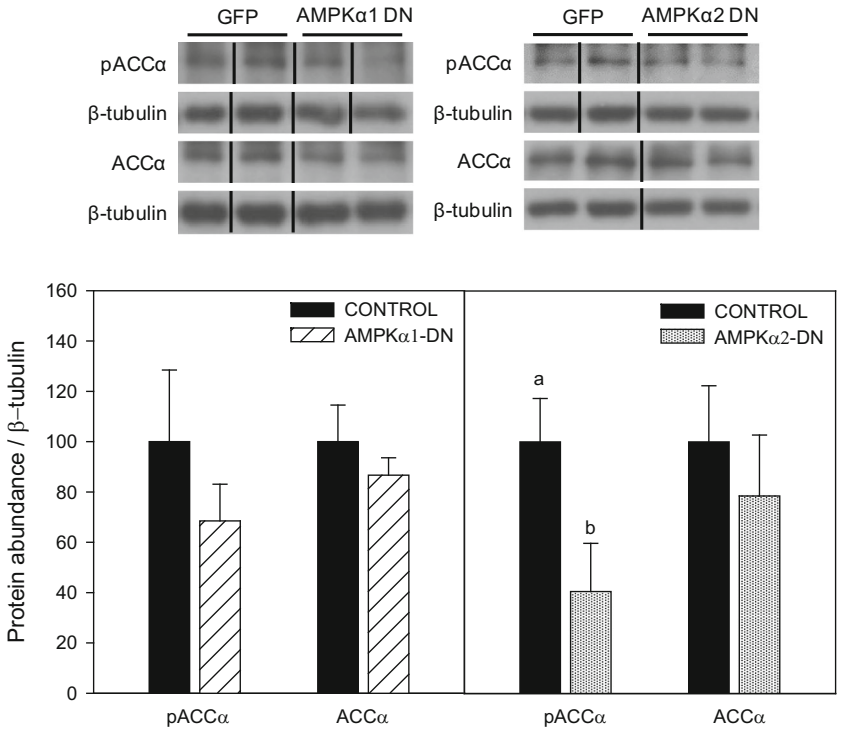

Fig. 3 pACCα and ACCα protein levels in hypothalamus after treatment with adenoviral vectors expressing AMPKα-DN isoforms. Western blot analysis of pACCα and ACCα protein levels in the hypothalamus of rainbow trout 13 days after intracerebroventricular administration of adenovirus tagged with GFP (Control) or adenovirus tagged with GFP expressing a dominant negative AMPKα1 isoform (AMPKα1-DN) or expressing a dominant negative AMPKα2 isoform (AMPKα2-DN). Ten micrograms of total protein was loaded on the gel per lane. Western blots were performed on six individual samples per treatment, and two representative blots are shown. Graphs represent the ratio between proteins of interest and β-tubulin. Each value is the mean ± SEM of $n = 6$ fish per treatment. Different letters indicate significant differences ($P < 0.05$) between groups

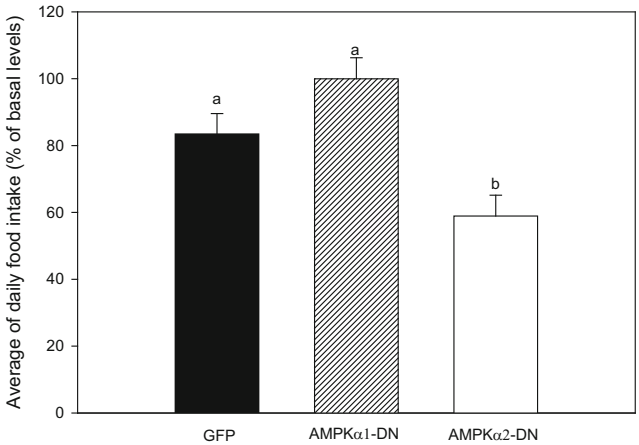

Fig. 4 Changes in food intake after adenoviral treatment. Average daily food intake registered in rainbow trout from day 4 to day 13 after intracerebroventricular administration of adenovirus tagged with GFP (Control) or adenovirus tagged with GFP expressing a dominant negative AMPKα1 isoform (AMPKα1-DN) or expressing a dominant negative AMPKα2 isoform (AMPKα2-DN). Food intake is displayed as the percentage of food ingested with respect to baseline levels (calculated as the average of food intake the 7 days previous to experiment). Each value is the mean ± SEM of $n = 14$ fish per treatment. Different letters indicate significant differences ($P < 0.05$) between groups

showed lower mRNA values in AMPKα1-DN and α2 than in controls (Fig. 8h). Finally, GDH (Fig. 9a) and GPT (Fig. 9b) displayed decreased activity in AMPKα2-DN compared with controls and also with α1 group in the case of GPT.

Discussion

In fish, we previously reported in rainbow trout the presence of nutrient-sensing mechanisms in hypothalamus changing in parallel with the expression of neuropeptides regulating food intake [71, 72]. These changes in hypothalamic function occur in parallel with decreased AMPKα phosphorylation status as recently reported in hypothalamus of rainbow trout fed with a lipid-enriched diet [37] or in hypothalamus of rainbow trout in vitro exposed to increased concentrations of oleate/octanoate [38] or glucose [39]. These changes support a role for AMPKα in the control of food intake in fish hypothalamus. To further support such a role, we assessed changes in hypothalamic AMPKα under conditions of reduced levels of nutrients, such as those occurring under food deprivation conditions. We indeed observed an increase in pAMPKα values in hypothalamus of fish deprived of food for 5 days, but this

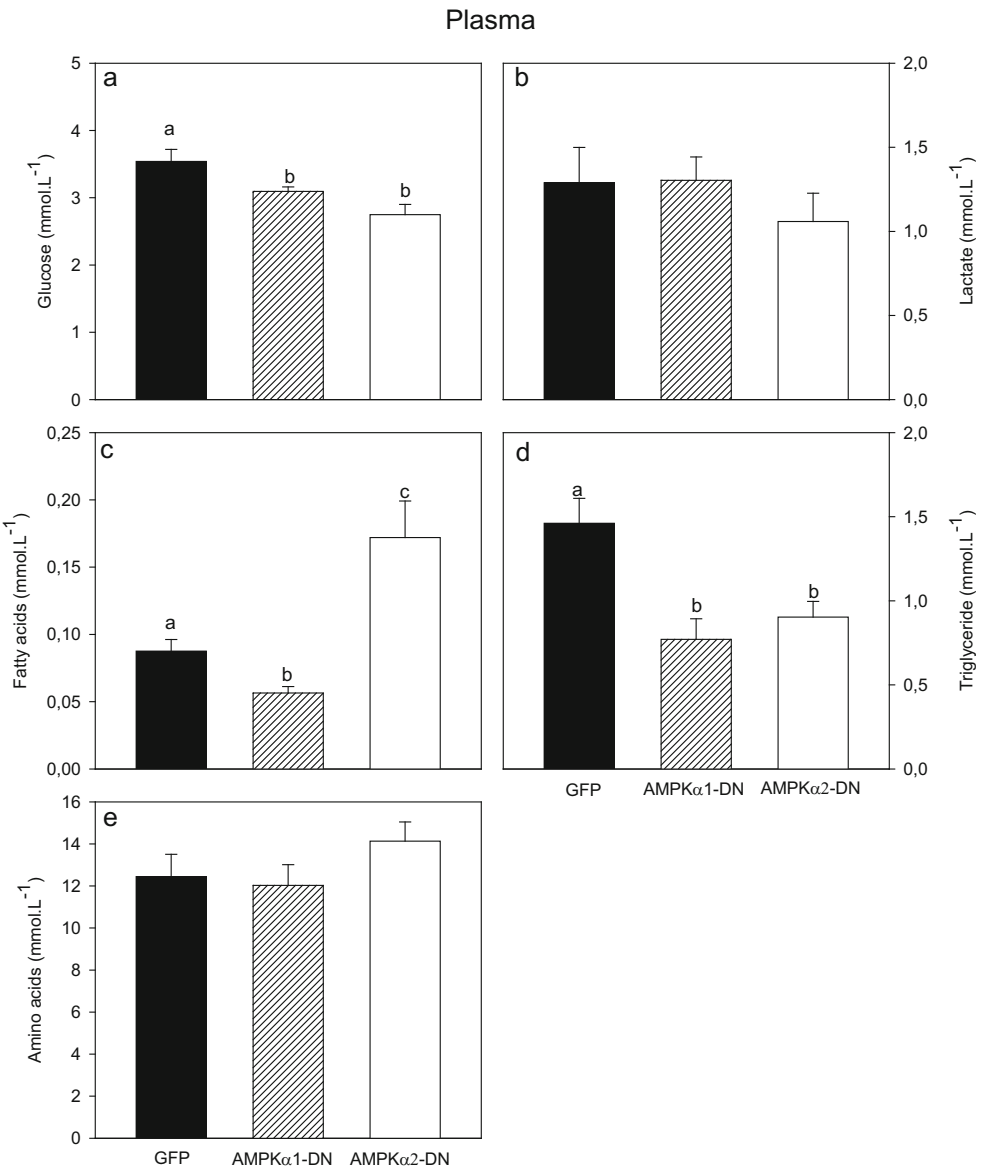

Fig. 5 Metabolite levels in plasma affected by treatment with AMPKα-DN. Levels of glucose (**a**), lactate (**b**), fatty acid (**c**), triglyceride (**d**), and amino acid (**e**) in plasma of rainbow trout, 13 days after intracerebroventricular administration of adenovirus tagged with GFP (Control) or adenovirus tagged with GFP expressing a dominant negative AMPKα1 isoform (AMPKα1-DN) or expressing a dominant negative AMPKα2 isoform (AMPKα2-DN). Each value is the mean ± SEM of $n = 10$ fish per treatment. Different letters indicate significant differences ($P < 0.05$) between groups

increase was not significant ($p = 0.194$). Considering fish resistance to food deprivation [46], it is likely that a significant increase would require a longer time of food deprivation. No other studies addressed this issue in fish hypothalamus before, though in peripheral tissues like liver and muscle [19–21] increased values of pAMPKα occurred from days 10–21 of food deprivation onwards. The trend to increase in fish would be also comparable to the increase reported in mammalian hypothalamus [1, 2] suggesting a conserved role for AMPK when comparing fish and mammals, with the main difference being the time period required to observe those changes, which in fish is longer than in mammals.

Our second aim was to elucidate the putative role of the two AMPKα isoforms in the control of food intake. Thus, we administered adenoviral vectors that express dominant negative (DN) AMPKα isoforms. The immunohistochemical analyses verified that the experimental design was appropriate since GFP fluorescence was present in all groups indicating that the adenoviruses were successful in transfecting neurons surrounding the ventricle close to key hypothalamic areas involved in food intake regulation [72]. Therefore, the differences observed between groups might relate to the action exerted by AMPKα-DN isoforms. The group treated with AMPKα2-DN displayed a decrease in food intake compared with control group. In contrast, a decrease did not occur in the group treated with AMPKα1-DN. We expected that inhibition of AMPK function would lead to reduced food intake since in the same species we have previously demonstrated a decrease in food intake after icv treatment with oleate/octanoate [73, 74], i.e., under conditions in which we also observed decreased phosphorylation status of AMPKα [38]. The novelty of the present study is the differential effect elicited by the two

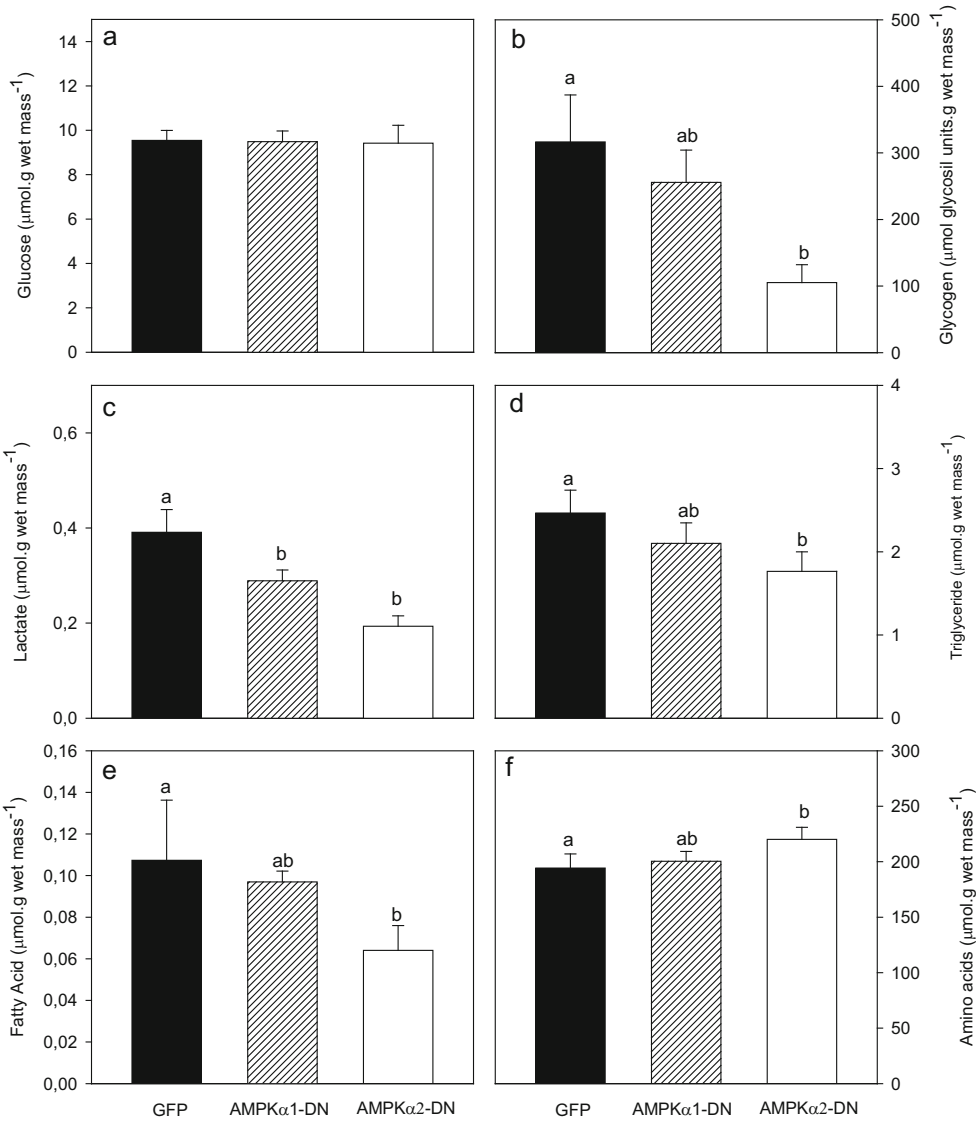

Fig. 6 Metabolite levels in liver affected by treatment with AMPKα-DN. Levels of glucose (**a**), glycogen (**b**), lactate (**c**), fatty acid (**d**), triglyceride (**e**), and amino acid (**f**) in the liver of rainbow trout 13 days after intracerebroventricular administration of adenovirus tagged with GFP (Control) or adenovirus tagged with GFP expressing a dominant negative AMPKα1 isoform (AMPKα1-DN) or expressing a dominant negative AMPKα2 isoform (AMPKα2-DN). Each value is the mean ± SEM of $n = 10$ fish per treatment. Different letters indicate significant differences ($P < 0.05$) between groups

isoforms assessed. It seems that only AMPKα2 is involved in the control of food intake with the expected decrease occurring only in the AMPKα2-DN group. To further support this effect, we also evaluated the phosphorylation status of ACCα in hypothalamus of the groups assessed. The phosphorylation levels of this protein are known to increase in hypothalamus when AMPK is activated [4, 5], and thus, we expected a decrease in pACCα levels under conditions of AMPK inhibition. Such a decrease was clearly observed for the AMPKα2-DN group but it was not significant ($p = 0.14$) for the AMPKα1-DN group. We cannot discard the possibility that the treatment with AMPKα1-DN was ineffective, though it seems that AMPK inhibition occurred with both treatments resulting in one case in decreased food intake (AMPKα2-DN) but not in the other (AMPKα1-DN). This result is comparable to that characterized in mice where the use of AMPK dominant negative isoforms decreased food intake and the mRNA expression of AgRP and NPY [5]. However, since both isoforms are present in fish brain [36, 44], a compensatory effect between the two isoforms cannot be discarded [11]. In this context, the present results allow us to suggest that AMPKα2 relates to the control of food intake in fish, and the lack of AMPKα1 could compensate for the presence of AMPKα2 in the AMPKα1-DN group whereas the contrary would be happening in the AMPKα2-DN group. The finding that results of food intake are similar when comparing rainbow trout and mice allows us to make an interesting question about evolutive changes in AMPK function. Fish evolved from a lineage more primitive than tetrapods like mammals, and the AMPKα2 isoform is involved in food intake regulation in both groups. Therefore, what is the role of the AMPKα1 isoform that in mammals mainly relates to a process, thermoregulation, which is not present in fish? A plausible hypothesis would be that the original function of α1 in fishes would relate to regulation of peripheral (hepatic) metabolism. In endotherms, the original function related to

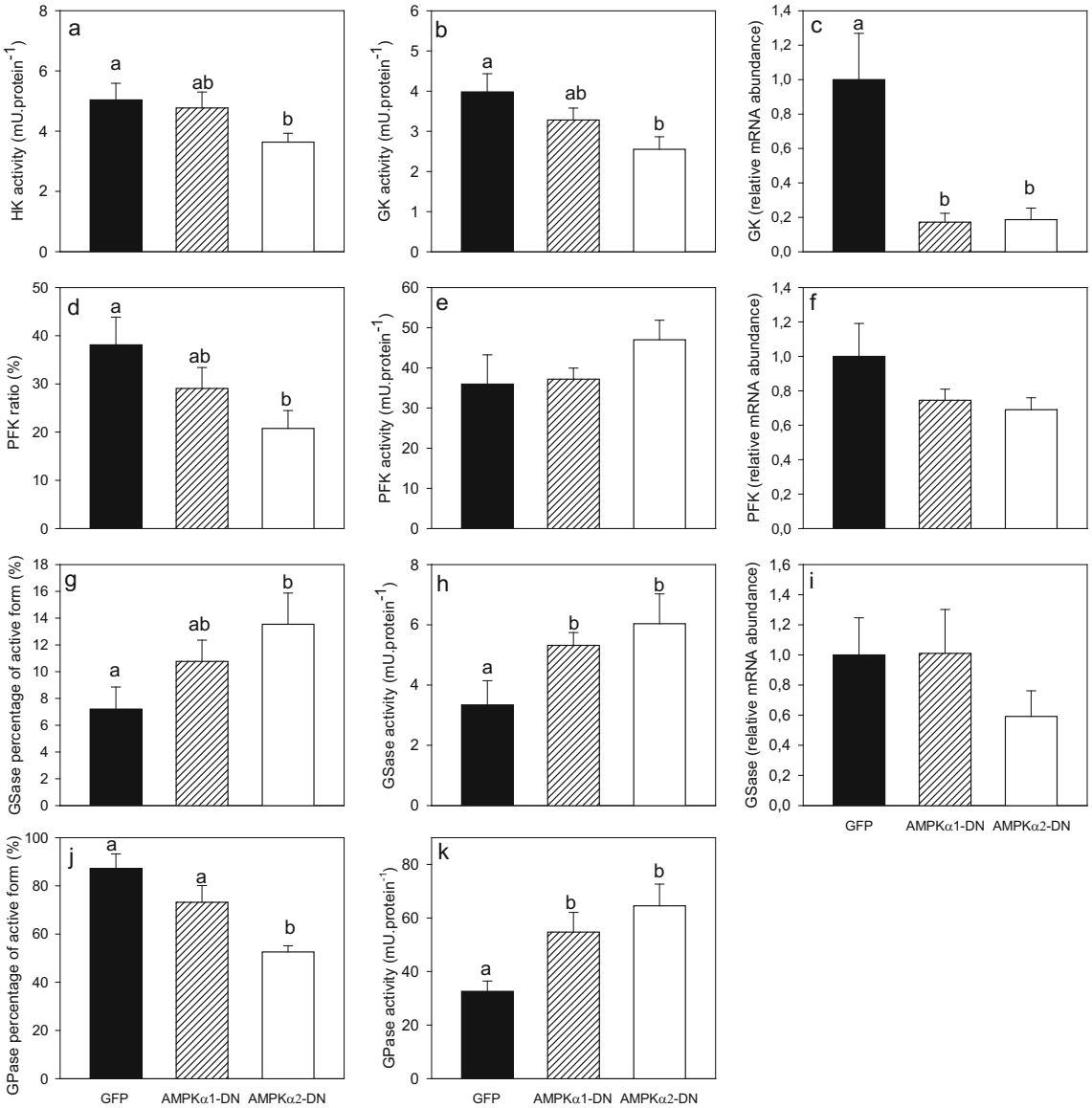

Fig. 7 Hepatic enzymes of glucose metabolism are affected by AMPKα-DN treatment. Enzyme activity (**a**, **b**, **e**, **h**, **k**), percentage of enzyme in active form (**d**, **g**, **j**), and mRNA abundance (**c**, **f**, **i**) of HK, GK, PFK, GPase, and GSase in the liver of rainbow trout 13 days after intracerebroventricular administration of adenovirus tagged with GFP (Control) or adenovirus tagged with GFP expressing a dominant negative AMPKα1 isoform (AMPKα1-DN) or expressing a dominant negative AMPKα2 isoform (AMPKα2-DN). Each value of enzyme activity and ratios is the mean ± SEM of $n = 10$ fish per treatment. Each value of mRNA abundance is the mean ± SEM of $n = 5$ fish per treatment. Gene expression results are standardized to the control group and are normalized by β-actin and EF1α expression. Different letters indicate significant differences ($P < 0.05$) between groups

regulation of peripheral metabolism would be maintained but adding a new one related to browning and thermogenesis.

Central activation of the AMPKα2 isoform might also be involved in the regulation of energy homeostasis acting on peripheral tissues. We now focused on liver since this is the main tissue involved in energy homeostasis in fish, a group lacking the brown adipose tissue that is the main target for AMPKα1 actions in mammals [2, 7]. In mammals, the inactivation of hypothalamic AMPKα1 or AMPKα2 relates to the activation of the sympathetic nervous system allowing an outflow to peripheral tissues, including liver [8, 13, 43]. Moreover, the hypothalamus-pituitary-adrenal (HPA) axis is also involved in the liver response to AMPK activation in hypothalamus [17]. In rainbow trout, downstream mechanisms by which the hypothalamus may modulate hepatic metabolism might be also based on sympathetic and parasympathetic systems (that innervate gastrointestinal tract and liver [75, 76]) as well as the hypothalamus-pituitary-interrenal axis (fish equivalent to mammalian HPA). A functional relationship between central presence of nutrients and peripheral effects on liver metabolism comes from previous studies in which we demonstrated that icv treatment with oleate or octanoate elicited changes in energy metabolism in liver [77].

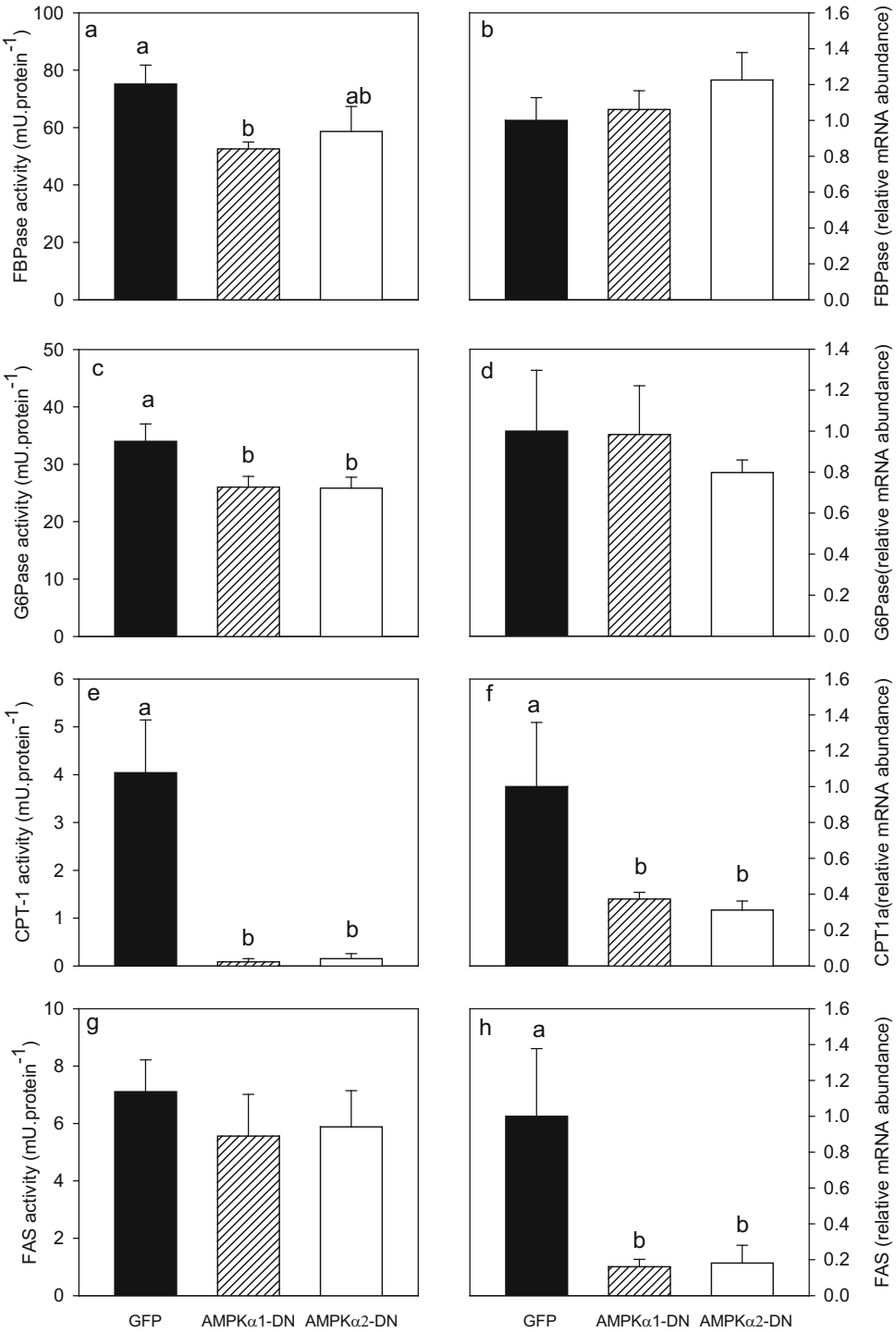

Fig. 8 Hepatic enzymes of glucose and lipid metabolism are affected by AMPKα-DN treatment. Enzyme activity (**a**, **c**, **e**, **g**) and mRNA abundance (**b**, **d**, **f**, **h**) of FBPase, G6Pase, CPT-1, and FAS in the liver of rainbow trout 13 days after intracerebroventricular administration of adenovirus tagged with GFP (Control) or adenovirus tagged with GFP expressing a dominant negative AMPKα1 isoform (AMPKα1-DN) or expressing a dominant negative AMPKα2 isoform (AMPKα2-DN). Each value of enzyme activity is the mean ± SEM of $n = 10$ fish per treatment. Each value of mRNA abundance is the mean ± SEM of $n = 5$ fish per treatment. Gene expression results are standardized to the control group and are normalized by β-actin and EF1α expression. Different letters indicate significant differences ($P < 0.05$) between groups

Glucose metabolism in liver is affected by central inhibition of AMPKα. A decreased glycolytic capacity is evident as supported by decreased activities of HK, GK, and PFK (activity and activity ratio) and mRNA abundance of GK in the AMPKα2-DN group while decreased GK mRNA abundance occurred in the AMPKα1-DN group. The gluconeogenic potential of liver also decreased after inhibition of both AMPKα isoforms as supported by changes in G6Pase and FBPase (not significant for α2) activities though the mRNA levels of both enzymes do not present any significant change. Glycogen levels clearly decreased in livers of AMPKα2-DN group while no changes occurred in the AMPKα1-DN group. This differential effect on levels of this metabolite might relate to changes in the activity of the two main enzymes involved in glycogen metabolism. However, contradictory changes were observed since increased activities of GSase and GPase were

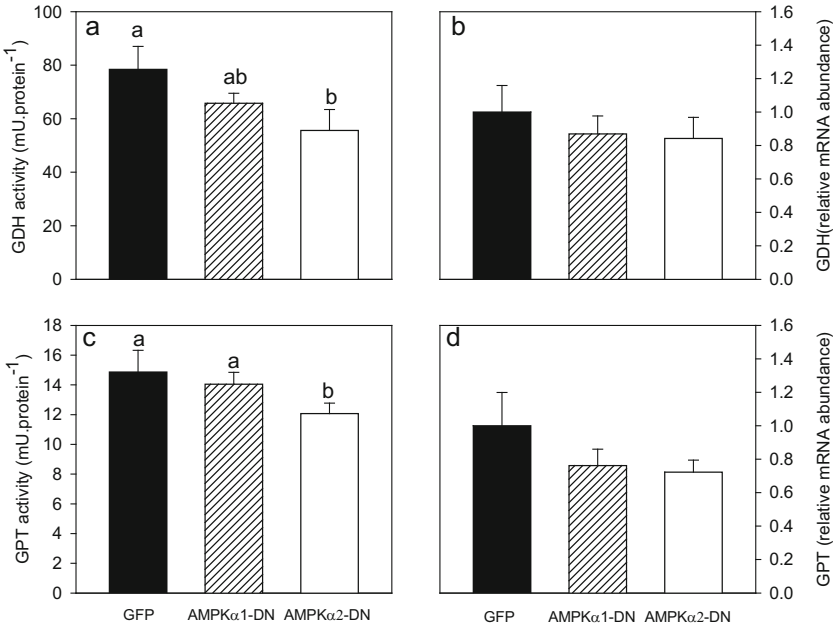

Fig. 9 Hepatic enzymes of amino acid metabolism are affected by AMPKα-DN treatment. Enzyme activity (**a**, **c**) and mRNA abundance (**b**, **d**) of GDH and GPT in liver of rainbow trout 13 days after intracerebroventricular administration of adenovirus tagged with GFP (Control) or adenovirus tagged with GFP expressing a dominant negative AMPKα1 isoform (AMPKα1-DN) or expressing a dominant negative AMPKα2 isoform (AMPKα2-DN). Each value of enzyme activity is the mean ± SEM of $n = 10$ fish per treatment. Each value of mRNA abundance is the mean ± SEM of $n = 5$ fish per treatment. Gene expression results are standardized to the control group and are normalized by β-actin and EF1α expression. Different letters indicate significant differences ($P < 0.05$) between groups

observed simultaneously. Overall, it seems that central inhibition of AMPKα2 results in decreased glucose production and use in liver, and this matches with the reduced levels observed in plasma while inhibition of the AMPKα1 isoform would result in a clear decrease in gluconeogenesis also concomitant with reduced plasma glucose levels. In mammals, molecular and pharmacological inhibition of hypothalamic AMPK also lowers hepatic glucose production [16, 78]. In addition, when AMPK is pharmacologically activated by AICAR, there is an increase in hormonal counterregulation, resulting in elevated endogenous glucose production in liver [15, 17, 79] and increased glycogen levels in muscle [80]. Additionally, deletion of LKB1, an important kinase upstream of AMPK, in POMC neurons leads to an impairment in peripheral glucose homeostasis [81]. It seems that the signal coming from the brain is suggesting the fish nutrient abundance and therefore is necessary to limit entry of nutrients in the circulation for use in other tissues. An indirect support to these findings comes from previous studies in the same species in which the rise in nutrient levels in the brain by icv treatment also resulted in decreased mRNA abundance of GK and FBPase in liver [82]. Moreover, in mammals, icv administration of glucose or oleate also resulted in liver with decreased hepatic glucose production [83]. Interestingly, the effects elicited by inhibition of the AMPKα2 isoform are more important than those elicited by the AMPKα1 isoform. A more important role for AMPKα2 in fish agrees with the mammalian model where this isoform is more important in regulation of glucose metabolism than AMPKα1. Thus, hypoglycemic conditions induced a rise in hypothalamic phosphorylation of AMPK in parallel with increased activity of AMPKα2 but not AMPKα1 [84]. Furthermore, AMPKα2 knockout mice presented impaired glucose tolerance, whereas no metabolic effects were detected in mice lacking AMPKα1 isoform [10, 11]. However, since some effects also occurred in AMPKα1-DN group in the present study, the response of mammals and fish is not exactly the same, and this might relate to the reduced importance of glucose metabolism in fish compared with mammals [45, 46].

In mammals, the sympathetic system as well as the hypothalamus-pituitary-adrenal axis are involved in the liver response to the activation of AMPK in hypothalamus [17]. Therefore, central pharmacological or molecular inhibition of AMPK reduced the sympathetic tone in liver together with reduced circulating levels of catecholamines and corticosterone [84]. In fish liver, catecholamines induce glycogenolysis [85], whereas cortisol (the main glucocorticoid in fish) activates gluconeogenesis [86]. A hypothetical decrease in the signal of these endocrine axes would match with the results observed in parameters related to gluconeogenesis but not in glycogen levels.

Lipid metabolism in liver was also affected by the inhibition of AMPKα in the brain. The lipogenic capacity of liver apparently decreased in both treated groups as supported by changes observed in FAS mRNA abundance. As suggested above for glucose, these changes would suggest a lower synthesis of lipid in the liver of treated groups, which is reflected

in the decreased levels of fatty acid and triglyceride in liver (though not significant for α1 group). This is in agreement with studies in mammals reporting that icv treatment with glucose or oleate, conditions decreasing AMPK activity in hypothalamus, inhibited hepatic lipogenesis [83]. However, this effect is opposite to that found in rodents, where genetic inhibition or ablation of hypothalamic AMPKα1 promotes hepatic lipogenesis [13]. Therefore, the existence of alternative mechanism, besides AMPK, mediating the actions of glucose and oleate cannot be excluded. This reduced capacity of liver to synthesize lipids would result in a decreased availability of fuels to be used in other tissues. This is supported by the decreased levels of triglyceride in plasma in treated groups as well as by the reduced levels of fatty acids in the AMPKα1-DN group though a striking increase occurred in the fatty acid levels in plasma of AMPKα2-DN group. The observed decrease in the potential of liver to oxidize fatty acids (reduced CPT-1 activity and CPT1a mRNA abundance) is surprising since rainbow trout fed a high lipid diet, with concomitant decreased phosphorylation status of AMPKα in hypothalamus, presented increased mRNA abundance of CPT1a, HOAD, and UCP2a in liver [37].

Central inhibition of AMPKα2 but not AMPKα1 resulted in decreased capacity of amino acid catabolism in liver as supported by decreased GDH and GPT activities in this tissue. This reduced use of amino acids coincides with the rise observed in total amino acid levels in the same tissue and with the absence of changes in plasma. Considering the importance of amino acids in the regulation of food intake in fish [87], as well as in metabolism for fueling purposes [46], it is very interesting that changes observed in amino acid metabolism paralleled in general those observed in glucose and lipid metabolism. It is also very relevant that in this case the effects can be only attributable to the isoform AMPKα2.

In conclusion, inhibiting AMPKα isoforms in rainbow trout brain provided information regarding putative differential roles of these isoforms in carnivore fish to compare with the other available vertebrate model (omnivore mammals). Both isoforms are apparently involved in the regulation of energy homeostasis though the way in which they operate appears to be different. AMPKα1 is not apparently involved in the regulation of food intake in contrast to the effects noticed for AMPKα2. This differential response is comparable to that known in mammals. However, the central inhibition of both isoforms elicited changes in hepatic metabolism, mainly a reduced synthesis and use of glucose and lipids with more important changes elicited by AMPKα2, which furthermore is the only isoform involved in changes in amino acid metabolism. Based on present results and available literature, we may suggest that from an evolutionary point of view, the function of AMPKα2 appears to be similar throughout the vertebrate lineage. The function of AMPKα1 in fish basically appears to relate to modulation of metabolism to maintain homeostasis. In contrast, in mammals, AMPKα1 acquired an additional role to deal with modulation of brown adipose tissue and thermogenesis [42, 56] but notably using hepatic lipid as fuel in a coordinate fashion [13]. Future studies are required to assess the exact molecular mechanism and autonomic and/or hormonal pathways mediating those actions.

Author's Contributions ML and JLS conceived the experiments. MC-S, SC, and CV conducted the experiments and carried out the assessment of mRNA abundance, enzyme activities, and metabolite levels. RA-O carried out the histochemical procedures. V.C and LL-P carried out Western blots. MC-S, VC, ML, and JLS analyzed the results. MC-S, ML, and JLS wrote the manuscript. All authors reviewed the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical Approval The experiments described comply with the Guidelines of the European Union Council (2010/63/UE) and of the Spanish Government (RD53/2013) for the use of animals in research and were approved by the Ethics Committee of the Universidade de Vigo.

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