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RESEARCH ARTICLE

Changes in the levels and phosphorylation status of Akt, AMPK, CREB and FoxO1 in hypothalamus of rainbow trout under conditions of enhanced glucosensing activity

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ABSTRACT

There is no available information about mechanisms linking glucosensing activation in fish and changes in the expression of brain neuropeptides controlling food intake. Therefore, we assessed in rainbow trout hypothalamus the effects of raised levels of glucose on the levels and phosphorylation status of two transcription factors, FoxO1 and CREB, possibly involved in linking these processes. We also aimed to assess the changes in the levels and phosphorylation status of two proteins possibly involved in the modulation of these transcription factors: Akt and AMPK. Therefore, in pooled preparations of hypothalamus incubated for 3 and 6 h in the presence of 2, 4 or 8 mmol l^{-1} D-glucose, we evaluated the response of parameters related to glucosensing mechanisms, neuropeptide expression and levels and phosphorylation status of the proteins of interest. The activation of hypothalamic glucosensing systems and the concomitant enhanced anorectic potential occurred in parallel with activation of Akt and inhibition of AMPK. The changes in these proteins relate to neuropeptide expression through changes in the level and phosphorylation status of transcription factors under their control, such as CREB and FoxO1, which displayed inhibitory (CREB) or activatory (FoxO1) responses to increased glucose.

KEY WORDS: Trout, Glucosensing, AMPK, CREB, FoxO1, Akt

INTRODUCTION

The detection of changes in nutrient levels in the vertebrate brain is an essential process involved in the regulation of food intake and energy expenditure as demonstrated in mammals (Blouet and Schwartz, 2010; Morton et al., 2014) and fish (Delgado et al., 2017). Accordingly, several mechanisms are present in brain areas – in particular, the hypothalamus – to detect changes in the levels of glucose, fatty acids and amino acids as demonstrated in mammals (Efeyan et al., 2015) and fish (Soengas, 2014; Conde-Sieira and Soengas, 2017). Glucose is one of the main nutrients whose levels are sensed through different mechanisms (Polakof et al., 2011a). In fish, evidence obtained in recent years suggested the presence in rainbow trout hypothalamus of glucosensing mechanisms including the canonical mechanism based on glucokinase (GK), glucose facilitative transporter 2 (GLUT2) and ATP-dependent inward rectified potassium channel (K_{ATP}) (see reviews by Polakof et al., 2011a; Soengas, 2014; Conde-Sieira and Soengas, 2017). Moreover, evidence has been obtained in rainbow trout hypothalamus regarding the presence of GK-independent glucosensing mechanisms based on liver X receptor (LXR) and sweet taste receptor (Otero-Rodiño et al., 2015, 2016; Balasubramanian et al., 2016). The activation of these mechanisms in the hypothalamus relates to the control of food intake. This occurs through the enhancement of cocaine- and amphetamine-related transcript (CART) and pro-opio melanocortin (POMC) expression, and the inhibition of agouti-related peptide (AgRP) and neuropeptide Y (NPY) expression. These changes result in decreased food intake (Conde-Sieira and Soengas, 2017; Delgado et al., 2017) in a way comparable to that observed in mammals (Belgardt et al., 2009; Blouet and Schwartz, 2010; Morton et al., 2014).

The mechanisms linking the function of glucosensing systems with changes in the expression of neuropeptides, which ultimately regulate food intake, are mostly unknown even in mammals. Changes in the expression of neuropeptides might relate to modulation of forkhead box protein O1 (FoxO1), cAMP response element binding protein (CREB) and/or brain homeobox transcription factor (BSX) (Belgardt et al., 2009; Diéguez et al., 2011). However, it is not clear how these transcription factors relate to the activity of the different nutrient-sensing systems. Several possibilities have been suggested in mammals (Diéguez et al., 2011; Gao et al., 2013; Morton et al., 2014), including modulation by AMP-activated protein kinase (AMPK), mechanistic target of rapamycin (mTOR) and protein kinase B (Akt). There are few studies addressing the effects in mammalian hypothalamus of raised glucose levels on the amount and/or phosphorylation status of these proteins, with some studies carried out on AMPK (Beall et al., 2012; Zhang et al., 2015; Oh et al., 2016) and Akt (Chalmers et al., 2014). In fish, no available studies have assessed the effects of raised levels of glucose on the amount and/or phosphorylation status of these proteins in the hypothalamus. The available studies in fish demonstrated a rise in protein levels of AMPK, Akt and mTOR in hypothalamus of fish fed a lipid-enriched diet (Librán-Pérez et al., 2015) and increased Akt protein levels in isolated hypothalamic cells incubated with leptin (Gong et al., 2016). In other tissues, like liver, the exposure to increased levels of glucose (induced by direct treatment or feeding a carbohydrate-enriched diet) resulted in increased levels of Akt in rainbow trout (Seiliez et al., 2011; Jin et al., 2014; Dai et al., 2014) and zebrafish (Jörgens et al., 2015) and decreased levels of AMPK in rainbow trout (Kamalam et al., 2012).

Therefore, we aimed to assess the effects of raised levels of glucose on the levels and phosphorylation status of two of the transcription factors, CREB and FoxO1, possibly involved in

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linking the activation of glucosensing systems with the modulation of food intake through the expression of brain neuropeptides. We also assessed the changes in the levels and phosphorylation status of two of the proteins, Akt and AMPK, possibly involved in the modulation of these transcription factors.

MATERIALS AND METHODS

Fish

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

Experimental design

Freshly obtained tissues were incubated as previously described (Polakof et al., 2007). Fish were fasted for 24 h before treatment to ensure basal hormone levels were achieved. Every morning of an experiment, fish were dip-netted from the tank, anaesthetized with 2-phenoxyethanol (Sigma, St Louis, MO, USA; 0.2% v/v), killed by decapitation and weighed. The hypothalamus was removed, rinsed with modified Hanks' medium (136.9 mmol l^{-1} NaCl, 5.4 mmol l^{-1} KCl, 5 mmol l^{-1} $NaHCO_3$, 1.5 mmol l^{-1} $CaCl_2$, 0.81 mmol l^{-1} $MgSO_4$, 0.44 mmol l^{-1} KH_2PO_4, 0.33 mmol l^{-1} Na_2HPO_4, 10 mmol l^{-1} Hepes, 50 U ml^{-1} penicillin and 50 µg ml^{-1} streptomycin sulphate, pH 7.4; referred to as basal medium), sliced on chilled Petri dishes, and placed in a chilled Petri dish containing 100 ml modified Hanks' medium g^{-1} tissue that was gassed with 0.5% CO_2/99.5% O_2. To ensure adequate mass, pooled tissue samples from 3–4 fish were prepared. Tissue samples were incubated in 48-well culture plates at 15°C for 3–6 h with 250 µl of modified Hanks' medium per well containing 25 mg of tissue and 2, 4 or 8 mmol l^{-1} D-glucose. The wells were gassed with a 0.5% CO_2/99.5% O_2 mixture. After 3 or 6 h of incubation, tissue samples were quickly removed, rinsed, frozen in liquid nitrogen and stored at −80°C until assayed. D-Glucose concentrations were selected based on previous *in vitro* studies in rainbow trout (Polakof et al., 2007). For each experiment, one set of four pooled tissue samples was assayed for mRNA levels, and another set of four pooled tissue samples was assayed for changes in protein levels by western blot. The number of independent experiments (one set of four pooled tissue samples each) carried out was six (N=6).

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). A 2 µg sample of total RNA was reverse transcribed using Superscript II reverse transcriptase (Promega) and random hexamers (Promega) in approximately 20 µl volume. 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 nmol l^{-1} of each primer. mRNA abundance of transcripts was determined as previously described in the same species by Panserat et al. (2000; GK), Leder and Silverstein (2006; POMC-a1), Cruz-García et al. (2009; LXRα), Conde-Sieira et al. (2010b; CART, NPY), Polakof and Soengas (2013; type 1 taste receptor subunit 3, T1R3) and MacDonald et al. (2014; AgRP). Sequences of the forward and reverse primers for each gene are shown in Table 1. Relative quantification of the target gene transcript was done using β-actin gene expression as a reference, which was stably expressed in this experiment. Thermal cycling was initiated with incubation at 95°C for 90 s using hot-start iTaq DNA polymerase activation followed by 35 cycles of heating at 95°C for 20 s and specific annealing and extension temperatures for 20 s. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C s^{-1} from 55 to 94°C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without RNA were run for each reaction as negative controls. Relative quantification of the target gene transcript with the β-actin reference gene transcript was made following the Pfaffl (2001) method.

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 the Bradford assay with bovine serum albumin as standard.

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

Gene	Forward (F)/reverse (R) primer	Annealing temperature (°C)	Database	Accession no.
β-actin	F: GATGGGCCAGAAAGACAGCTA R: TCGTCCCAGTTGGTGACGAT	59	GenBank	NM_ 001124235.1
AgRP	F: ACCAGCAGTCCTGTCTGGGTAA R: AGTAGCAGATGGAGCCGAACA	60	GenBank	CR376289
CART	F: ACCATGGAGAGCTCCAG R: GCGCACTGCTCTCCAA	60	GenBank	NM_001124627
GK	F: GCACGGCTGAGATGCTCTTTG R: GCCTTGAACCCTTTGGTCCAG	60	GenBank	AF053331
LXRα	F: TGCAGCAGCCGTATGTGGA R: GCGGCGGGAGCTTCTTGTC	62	GenBank	FJ470291
NPY	F: CTCGTCTGGACCTTTATATGC R: GTTCATCATATCTGGACTGTG	58	GenBank	NM_001124266
POMCa1	F: CTCGCTGTCAAGACCTCAACTCT R: GAGTTGGGTTGGAGATGGACCTC	60	Tigr	TC86162
T1R3	F: GCCCTGTGGAGCCCATCTTA R: CCACACAGTAGGTCAGGGTGGA	60	Sigenae	GAY7CUQ01EHKNI.s.om.10

AgRP, agouti-related peptide; CART, cocaine- and amphetamine-related transcript; GK, glucokinase; LXRα, liver X receptor α; NPY, neuropeptide Y; POMCa1, pro-opio melanocortin a1; T1R3, type 1 taste receptor subunit 3.

Hypothalamus protein lysates (20 µg) were western blotted using appropriate antibodies from Cell Signaling Technology (Saint Quentin Yvelings, France): 1:1000 anti-phospho Akt (Ser473), 1:1000 anti-carboxyl terminal Akt, 1:500 anti-phospho AMPK (Thr172), 1:500 anti-AMPK, 1:500 anti-phospho CREB (Ser133), 1:500 anti-CREB (48h2), 1:250 anti-phospho-FoxO1 (Thr24), 1:250 anti-FoxO1 (L27) and 1:1000 anti-tubulin (used as control protein for normalization). All these antibodies cross-react successfully with rainbow trout proteins of interest (Skiba-Cassy et al., 2009; Kamalam et al., 2012; Velasco et al., 2016). After washing, membranes were incubated with an IgG-HRP secondary antibody (Bio-Rad) and bands were quantified by Image Lab software version 5.2.1 (Bio-Rad).

Statistics

Comparisons among groups were carried out using two-way ANOVA with glucose concentration (2, 4 and 8 mmol l^{-1}) and time (3 and 6 h) as main factors. The normal distribution of variables and homoscedasticity were analysed by Kolmogorov–Smirnov and Levene tests, respectively. In those cases where a significant effect was observed for a factor, comparisons were carried out by a Student–Newman–Keuls (SNK) test. Differences were considered statistically significant at $P<0.05$. Comparisons were carried out with the SigmaStat statistical package.

RESULTS

Changes in mRNA abundance of transcripts is shown in Table 2. GK mRNA levels after 6 h incubation increased under 4 mmol l^{-1} glucose compared with 2 mmol l^{-1} and 8 mmol l^{-1} glucose whereas those under 8 mmol l^{-1} glucose were lower than those under 2 mmol l^{-1} glucose; furthermore, the mRNA level under 4 mmol l^{-1} glucose after 6 h was also higher than that after 3 h at the same concentration. The mRNA level of LXRα after 3 h incubation was higher under 8 mmol l^{-1} glucose than under 4 mmol l^{-1} glucose whereas levels after 6 h incubation were higher under 8 mmol l^{-1} glucose than at both the other glucose concentrations. T1R3 mRNA levels after 6 h incubation were higher at 2 mmol l^{-1} glucose than at the other concentrations; the level at 4 mmol l^{-1} glucose was lower after 6 h than after 3 h of incubation. The mRNA levels of AgRP after 3 h incubation were higher at 4 mmol l^{-1} glucose than at the other glucose concentrations; after 6 h, levels at 2 mmol l^{-1} glucose were lower than those under 4 and 8 mmol l^{-1} glucose; the level after 6 h incubation for 2 mmol l^{-1} glucose was lower than that after 3 h at the same concentration. NPY mRNA level after 6 h incubation was higher at 4 mmol l^{-1} glucose than at 2 and 8 mmol l^{-1} glucose. The mRNA levels of POMC-a1 at 4 and 8 mmol l^{-1} glucose were higher than those at 2 mmol l^{-1} glucose after 3 and 6 h incubation; the level at 8 mmol l^{-1} glucose after 6 h incubation was also higher than that at 4 mmol l^{-1} glucose at the same time, and the values after 6 h incubation at 2 and 8 mmol l^{-1} glucose were higher than those for the same concentrations after 3 h incubation. CART mRNA levels after 3 h incubation were higher at 8 mmol l^{-1} glucose than at 2 mmol l^{-1} glucose whereas a progressive increase with glucose concentration was observed after 6 h incubation; the levels after 6 h incubation were lower (2 and 4 mmol l^{-1} glucose) or higher (8 mmol l^{-1} glucose) than those observed at the same concentrations after 3 h incubation.

The abundance of phosphorylated Akt (P-Akt) decreased at 4 and 8 mmol l^{-1} glucose compared with 2 mmol l^{-1} glucose after 6 h incubation; the values at 4 and 8 mmol l^{-1} glucose after 6 h were also lower than those observed after 3 h at the same concentrations (Fig. 1A). The abundance of Akt decreased under 4 and 8 mmol l^{-1} glucose compared with 2 mmol l^{-1} glucose after both 3 and 6 h incubation (Fig. 1B) and all values after 6 h were also lower those after 3 h. The phosphorylation state of Akt (Fig. 1C) increased under 4 and 8 mmol l^{-1} glucose after 3 h incubation; the values observed after 6 h incubation for all concentrations assessed were lower than their respective counterparts after 3 h incubation.

The abundance of phosphorylated AMPK (P-AMPK; Fig. 2A) decreased with increases in glucose concentration after 3 h incubation, and at 4 mmol l^{-1} glucose compared with 2 mmol l^{-1} glucose after 6 h of incubation; the values after 6 h incubation were lower than those after 3 h at all concentrations assessed. The abundance of AMPK (Fig. 2B) decreased at 4 and 8 mmol l^{-1} glucose compared with 2 mmol l^{-1} glucose after both 3 and 6 h of incubation; the values observed after 6 h were lower than those observed after 3 h at the same concentrations. The phosphorylation status of AMPK (Fig. 2C) increased at 4 mmol l^{-1} glucose after 3 h incubation; after 6 h incubation, the value at 8 mmol l^{-1} glucose was lower than that at 2 mmol l^{-1} glucose; the value at 2 mmol l^{-1} glucose after 6 h was higher than that after 3 h at the same glucose concentration.

The abundance of phosphorylated CREB (P-CREB; Fig. 3A) decreased after 6 h incubation at 4 and 8 mmol l^{-1} glucose compared with 2 mmol l^{-1} glucose and these values were also

Table 2. Changes in mRNA abundance of selected transcripts in rainbow trout hypothalamus incubated *in vitro* for 3 or 6 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} glucose

	3 h incubation			6 h incubation		
	2 mmol l^{-1} glucose	4 mmol l^{-1} glucose	8 mmol l^{-1} glucose	2 mmol l^{-1} glucose	4 mmol l^{-1} glucose	8 mmol l^{-1} glucose
Glucosensing parameters						
GK	1.00±0.22	0.95±0.24	1.07±0.20	1.36±0.16^{a}	1.68±0.11^{b,*}	0.72±0.25^{c}
LXRα	1.00±0.08^{a,b}	0.84±0.03^{a}	1.16±0.12^{b}	0.91±0.07^{a}	0.96±0.11^{a}	1.36±0.13^{b}
T1R3	1.00±0.15	1.20±0.33	0.59±0.17	1.47±0.26^{a}	0.50±0.09^{b,*}	0.38±0.06^{b}
Neuropeptides						
AgRP	1.00±0.06^{a}	2.29±0.38^{b}	1.44±0.29^{a}	0.68±0.11^{a,*}	1.60±0.23^{b}	1.67±0.27^{b}
NPY	1.00±0.16	1.15±0.24	1.19±0.14	0.66±0.08^{a}	1.54±0.22^{b}	0.91±0.25^{a}
POMCa1	1.00±0.18^{a}	3.37±0.85^{b}	2.67±0.55^{b}	1.95±0.07^{a,*}	4.10±0.96^{b}	7.43±1.49^{c,*}
CART	1.00±0.23^{a}	1.28±0.13^{a,b}	1.57±0.07^{b}	0.39±0.02^{a,*}	0.92±0.13^{b,*}	2.58±0.13^{c,*}

Data represent means±s.e.m. of six independent experiments carried out on hypothalamus pooled from 3–4 different fish. Data are normalized to the group incubated for 3 h with 2 mmol l^{-1} glucose (results were previously normalized by β-actin mRNA levels, which did not show changes among groups). Different letters indicate significant differences from levels at other glucose concentrations at the same time [two-way ANOVA $P<0.05$, *post hoc* Student–Newman–Keuls (SNK) test $P<0.05$]. *Significantly different ($P<0.05$) from 3 h at the same glucose concentration (two-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$).

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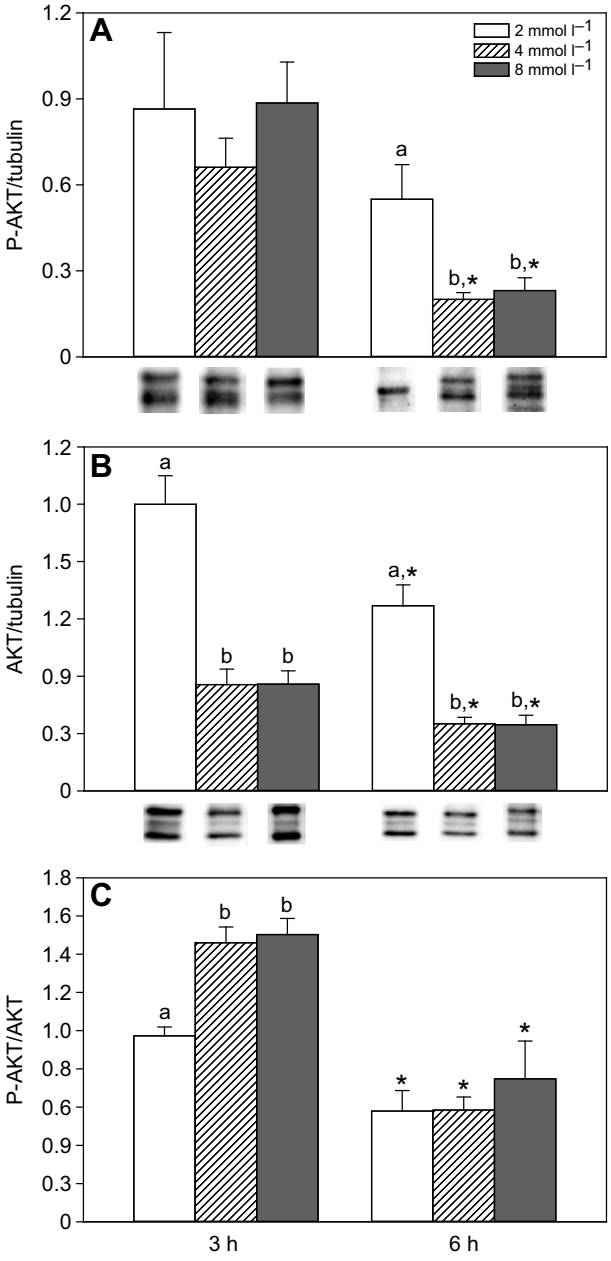

Fig. 1. Abundance and phosphorylation state of protein kinase B (Akt) in the hypothalamus. Western blot analysis of phosphorylated Akt (P-Akt; A) and Akt (B) in rainbow trout hypothalamus incubated *in vitro* for 3 or 6 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} glucose; 20 μg of total protein was loaded on the gel per lane, and results were normalized by tubulin abundance. Western blots were performed on six individual samples per treatment and one representative blot per treatment is shown here. (C) Ratio of phosphorylated protein to total amount of the target protein. Each value is the mean+s.e.m. of six independent experiments carried out on hypothalamus pooled from 3–4 different fish. Different letters indicate a significant difference from values at other glucose concentrations at the same time (two-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$). *Significantly different ($P<0.05$) from 3 h at the same glucose concentration [two-way ANOVA $P<0.05$, *post hoc* Student–Newman–Keuls (SNK) test $P<0.05$].

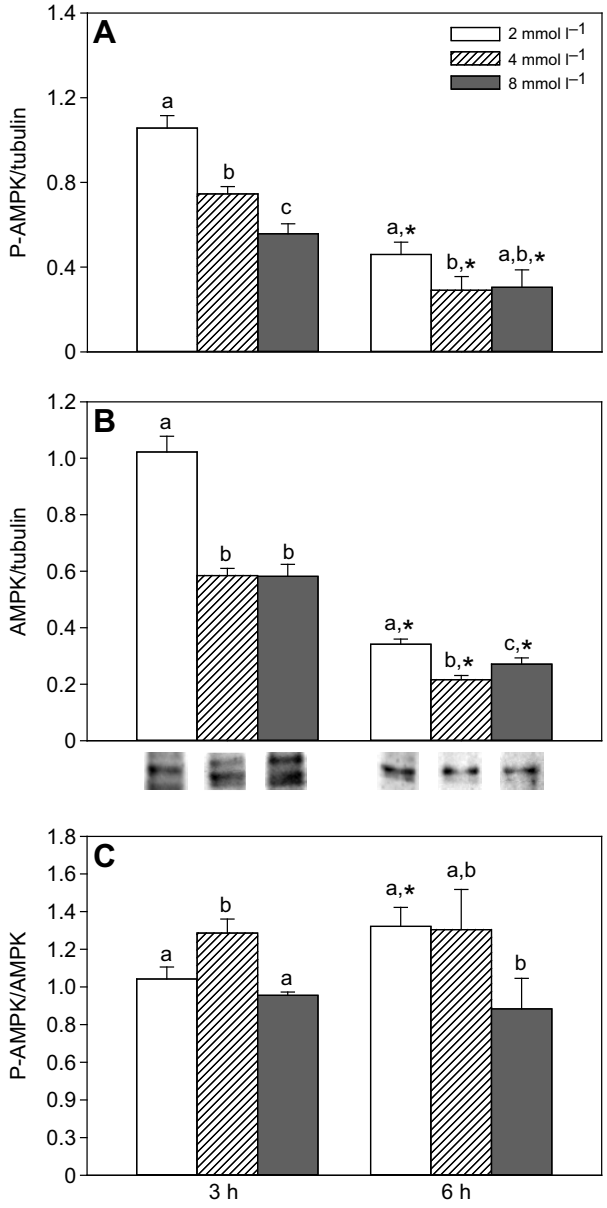

Fig. 2. Abundance and phosphorylation state of AMP-activated protein kinase (AMPK) in the hypothalamus. Western blot analysis of phosphorylate AMPK (P-AMPK; A) and AMPK (B) in rainbow trout hypothalamus incubated *in vitro* for 3 or 6 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} glucose; 20 μg of total protein was loaded on the gel per lane, and results were normalized by tubulin abundance. Western blots were performed on six individual samples per treatment and one representative blot per treatment is shown here. (C) Ratio of phosphorylated protein to total amount of the target protein. Each value is the mean+s.e.m. of six independent experiments carried out with on hypothalamus pooled from 3–4 different fish. Different letters indicate significant differences from values at other glucose concentrations at the same time (two-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$). *Significantly different ($P<0.05$) from 3 h at the same glucose concentration (two-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$).

lower than those after 3 h at the same concentrations. The abundance of CREB (Fig. 3B) decreased at 4 and 8 mmol l^{-1} glucose compared with 2 mmol l^{-1} glucose for tissues incubated for 3 and 6 h. The phosphorylation ratio of CREB (Fig. 3C) increased at 4 and 8 mmol l^{-1} glucose compared with 2 mmol l^{-1} glucose after 3 h incubation, and these values were also higher than those observed at the same concentrations after 6 h.

The abundance of P-FoxO1 did not show changes in parallel with glucose concentration, although values after 6 h incubation were higher than those observed after 3 h at all glucose concentrations (Fig. 4A). The samples corresponding to

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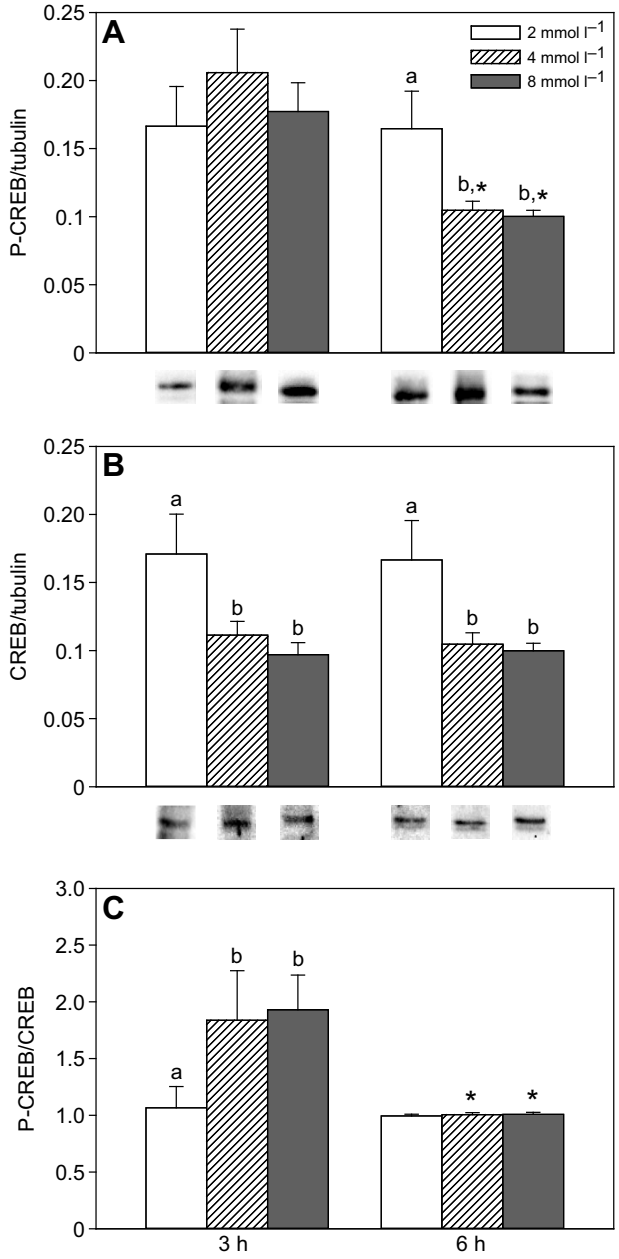

Fig. 3. Abundance and phosphorylation state of cAMP response element binding protein (CREB) in the hypothalamus. Western blot analysis of phosphorylated CREB (P-CREB; A) and CREB (B) in rainbow trout hypothalamus incubated *in vitro* for 3 or 6 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} glucose; 20 μg of total protein was loaded on the gel per lane, and results were normalized by tubulin abundance. Western blots were performed on five individual samples per treatment and one representative blot per treatment is shown here. (C) Ratio of phosphorylated protein to total amount of the target protein. Each value is the mean+s.e.m. of six independent experiments carried out on hypothalamus pooled from 3–4 different fish. Different letters indicate significant differences from values at other glucose concentrations at the same time (two-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$). *Significantly different ($P<0.05$) from 3 h at the same glucose concentration (two-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$).

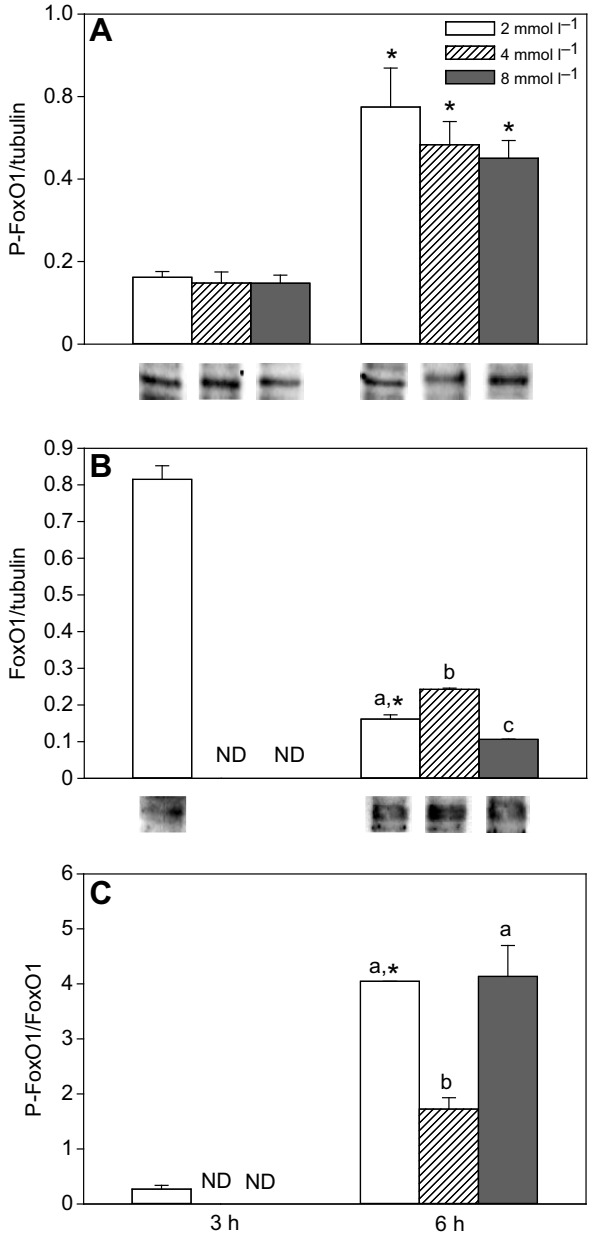

Fig. 4. Abundance and phosphorylation state of forkhead box protein O1 (FoxO1) in the hypothalamus. Western blot analysis of phosphorylated FoxO1 (P-FoxO1; A) and FoxO1 (B) in rainbow trout hypothalamus incubated *in vitro* for 3 or 6 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} glucose; 20 μg of total protein was loaded on the gel per lane, and results were normalized by tubulin abundance. Western blots were performed on six individual samples per treatment and one representative blot per treatment is shown here. (C) Ratio of phosphorylated protein to total amount of the target protein. Each value is the mean+s.e.m. of six independent experiments carried out on hypothalamus pooled from 3–4 different fish. Different letters indicate significant differences from values at other glucose concentrations at the same time (two-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$). *Significantly different ($P<0.05$) from 3 h at the same glucose concentration (two-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$). ND, not determined.

FoxO1 after 3 h incubation under 4 and 8 mmol l^{-1} glucose were lost and therefore are not shown (Fig. 4B); after 6 h incubation, the value at 4 mmol l^{-1} glucose was higher than that at the other glucose concentrations, and the value at 8 mmol l^{-1} was also lower than that at 2 mmol l^{-1} glucose; finally, the value at 2 mmol l^{-1} glucose after 6 h was also lower than that after 3 h at the same concentration. The phosphorylation status of FoxO1 (Fig. 4C) could not be determined (see above) for 3 h incubation; after 6 h, the value at 4 mmol l^{-1} glucose was lower than those at 2 and 8 mmol l^{-1} glucose; the value at

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2 mmol l^{-1} glucose after 6 h was higher than that after 3 h at the same concentration.

The abundance of tubulin under the same conditions is shown in Fig. 5. No significant changes were noticed and therefore we used these values for normalization.

DISCUSSION

Exposure to raised levels of glucose induced changes in mRNA abundance of several parameters related to glucosensing mechanisms present in rainbow trout hypothalamus (Conde-Sieira and Soengas, 2017). These include the rise in mRNA levels of GK and LXRα as well as the decrease in mRNA levels of T1R3. These changes were comparable to those previously observed in rainbow trout hypothalamus under comparable experimental conditions (Polakof et al., 2007; Aguilar et al., 2011; Conde-Sieira et al., 2011, 2012; Otero-Rodiño et al., 2016), thus validating the experimental design. As for neuropeptides involved in the control of food intake, a sharp increase occurred in the mRNA abundance of anorexigenic neuropeptides POMC-a1 and CART with an increase in glucose levels in the medium. This is in agreement with prior evidence obtained in rainbow trout hypothalamus under similar experimental conditions (Conde-Sieira et al., 2011, 2012; Otero-Rodiño et al., 2016). For the orexigenic neuropeptides NPY and AgRP, the expected decrease in mRNA in parallel with increased glucose levels was only observed when comparing 4 and 8 mmol l^{-1} glucose after 3 h incubation for AgRP and after 6 h incubation for NPY but not in the other conditions. However, as a whole, considering changes in mRNA abundance of the four neuropeptides, an increase in the anorexigenic potential occurred with an increase in glucose levels in the medium, thus validating the experimental design. The increased anorexigenic potential is also in agreement with decreased food intake observed *in vivo* in the same species when subjected to raised glucose levels (Ruibal et al., 2002; Polakof et al., 2008a,b; Conde-Sieira et al., 2010a). Therefore, we aimed to evaluate whether, under these conditions of activation of glucosensing systems, eliciting changes in the expression of neuropeptides, changes occurred in the level and phosphorylation state of proteins that could be involved in linking these processes.

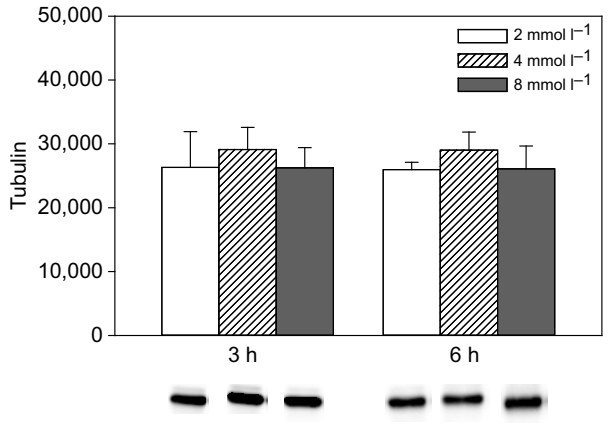

Fig. 5. Abundance of tubulin in the hypothalamus. Western blot analysis of tubulin in rainbow trout hypothalamus incubated *in vitro* for 3 or 6 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} glucose; 20 μg of total protein was loaded on the gel per lane. Western blots were performed on six individual samples per treatment and one representative blot per treatment is shown here. Each value is the mean+s.e.m. of six independent experiments carried out on hypothalamus pooled from 3–4 different fish. No significant differences were observed.

CREB is one of the molecules hypothesized to connect changes in brain metabolism with expression of neuropeptides. In mammals, decreased CREB levels resulted in decreased mRNA abundance of AgRP and NPY, thus favouring decreased food intake (Belgardt et al., 2009; Blanco de Morentín et al., 2011). Accordingly, in the present study, the abundance of CREB clearly decreased with an increase of glucose in the medium, and this was evident for both the phosphorylated and unphosphorylated forms of the protein. This is the first time that changes in CREB abundance have been assessed in fish hypothalamus under any condition. The presence of CREB has been characterized in goldfish liver (Yan et al., 2016); in zebrafish liver (Craig and Moon, 2011), food deprivation (a situation opposite to the raised levels of glucose in the present study) resulted in increased mRNA levels of CREB. Even in mammals, as far as we are aware, there are no comparable studies available regarding the effects of glucose on the level and/or phosphorylation status of this protein. However, the abundance of this protein is known to decrease under conditions in which food intake is inhibited, resulting in decreased expression of NPY/AgrP and thus increasing anorexigenic potential (Diéguez et al., 2011; Blanco de Morentín et al., 2011; Kwon et al., 2016). Given that the incubation of rainbow trout hypothalamus with glucose also resulted in increasing anorexigenic potential (Aguilar et al., 2011; Conde-Sieira et al., 2011; Otero-Rodiño et al., 2016; this study), we suggest that a comparable situation may be present in rainbow trout hypothalamus.

FoxO1 is another molecule hypothesized to connect changes in brain nutrient sensing with expression of neuropeptides. Increased FoxO1 resulted in increased mRNA abundance of POMC and CART, thus favouring decreased food intake (Belgardt et al., 2009; Blanco de Morentín et al., 2011). In the present study, glucose treatment did not affect the abundance of P-FoxO1 whereas we could only show the phosphorylation ratio for samples incubated for 6 h. The main changes occurred after 6 h incubation, at which time the phosphorylation ratio was lower at 4 mmol l^{-1} compared with that at 2 and 8 mmol l^{-1} glucose. The only available studies regarding this protein in fish hypothalamus demonstrated no main changes in fish treated with the orexigenic hormone ghrelin (Velasco et al., 2017), while in other tissues like liver, a decrease in the abundance of phosphorylated FoxO1 occurred under refeeding conditions (Dai et al., 2013). The increase observed when comparing values at 4 and 8 mmol l^{-1} glucose is in agreement with the increased ratio observed in mammalian hypothalamus under anorexigenic conditions eliciting increased expression of POMC and CART (Kwon et al., 2016).

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

This is the first study demonstrating the effects of raised glucose levels on Akt abundance and phosphorylation status in fish hypothalamus. The abundance of Akt displayed a decrease in parallel with the increase of glucose in the medium. However, when the phosphorylation status was assessed, we found an increase after 3 h incubation in tissues incubated under 4 and 8 mmol l^{-1} compared with those under 2 mmol l^{-1} glucose. This increased phosphorylation status of Akt is comparable to that observed in mammalian hypothalamus under increased glucose concentrations *in vitro* (Chalmers et al., 2014) and is also the expected response

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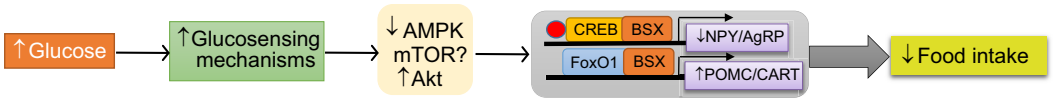

Fig. 6. Schematic drawing summarizing responses obtained for transcription factors and proteins involved in cellular signalling under conditions eliciting activation of glucosensing systems and inhibition of food intake. AgRP, agouti-related peptide; Akt, protein kinase B; AMPK, AMP-activated protein kinase; BSX, brain homeobox transcription factor; CART, cocaine- and amphetamine-related transcript; CREB, cAMP response element binding protein; FoxO1, forkhead box protein O1; mTOR, mechanistic target of rapamycin; NPY, neuropeptide Y; POMC, pro-opio melanocortin.

under situations eliciting raised POMC mRNA abundance and therefore decreased food intake (Kwon et al., 2016). In this way, it is interesting that intracerebroventricular treatment in rainbow trout with the orexigenic peptide ghrelin resulted in decreased phosphorylation ratio of Akt (Velasco et al., 2017), i.e. the converse response to that herein observed under an anorexigenic situation. The increase is also comparable to that observed in rainbow trout liver (Dai et al., 2014; Jin et al., 2014) or in the whole embryo of zebrafish (Jörgens et al., 2015) exposed to raised levels of glucose. Interestingly, in other situations in which levels of glucose increase, such as under refeeding conditions or after feeding carbohydrate-enriched diets, increased phosphorylation status of Akt also occurred in peripheral tissues, as demonstrated in liver of rainbow trout (Lansard et al., 2009; Seiliez et al., 2011) and barramundi (Wade et al., 2014), and muscle of Senegalese sole (Borges et al., 2014). The activation of Akt induces FoxO1 phosphorylation (Gross et al., 2009). Those situations in which Akt is activated, as in the present study, should presumably result in increased FoxO1 phosphorylation (Belgardt et al., 2009), which is known to promote expression of POMC and CART (Kwon et al., 2016). Thus, a possible relationship may exist between Akt and FoxO1 activation in the present study.

AMPK levels can be used to detect lowered cell energy status coupled to intrinsic cell mechanisms designed to restore energy balance (López, 2017). In the present study, the abundance of AMPK clearly decreased in the presence of 4 and 8 mmol l^{-1} glucose, and this was evident for both the phosphorylated and unphosphorylated forms. This decrease was not so clear for the phosphorylation status, which showed lower levels under 8 mmol l^{-1} conditions. This is again the first time in which changes in AMPK abundance have been assessed in fish hypothalamus exposed to glucose. In mammalian hypothalamus, comparable experimental approaches eliciting raised glucose levels resulted in decreased levels of P-AMPK (Beall et al., 2012; Zhang et al., 2015) and reduced phosphorylation status (Cai et al., 2007; Chalmers et al., 2014; Oh et al., 2016). These results are also comparable to those obtained in liver of rainbow trout exposed to situations resulting in higher glucose levels such as refeeding or feeding carbohydrate-enriched diet (Polakof et al., 2011b; Kamalam et al., 2012). As a whole, the changes displayed in hypothalamus fit with the role of AMPK as an energy gauge, with decreasing values under situations of enhanced energy availability, which usually result in decreased food intake (López, 2017).

Conclusions

We have demonstrated for the first time in fish that the activation of glucosensing systems in the hypothalamus resulting in increased anorectic potential occurs in parallel with changes in the abundance and phosphorylation status of transcription factors and cellular signals. Based on these results, we suggest a relationship among those processes. Thus, the expected enhancement of the ATP/ADP ratio elicited in the cell by the activation of the glucosensing systems (Conde-Sieira and Soengas, 2017), especially through the GK-dependent mechanism, would result in Akt activation and AMPK inhibition. The changes in these proteins would control phosphorylation of the transcription factors CREB, FoxO1 and BSX, as suggested for mammals (Belgardt et al., 2009; Diéguez et al., 2011; Blanco de Morentín et al., 2011), ultimately leading to food intake inhibition through changes in the expression of neuropeptides. However, the precise mechanisms involved in these mechanisms still need to be evaluated, as well as the role of other components not assessed yet such as mTOR or BSX. A diagram summarizing these relationships is shown in Fig. 6.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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