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RESEARCH ARTICLE

In vitro evidence supports the presence of glucokinase-independent glucosensing mechanisms in hypothalamus and hindbrain of rainbow trout

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ABSTRACT

We previously obtained evidence in rainbow trout for the presence and response to changes in circulating levels of glucose (induced by intraperitoneal hypoglycaemic and hyperglycaemic treatments) of glucosensing mechanisms based on liver X receptor (LXR), mitochondrial production of reactive oxygen species (ROS) leading to increased expression of uncoupling protein 2 (UCP2), and sweet taste receptor in the hypothalamus, and on sodium/glucose co-transporter 1 (SGLT-1) in hindbrain. However, these effects of glucose might be indirect. Therefore, we evaluated the response of parameters related to these glucosensing mechanisms in a first experiment using pooled sections of hypothalamus and hindbrain incubated for 6 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} D-glucose. The responses observed in some cases were consistent with glucosensing capacity. In a second experiment, pooled sections of hypothalamus and hindbrain were incubated for 6 h at 15°C in modified Hanks' medium with 8 mmol l^{-1} D-glucose alone (control) or containing 1 mmol l^{-1} phloridzin (SGLT-1 antagonist), 20 µmol l^{-1} genipin (UCP2 inhibitor), 1 µmol l^{-1} trolox (ROS scavenger), 100 µmol l^{-1} bezafibrate (T1R3 inhibitor) and 50 µmol l^{-1} geranyl-geranyl pyrophosphate (LXR inhibitor). The response observed in the presence of these specific inhibitors/antagonists further supports the proposal that critical components of the different glucosensing mechanisms are functioning in rainbow trout hypothalamus and hindbrain.

KEY WORDS: Rainbow trout, Glucosensing, Hypothalamus, Hindbrain

INTRODUCTION

Glucosensing mechanisms in brain areas like the hypothalamus and hindbrain are related to the control of food intake and to counter-regulatory mechanisms to restore plasma levels of metabolites (Blouet and Schwartz, 2010; Polakof et al., 2011a). In mammals, the best-characterized glucosensing mechanism is that dependent on glucokinase (GK), glucose facilitative transporter type 2 (GLUT2) and ATP-dependent inward rectifier potassium channel (K_{ATP}) (Polakof et al., 2011a). However, this is not the only mechanism present in neurons (Fioramonti et al., 2004; O'Malley et al., 2006), as evidence for alternative glucosensing mechanisms has been provided (Blouet and Schwartz, 2010): (i) the expression of liver X receptor (LXR) is stimulated by high glucose concentrations and inhibits gluconeogenesis (Mitro et al., 2007; Anthonisen et al., 2010; Archer et al., 2014); (ii) the stimulation of sweet taste receptor [heterodimer of type 1 taste receptor subunits (T1Rs) formed by T1R2+T1R3 and α-gustducin] by glucose activates an intracellular signaling cascade (Ren et al., 2009); (iii) the expression of sodium/glucose co-transporter 1 (SGLT-1) increases when glucose levels increase (Díez-Sampedro et al., 2003; González et al., 2009); and (iv) intracellular glucose metabolism stimulates the production of reactive oxygen species (ROS), resulting in an increase in the expression of uncoupling protein 2 (UCP2) (Diano and Horvath, 2012; Toda and Diano, 2014).

Even though in carnivorous fish species protein and lipid are more important for energy purposes than glucose (Polakof et al., 2012), glucose metabolism is still important in tissues like the brain (Polakof et al., 2012) and glucose homeostasis is maintained (Polakof et al., 2011a; Soengas and Polakof, 2013). Accordingly, we have demonstrated the presence in rainbow trout hypothalamus and hindbrain of a glucosensing mechanism dependent on GK–GLUT2–K_{ATP}, which is involved in both counter-regulatory mechanisms and the control of food intake (Polakof et al., 2011a, 2012; Soengas and Polakof, 2013; Soengas, 2014). In a recent study (Otero-Rodiño et al., 2015), we obtained evidence for the first time in fish for the presence and response to changes in circulating levels of glucose (induced by intraperitoneal hypoglycaemic and hyperglycaemic treatments) of glucosensing mechanisms in the hypothalamus dependent on mitochondrial activity, LXR and sweet taste receptor, whereas evidence for the presence of a SGLT-1-dependent mechanism was observed in the hindbrain. However, these effects of glucose on the hypothalamus and hindbrain might be indirect, mediated by changes in circulating levels of hormones and/or metabolites. Therefore, we evaluated whether the response to changes in glucose levels of the hypothalamus and hindbrain *in vitro* (without external influences) is similar to that *in vivo*. Additionally, in a second experiment, we evaluated the response of these parameters in the hypothalamus and hindbrain to glucose in the presence of specific inhibitors/antagonists related to components of alternative glucosensing mechanisms. These included phloridzin (SGLT-1 antagonist), genipin (UCP2 inhibitor), trolox (ROS scavenger), bezafibrate (T1R3 inhibitor) and geranyl-geranyl pyrophosphate (LXR inhibitor).

In all these experiments, we evaluated the response of parameters related to glucosensor mechanisms. The mechanism based on LXR was assessed by changes in mRNA abundance of liver X receptor α (LXRα), fructose 1,6-bisphosphatase (FBPase), peroxisome proliferator-activated receptor type γ (PPARγ) and sterol regulatory element-binding protein type 1c (SREBP1c), and

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FBPase activity. The mitochondrial activity mechanism was assessed by changes in the mRNA abundance of carnitine palmitoyltransferase 1c (CPT1c), CPT1d, hydroxyacil-CoA dehydrogenase (HOAD), cytochrome *c* oxidase subunit 4 (COX4) and UCP2a, and the activity of HOAD and CPT1. The sweet taste receptor-dependent mechanism was assessed by changes in mRNA abundance of α-gustducin (Gnat3) and type 1 taste receptor subunits 2 (T1R2) and 3 (T1R3). The mechanism based on SGLT-1 was evaluated through changes in its mRNA abundance. We have also evaluated, as a positive control, changes in parameters related to the mechanism based on GK including the mRNA abundance of GK, sulfonylurea receptor-like (SUR-like), GLUT2, 6-phosphofructo 1-kinase (PFK), pyruvate kinase (PK) and inward rectifier K^+ channel pore type 6.x-like (Kir6.x-like), and GK and PK activity.

MATERIALS AND METHODS

Fish

Rainbow trout obtained from a local fish farm (A Estrada, Spain) were maintained for 1 month in 100 l tanks under laboratory conditions, 12 h:12 h light:dark photoperiod and dechlorinated tap water at 15°C. Fish mass was 101±3 g. Trout were fed to satiety with commercial dry fish pellets once daily (Dibaq-Diproteg SA, Spain; proximate food analysis: 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. The Ethics Committee of the Universidade de Vigo approved the procedures.

Experimental design

Experiment 1. *In vitro* incubation with increasing concentrations of glucose

Freshly obtained hypothalamus and hindbrain were incubated as previously described (Polakof et al., 2007b). 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 and hindbrain were 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 a basal medium), sliced on chilled Petri dishes, and placed in a chilled Petri dish containing modified Hanks' medium (100 ml g^{-1} tissue, gassed with 0.5% CO_2/99.5% O_2). To obtain enough mass, tissues were pooled from different fish (3–4 for hypothalamus and hindbrain). Tissues were incubated in 48-well culture plates at 15°C for 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 incubation, tissues were quickly removed, rinsed, frozen and stored at −80°C until assayed. D-Glucose concentrations were selected as previously described (Polakof et al., 2007b). Four lots of pooled tissue per tissue type were assessed for enzyme activity, another four were used to assay tissue metabolites, and another four were used to assay mRNA levels. The number of independent experiments (comprising four lots of pooled tissue each) carried out was four (*N*=4).

Experiment 2. *In vitro* incubation with glucose alone or in the presence of inhibitors/antagonists

Tissues were incubated as described in experiment 1, with control wells containing medium with 8 mmol l^{-1} D-glucose. Treated wells contained 8 mmol l^{-1} D-glucose and specific inhibitors/antagonists including 1 mmol l^{-1} phloridzin [1-{2,4-dihydroxy-6-[(2*S*,3*R*,4*R*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]oxy-phenyl}-3-(4-hydroxyphenyl)propan-1-one], 20 µmol l^{-1} genipin [methyl (1*R*,2*R*,6*S*)-2-hydroxy-9-(hydroxymethyl)-3-oxabicyclo[4.3.0]nona-4,8-diene-5-carboxylate], 1 µmol l^{-1} trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 100 µmol l^{-1} bezafibrate [2-(4-{2-[(4-chlorobenzoyl)amino]ethyl}phenoxy)-2-methylpropanoic acid] and 50 µmol l^{-1} geranyl-geranyl pyrophosphate [(2*E*,4*E*,6*E*)-3,5,7,11-tetramethyldodeca-2,4,6,10-tetraene-1-pyrophosphate]. The inhibitors/antagonists were previously dissolved in modified Hanks' medium (phloridzin, bezafibrate and geranyl-geranyl pyrophosphate), ethanol (trolox), or DMSO (genipin); no effects were observed due to the vehicle alone (data not shown). The concentrations of inhibitors/antagonists was selected based on previous studies in fish (Soengas and Moon, 1998; Librán-Pérez et al., 2013) and mammals (Forman et al., 1997; Le Foll et al., 2009; Maillet et al., 2009). After 6 h incubation, tissues were quickly removed, rinsed, frozen and stored at −80°C until assayed. For each experiment, four lots of pooled tissue per tissue type were assessed for enzyme activity, another four were used to assay tissue metabolites, and another four were used to assay mRNA levels. The number of independent experiments (comprising four lots of pooled tissue each) carried out was four (*N*=4).

Assessment of metabolite level and enzyme activity

Tissue samples used to evaluate metabolite levels in hypothalamus and hindbrain were homogenized 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. Tissue glycogen levels were assessed using the method of Keppler and Decker (1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercial kit (Biomérieux).

Samples for the assessment of enzyme activity were homogenized by ultrasonic disruption in 10 volumes of ice-cold phosphorylation–dephosphorylation 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, P-2714). The homogenate was centrifuged (10,000 ***g***) and the supernatant used for enzyme assays. Enzyme activities were determined using a microplate reader (INFINITE 200 Pro, Tecan) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm or, in the case of CPT1 activity, of 5,5′-dithiobis(2-nitrobenzoic acid)-CoA complex at 412 nm. The reactions were started by the addition of 15 µl supernatant at a pre-established protein concentration, omitting the substrate in control wells (final volume 265–295 µl), and reactions were allowed to proceed at 20°C for pre-established time periods. Enzyme activity is expressed per protein level (quantified according to the bicinchoninic acid method with bovine serum albumin, Sigma, as standard). The activity of enzymes was assessed at maximum rates (determined in preliminary tests) to determine optimal substrate concentrations. CPT1 (EC 2.3.1.21), FBPase (EC 3.1.3.11), GK (EC 2.7.1.2), HOAD (EC 1.1.1.35) and PK (EC

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2.7.1.40) activities were determined as described previously (Polakof et al., 2007a,b, 2008a,b,c; Librán-Pérez et al., 2013).

mRNA abundance analysis by real-time quantitative RT-PCR

Total RNA was extracted from tissues using Trizol reagent (Life Technologies, Grand Island, NY, USA) and treated with RQ1-DNAse (Promega, Madison, WI, USA). Then, 2 µg total RNA were reverse transcribed into cDNA using Superscript II reverse transcriptase (Promega) and random hexaprimers (Promega). The expression levels of transcripts of interest were assessed by real-time quantitative RT-PCR (qPCR) using the iCycler iQ (Bio-Rad, Hercules, CA, USA). The analyses were carried out with 1 µl cDNA using the MAXIMA SYBR Green qPCR Mastermix (Thermo Scientific, Waltham, MA, USA), in 15 µl of PCR reaction containing 50–500 nmol l^{-1} of each primer. The mRNA abundance of transcripts of interest (COX4, CPT1c, CPT1d, FBPase, GLUT2, GK, Gnat3, HOAD, Kir6.x-like, LXRα, PK, PPARγ, SGLT-1, SREBP1c, SUR-like, T1R2, T1R3 and UCP2a) was assessed as described in the same species (Panserat et al., 2000, 2001; Polakof et al., 2008c, 2011b; Kolditz et al., 2008; Lansard et al., 2009; Cruz-Garcia et al., 2009; Conde-Sieira et al., 2010; Sánchez-Gurmaches et al., 2012; Polakof and Soengas, 2013; Librán-Pérez et al., 2013). The sequences and accession numbers of the primers used for each transcript are shown in Table 1. Relative quantification of the target gene transcripts was achieved using the reference gene β-actin (stably expressed throughout the experiment).

Thermal cycling was initiated by incubation at 95°C for 90 s using hot-start iTaq DNA polymerase activation; 40 steps of PCR were performed, each one consisting of denaturation at 95°C for 15 s, annealing at primer-specific temperatures for 30 s, and extension at 72°C for 30 s. Following the final PCR cycle, melting curves were systematically monitored (temperature gradient from 55 to 95°C) to ensure amplification of only one fragment. Each sample was assessed in triplicate. Samples without reverse transcriptase and samples without RNA were run for each reaction as negative controls. Only efficiency values between 85% and 100% were accepted (the R^2 was always higher than 0.985 for all the genes assessed). Relative quantification of the target gene transcript with the β-actin reference gene transcript was carried out following the Pfaffl method (2001).

Statistics

A one-way ANOVA followed by a Student–Newman–Keuls (SNK) test was used to assess the significance ($P<0.05$) of differences among groups.

RESULTS

In vitro incubation with increasing concentrations of glucose

The parameters related to LXR are shown in Fig. 1. In the hypothalamus, mRNA abundance of LXRα (Fig. 1A) increased in the presence of 8 mmol l^{-1} glucose compared with 4 mmol l^{-1} glucose. The mRNA abundance of FBPase increased at 2 mmol l^{-1} glucose compared with 4 mmol l^{-1} glucose (Fig. 1C). The mRNA abundance of SREBP1c (Fig. 1E) increased in the presence of 2 and 8 mmol l^{-1} glucose compared with 4 mmol l^{-1} glucose, whereas no changes were noted for the remaining parameters. In the hindbrain, a decrease in activity (Fig. 1G) and mRNA abundance (Fig. 1H) of FBPase was observed in parallel with the increase of glucose in the medium. PPARγ mRNA abundance increased with 8 mmol l^{-1} glucose compared with 4 mmol l^{-1} glucose (Fig. 1I). No significant differences were observed in the remaining parameters.

The parameters associated with the glucosensing mechanism based on mitochondrial activity are shown in Fig. 2. CPT1 activity in the presence of 2 and 8 mmol l^{-1} glucose was lower than that in 4 mmol l^{-1} glucose in the hypothalamus (Fig. 2A) and hindbrain (Fig. 2H). mRNA abundance of CPT1d (Fig. 2J) and COX4 (Fig. 2M) in the hindbrain, and UCP2a in the hypothalamus (Fig. 2G) and hindbrain (Fig. 2N) increased in the group incubated with 8 mmol l^{-1} glucose. No significant differences were observed in the remaining parameters.

The parameters related to the mechanism based on sweet taste receptors are shown in Fig. 3. In the hypothalamus, a decrease in

Table 1. Nucleotide sequences of the probes used to evaluate mRNA abundance using real-time quantitative RT-PCR (qPCR)

	Forward primer	Reverse primer	Database	Accession number
β-Actin	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	GenBank	NM 001124235.1
COX4	TACGTGGGGGACATGGTGTT	CCCAGGAGCCCTTCTCCTTC	Sigenae	tcav0004c.i.22_3.1.s.om.8
CPT1c	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	GenBank	AJ619768
CPT1d	CCGTTCCTAACAGAGGTGCT	ACACTCCGTAGCCATCGTCT	GenBank	AJ620356
FBPase	GCTGGACCCTTCCATCGG	CGACATAACGCCCACCATAGG	GenBank	AF333188
GK	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCCTTTGGTCCAG	GenBank	AF053331
GLUT2	GTGGAGAAGGAGGCGCAAGT	GCCACCGACACCATGGTAAA	GenBank	AF321816
Gnat3	GCAAGACGTGCTGAGGACCA	ATGGCGGTGACTCCCTCAAA	Sigenae	CU073912
HOAD	GGACAAAGTGGCACCAGCAC	GGGACGGGGTTGAAGAAGTG	Sigenae	tcad0001a.i.15 3.1.om
Kir6.x-like	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	Sigenae	CA346261.1.s.om.8:1:773:1
LXRα	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	GenBank	FJ470291
PK	CCATCGTCGCGGTAACAAGA	ACATAGGAAAGGCCAGGGGC	GenBank	AF246146
PPARγ	GACGGCGGGTCAGTACTTTA	ATGCTCTTGGCGAACTCTGT	DFCI	CA345564
SGLT-1	GGGCTGAACATCTACCTTGCT	CTCATAACCTCCCACCTCATTG	GenBank	AY210436
SREBP1c	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	GenBank	CA048941.1
SUR-like	CGAGGACTGGCCCCAGCA	GACTTTCCACTTCCTGTGCGTCC	Sigenae	tcce0019d.e.20_3.1.s.om.8
T1R2	GATGAGTGGGCCAGGAATGG	CCTCCCACCGGCTGACTTTA	Sigenae	FYV3OTN01AKLI0.s.om.10
T1R3	GCCCTGTGGAGCCCATCTTA	CCACACAGTAGGTCAGGGTGGA	Sigenae	GAY7CUQ01EHKNI.s.om.10
UCP2a	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	GenBank	DQ295324

Cytochrome *c* oxidase subunit 4 (COX4), carnitine palmitoyltransferase 1 (CPT1), fructose 1,6-bisphosphatase (FBPase), glucokinase (GK), glucose facilitative transporter type 2 (GLUT2), α-gustducin (Gnat3), hydroxyacil-CoA dehydrogenase (HOAD), inward rectifier K^+ channel pore type 6.x-like (Kir6.x-like), liver X receptor α (LXRα), pyruvate kinase (PK), peroxisome proliferator-activated receptor type γ (PPARγ), sodium–glucose linked transporter type 1 (SGLT-1), sterol regulatory element-binding protein type 1c (SREBP1c), sulfonylurea receptor-like (SUR-like), type 1 taste receptor subunit 2 (T1R2), type 1 taste receptor subunit 3 (T1R3) and mitochondrial uncoupling protein 2a (UCP2a).

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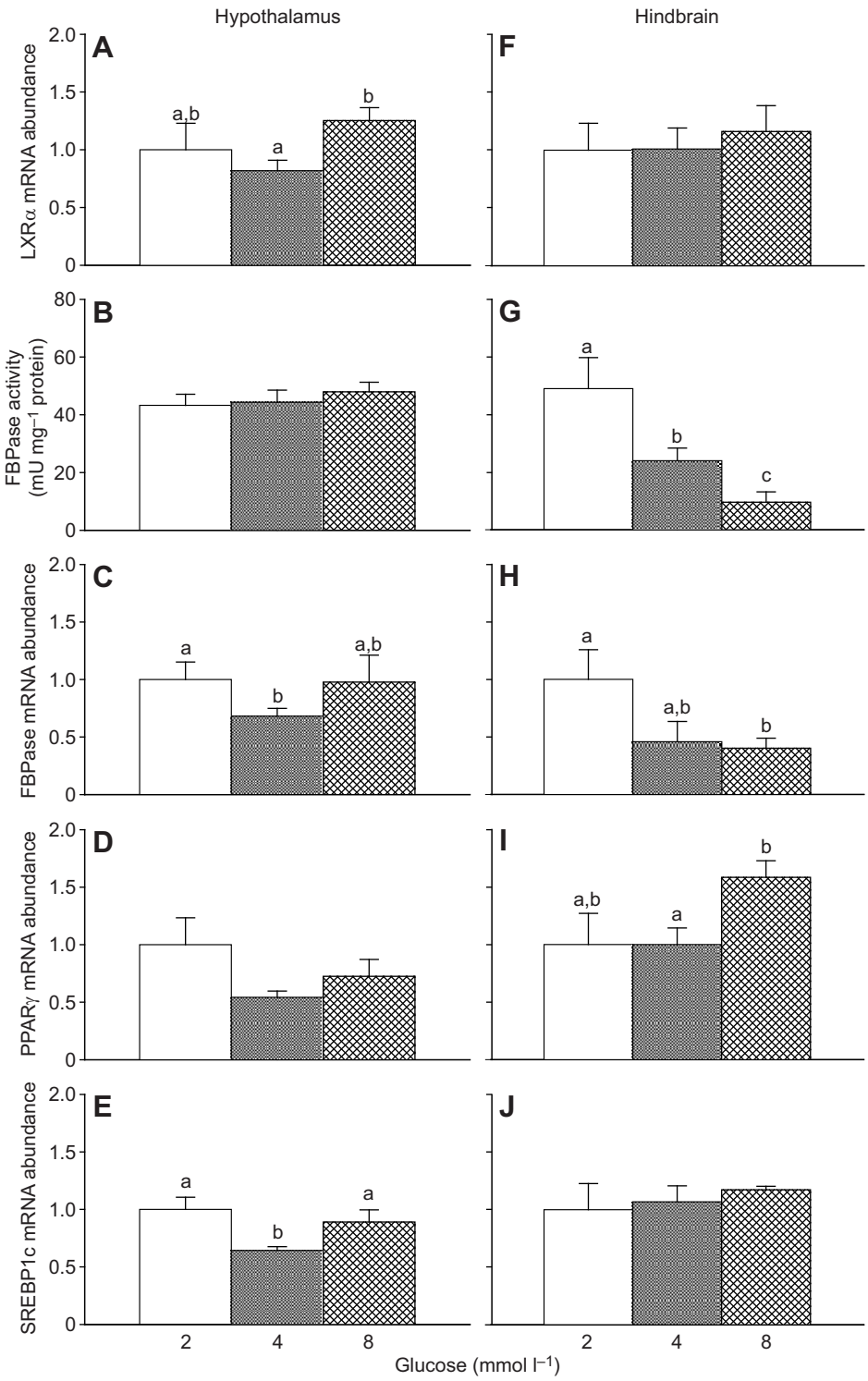

Fig. 1. Parameters related to glucosensing based on liver X receptor (LXR) in rainbow trout brain. mRNA abundance of LXRα (A,F), fructose 1,6-bisphosphatase (FBPase; C,H), peroxisome proliferator-activated receptor type γ (PPARγ; D,I) and sterol regulatory element-binding protein type 1c (SREBP1c; E,J), and activity of FBPase (B,G) in the hypothalamus (A–E) and hindbrain (F–J) of rainbow trout incubated for 6 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} D-glucose. Data represent means+s.e.m. of four independent experiments carried out with hypothalamus/hindbrain tissue pooled from 3–4 different fish. mRNA abundance data are expressed as fold-induction with respect to the group incubated 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 between groups [one-way ANOVA $P<0.05$, *post hoc* Student–Newman–Keuls (SNK) test $P<0.05$].

mRNA abundance of T1R2 (Fig. 3A) and T1R3 (Fig. 3B) was observed in parallel with the increase of glucose in the medium, whereas mRNA abundance of Gnat3 was lower in the presence of 4 mmol l^{-1} glucose (Fig. 3C). In the hindbrain, mRNA abundance of T1R2 (Fig. 3D) and T1R3 (Fig. 3E) was lower with 4 mmol l^{-1} glucose.

SGLT-1 mRNA abundance in the hypothalamus (Fig. 4A) was higher in the presence of 2 mmol l^{-1} glucose, whereas in the hindbrain (Fig. 4B), it was lower in 4 mmol l^{-1} glucose.

In vitro incubation with glucose alone or in the presence of inhibitors/antagonists

To simplify the description of these results, only differences observed between treated groups and the control are commented on in this section; all differences observed among treated groups are presented in Tables 2 and 3.

The results obtained in the hypothalamus are shown in Table 2. Glycogen levels decreased after treatment with all inhibitors/antagonists assessed. LXR mRNA abundance decreased in the

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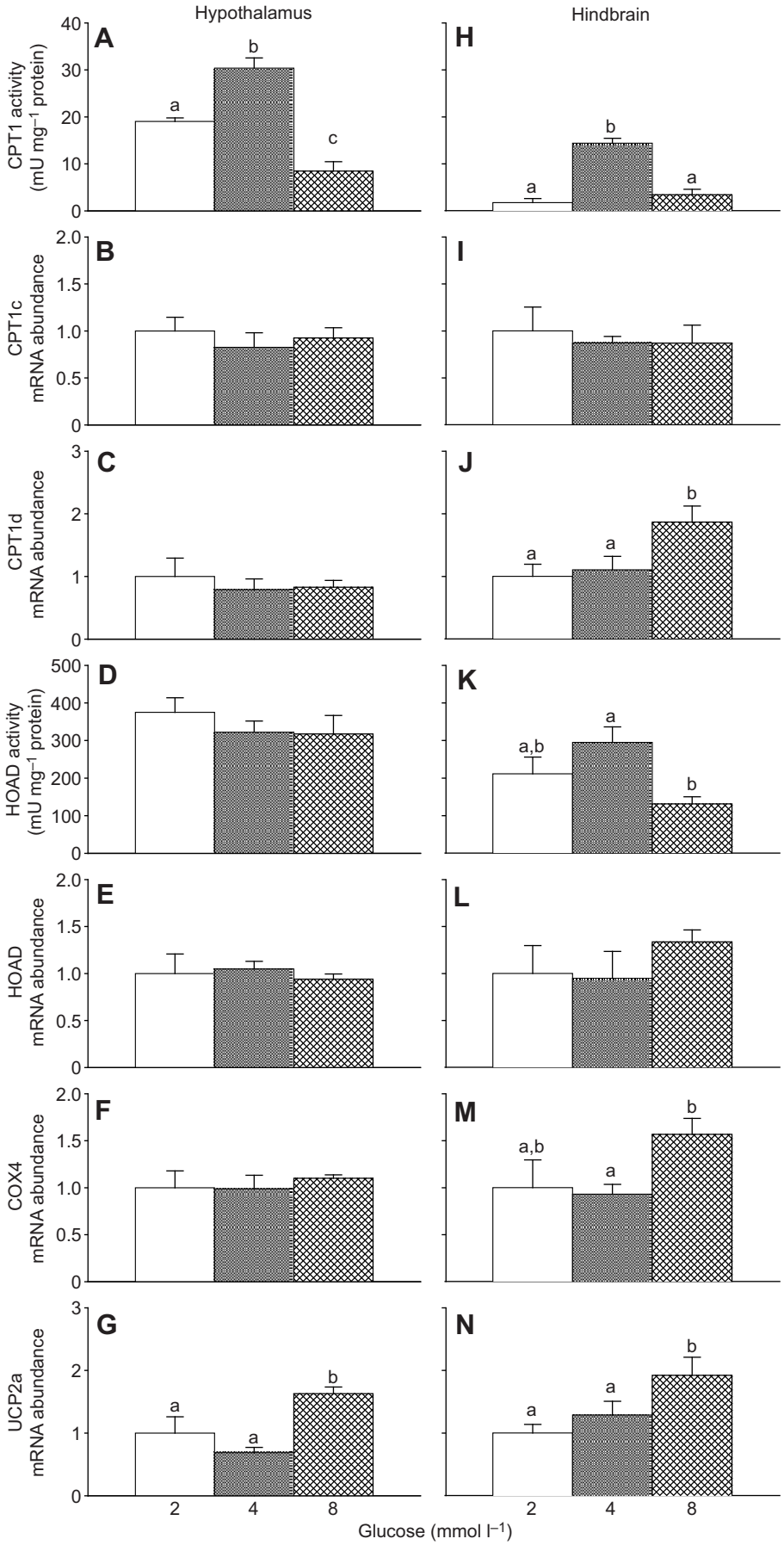

Fig. 2. Parameters related to glucosensing based on mitochondrial activity in rainbow trout brain. Activity of carnitine palmitoyltransferase 1 (CPT1; A,H) and hydroxyacil-CoA dehydrogenase (HOAD; D,K), and mRNA abundance of CPT1c (B,I), CPT1d (C,J), HOAD (E,L), cytochrome *c* oxidase subunit 4 (COX4; F,M) and mitochondrial uncoupling protein 2a (UCP2a; G,N) in the hypothalamus (A–G) and hindbrain (H–N) of rainbow trout incubated for 6 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} D-glucose. Data represent means+s.e.m. of four independent experiments carried out with hypothalamus/hindbrain tissue pooled from 3–4 different fish. mRNA abundance data are expressed as fold-induction with respect to the group incubated 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 between groups (one-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$).

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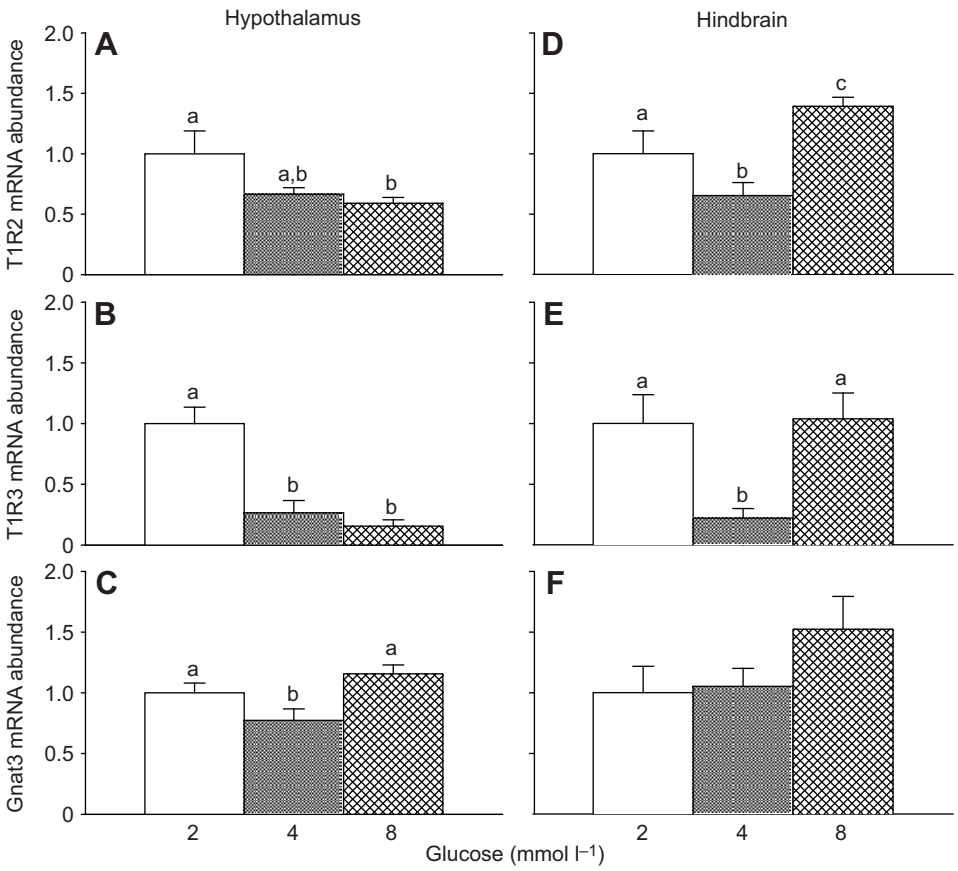

Fig. 3. Parameters related to glucosensing based on sweet taste receptors in rainbow trout brain. mRNA abundance of type 1 taste receptor subunit 2 (T1R2; A,D), T1R3 (B,E) and α-gustducin (Gnat3; C,F) in the hypothalamus (A–C) and hindbrain (D–F) of rainbow trout incubated for 6 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} D-glucose. Data represent means+s.e.m. of four independent experiments carried out with hypothalamus/hindbrain tissue pooled from 3–4 different fish. mRNA abundance data are expressed as fold-induction with respect to the group incubated 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 between groups (one-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$).

presence of geranyl-geranyl pyrophosphate. FBPase activity decreased compared with controls after treatment with all inhibitors except bezafibrate; FBPase mRNA abundance was not modified by treatments compared with the control group. PPARγ mRNA abundance increased in the presence of all substances except phloridzin. SREBP1c mRNA abundance decreased after treatment with phloridzin and geranyl-geranyl pyrophosphate. CPT1 activity was inhibited in the presence of phloridzin. CPT1c mRNA abundance was not affected by treatments compared with control. CPT1d mRNA abundance decreased in the presence of phloridzin and geranyl-geranyl pyrophosphate. HOAD activity decreased after treatment with genipin, trolox and geranyl-geranyl pyrophosphate; HOAD mRNA abundance decreased in the presence of geranyl-geranyl pyrophosphate. COX4 mRNA abundance decreased in the presence of phloridzin. UCP2a mRNA abundance decreased after treatment with genipin and geranyl-geranyl pyrophosphate. T1R2 and T1R3 mRNA abundance decreased in the presence of trolox. Gnat3 mRNA abundance decreased in the presence of phloridzin. Finally, SGLT-1 mRNA abundance decreased after phloridzin, trolox, bezafibrate or geranyl-geranyl pyrophosphate treatment.

The results obtained in hindbrain are shown in Table 3. Glycogen levels decreased in the presence of phloridzin. LXRα mRNA abundance decreased after treatment with genipin, trolox and bezafibrate. The activity of FBPase increased in the presence of

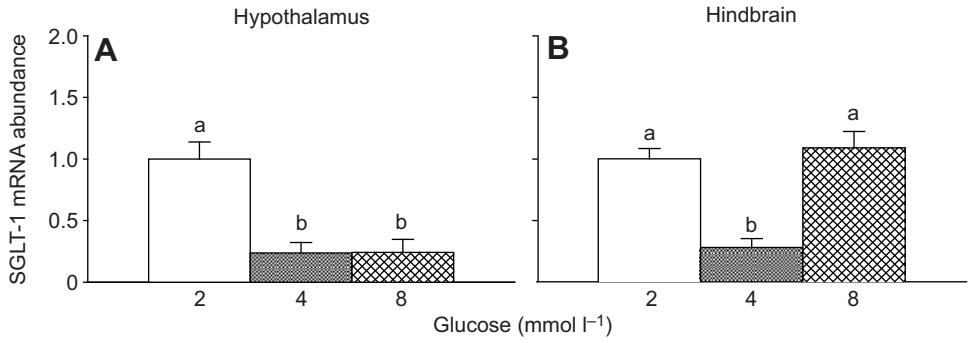

Fig. 4. Parameters related to glucosensing based on sodium–glucose linked transporter type 1 (SGLT-1) in rainbow trout brain. mRNA abundance of SGLT-1 in the hypothalamus (A) and hindbrain (B) of rainbow trout incubated for 6 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} D-glucose. Data represent means+s.e.m. of four independent experiments carried out with hypothalamus/hindbrain tissue pooled from 3–4 different fish. mRNA abundance data are expressed as fold-induction with respect to the group incubated 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 between groups (one-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$).

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Table 2. Response to inhibitors and antagonists of the glucosensing mechanisms of the hypothalamus

	Treatment					
Parameter	Control	Phloridzin	Genipin	Trolox	Bezafibrate	GGP
Glycogen level (μmol glycosyl units g^{-1})	2.12±0.20^{a}	1.52±0.25^{b}	0.91±0.26^{b,c}	0.47±0.09^{c}	0.68±0.20^{c}	1.09±0.10^{b}
Glucosensing based on LXR						
LXR mRNA abundance	1.00±0.17^{a}	0.65±0.07^{a,b}	1.11±0.11^{a}	0.93±0.20^{a,b}	1.07±0.22^{a}	0.56±0.04^{b}
FBPase activity (mU mg^{-1} protein)	47.9±3.29^{a}	18.0±3.94^{b,c}	24.1±3.09^{b}	23.2±5.27^{b,c}	40.4±3.63^{a}	13.1±3.50^{c}
FBPase mRNA abundance	1.00±0.16^{a,b,c}	0.71±0.09^{b,c}	1.21±0.12^{a}	1.12±0.24^{a,b}	0.55±0.14^{c}	0.54±0.10^{c}
PPARγ mRNA abundance	1.00±0.20^{a}	0.51±0.20^{a}	2.36±0.58^{b}	1.65±0.27^{b}	3.31±0.30^{c}	1.80±0.31^{b}
SREBP1c mRNA abundance	1.00±0.12^{a}	0.71±0.07^{b}	1.13±0.20^{a}	1.09±0.11^{a}	1.32±0.17^{a}	0.79±0.04^{b}
Glucosensing based on mitochondrial activity						
CPT1 activity (mU mg^{-1} protein)	8.46±1.49^{a,d}	2.29±1.53^{b}	5.22±1.33^{a,b}	11.5±1.63^{a,c}	8.10±0.46^{a,c,d}	10.8±1.13^{a,c}
CPT1c mRNA abundance	1.00±0.12^{a,b}	0.69±0.15^{b}	0.99±0.03^{a,b}	1.32±0.21^{a}	1.12±0.07^{a}	1.14±0.04^{a}
CPT1d mRNA abundance	1.00±0.13^{a,c}	0.54±0.10^{b}	1.19±0.07^{a,c}	1.01±0.19^{a,c}	1.36±0.13^{a}	0.79±0.15^{c}
HOAD activity (mU mg^{-1} protein)	317.4±49.2^{a}	264.7±27.4^{a}	185.9±25.1^{b,c}	193.7±25.6^{b,c}	240.8±16.2^{a}	192.8±15.4^{c}
HOAD mRNA abundance	1.00±0.17^{a}	0.72±0.05^{a}	1.05±0.16^{a}	0.71±0.05^{a}	0.86±0.09^{a}	0.54±0.06^{b}
COX4 mRNA abundance	1.00±0.03^{a}	0.61±0.07^{b}	1.28±0.23^{a}	0.91±0.08^{a}	1.16±0.19^{a}	1.14±0.24^{a}
UCP2a mRNA abundance	1.00±0.06^{a}	0.78±0.13^{a,b}	0.64±0.16^{b}	1.12±0.22^{a}	0.76±0.12^{a,b}	0.64±0.07^{b}
Glucosensing based on sweet taste receptor						
T1R2 mRNA abundance	1.00±0.08^{a}	0.83±0.19^{a,b}	1.17±0.20^{a}	0.48±0.10^{b}	1.30±0.28^{a}	0.97±0.14^{a}
T1R3 mRNA abundance	1.00±0.13^{a,b}	0.60±0.26^{a,c}	1.20±0.27^{a,b}	0.52±0.14^{c}	1.28±0.23^{b}	1.46±0.27^{b}
Gnat3 mRNA abundance	1.00±0.18^{a}	0.57±0.10^{b}	1.29±0.27^{a}	1.03±0.12^{a}	0.96±0.07^{a}	0.88±0.12^{a}
Glucosensing based on SGLT-1						
SGLT-1 mRNA abundance	1.00±0.21^{a}	0.51±0.12^{b}	0.76±0.13^{a,b}	0.33±0.13^{b,c}	0.34±0.18^{b,c}	0.21±0.06^{c}

Rainbow trout hypothalamus was incubated *in vitro* for 6 h at 15°C in modified Hanks' medium containing 8 mmol l^{-1} glucose alone (control) or selected inhibitors/antagonists related to different glucosensing mechanisms: 1 mmol l^{-1} phloridzin (SGLT-1 antagonist), 20 μmol l^{-1} genipin (UCP2 inhibitor), 1 μmol l^{-1} trolox (ROS scavenger), 100 μmol l^{-1} bezafibrate (T1R3 inhibitor) and 50 μmol l^{-1} geranyl-geranyl pyrophosphate (GGP, LXR inhibitor). Data represent means± s.e.m. of four independent experiments carried out with hypothalamus pooled from 3–4 different fish. mRNA abundance data are referred to the control group (results were previously normalized by β-actin mRNA levels, which did not show changes among groups). Different letters indicate significant differences between groups [one-way ANOVA $P<0.05$, *post hoc* Student–Newman–Keuls (SNK) test $P<0.05$].

bezafibrate. PPARγ mRNA abundance decreased after treatment with phloridzin, genipin, trolox and geranyl-geranyl pyrophosphate. mRNA abundance of SREBP1c, CPT1d, COX4 and SGLT-1 decreased after treatment with all substances. The activity of CPT1 decreased in the presence of phloridzin. FBPase and CPT1c mRNA abundance, and HOAD activity were not affected by treatments. UCP2a mRNA abundance decreased in the presence of genipin. T1R2 mRNA abundance decreased after treatment with all

Table 3. Response to inhibitors and antagonists of the glucosensing mechanisms of the hindbrain

	Treatment					
Parameter	Control	Phloridzin	Genipin	Trolox	Bezafibrate	GGP
Glycogen levels (μmol glycosyl units g^{-1})	0.83±0.15^{a}	0.45±0.08^{b}	0.54±0.10^{a,b}	0.74±0.12^{a}	0.49±0.11^{a,b}	0.40±0.07^{b}
Glucosensing based on LXR						
LXR mRNA abundance	1.00±0.10^{a}	0.84±0.09^{a}	0.40±0.04^{b}	0.54±0.05^{b}	0.69±0.04^{c}	0.82±0.10^{a,c}
FBPase activity (mU mg^{-1} protein)	9.53±3.60^{a}	25.9±10.9^{a}	18.2±5.7^{a}	8.66±2.70^{a,c}	21.7±1.93^{b}	4.42±1.38^{a,c}
FBPase mRNA abundance	1.00±0.22^{a,b}	0.76±0.28^{a,b}	0.57±0.09^{b}	1.12±0.18^{a}	0.82±0.17^{a,b}	0.86±0.26^{a,b}
PPARγ mRNA abundance	1.00±0.09^{a}	0.62±0.16^{b,c}	0.37±0.07^{b}	0.70±0.02^{c}	1.03±0.18^{a,c}	0.55±0.11^{b,c}
SREBP1c mRNA abundance	1.00±0.20^{a}	0.66±0.11^{b}	0.33±0.03^{c}	0.63±0.08^{b}	0.60±0.09^{b}	0.57±0.06^{b}
Glucosensing based on mitochondrial activity						
CPT1 activity (mU mg^{-1} protein)	3.38±1.15^{b,c}	8.98±0.86^{a}	4.24±0.92^{b,c}	5.70±1.23^{c}	4.74±1.19^{b,c}	2.94±0.49^{b}
CPT1c mRNA abundance	1.00±0.22	0.85±0.08	0.71±0.18	0.93±0.14	0.65±0.18	0.98±0.17
CPT1d mRNA abundance	1.00±0.14^{a}	0.50±0.05^{b}	0.34±0.06^{c}	0.58±0.05^{b}	0.52±0.06^{b}	0.49±0.07^{b,c}
HOAD activity (mU mg^{-1} protein)	130.9±18.9^{a,b}	121.9±11.7^{a,b}	119.1±14.1^{a,b}	96.8±19.8^{a,b}	95.9±13.0^{a}	142.6±17.8^{b}
HOAD mRNA abundance	1.00±0.09^{a}	0.59±0.03^{b}	0.48±0.15^{b}	0.43±0.05^{b}	0.58±0.13^{b}	0.55±0.09^{b}
COX4 mRNA abundance	1.00±0.11^{a}	0.51±0.03^{b}	0.40±0.14^{b,c}	0.32±0.05^{c}	0.60±0.14^{b,c}	0.49±0.08^{b,c}
UCP2a mRNA abundance	1.00±0.15^{a}	0.96±0.18^{a,b}	0.57±0.13^{b}	0.96±0.19^{a,b}	0.78±0.07^{a,b}	0.91±0.06^{a}
Glucosensing based on sweet taste receptor						
T1R2 mRNA abundance	1.00±0.05^{a}	0.43±0.11^{b}	0.27±0.10^{b}	0.28±0.03^{b}	0.78±0.23^{a}	0.37±0.05^{b}
T1R3 mRNA abundance	1.00±0.20^{a}	0.20±0.09^{b}	0.18±0.05^{b}	0.21±0.06^{b}	0.41±0.11^{b}	0.73±0.11^{a}
Gnat3 mRNA abundance	1.00±0.18^{a}	0.56±0.04^{b}	0.42±0.06^{b}	0.47±0.02^{b}	0.74±0.11^{a}	0.80±0.07^{a}
Glucosensing based on SGLT-1						
SGLT-1 mRNA abundance	1.00±0.12^{a}	0.33±0.07^{b}	0.62±0.05^{d}	0.43±0.13^{b,d}	1.85±0.30^{c}	0.46±0.08^{b,d}

Rainbow trout hindbrain was incubated *in vitro* for 6 h at 15°C in modified Hanks' medium containing 8 mmol l^{-1} glucose alone (control) or selected inhibitors/antagonists related to different glucosensing mechanisms: 1 mmol l^{-1} phloridzin (SGLT-1 antagonist), 20 μmol l^{-1} genipin (UCP2 inhibitor), 1 μmol l^{-1} trolox (ROS scavenger), 100 μmol l^{-1} bezafibrate (T1R3 inhibitor) and 50 μmol l^{-1} geranyl-geranyl pyrophosphate (GGP, LXR inhibitor). Data represent means±s.e.m. of four independent experiments carried out with hypothalamus pooled from 3–4 different fish. mRNA abundance data are referred to the control group (results were previously normalized by β-actin mRNA levels, which did not show changes among groups). Different letters indicate significant differences between groups (one-way ANOVA $P<0.05$, *post hoc* SNK test $P<0.05$).

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substances except bezafibrate. T1R3 mRNA abundance decreased after all treatments except geranyl-geranyl pyrophosphate. Gnat3 mRNA abundance decreased in the presence of phloridzin, genipin and trolox.

DISCUSSION

Changes in the parameters involved in glucosensing based on GK–GLUT2–K_{ATP} channel (glycogen levels, mRNA abundance of components of the K_{ATP} channel, GK, PFK and GLUT2, and activity of PK and GK) in the hypothalamus and hindbrain parallel the changes in glucose (data not shown). These results are comparable to those carried out previously (Polakof et al., 2011a, 2012; Soengas and Polakof, 2013; Soengas, 2014), therefore supporting the experimental design.

The glucosensing system based on LXR was apparently active in the hypothalamus as demonstrated by the increased mRNA abundance of LXRα and SREBP1c in response to increased glucose levels and the increased mRNA abundance of FBPase mRNA under low glucose concentration. These changes are similar to those already observed when glucose levels were changed by intraperitoneal hypoglycaemic and hyperglycaemic treatments (Otero-Rodiño et al., 2015). The changes suggest that gluconeogenic capacity is inhibited in parallel with the increase in glucose, in a way comparable to that occurring in mammalian liver (Steffensen and Gustafsson, 2004; Mitro et al., 2007) and to the decreased mRNA abundance of PEPCK in zebrafish exposed to glucose (Elo et al., 2007). Moreover, SREBP1c mRNA abundance increased in response to the rise in glucose levels, a response similar to that of mammalian liver (Archer et al., 2014) and to that observed in rainbow trout fed a carbohydrate-enriched diet (Craig and Moon, 2013), which could relate to the activation of LXR as in Atlantic salmon liver (Carmona-Antoñanzas et al., 2014). Furthermore, the incubation of the hypothalamus in the presence of a specific inhibitor of LXRα, such as geranyl-geranyl pyrophosphate, clearly inhibited the response of several parameters to raised levels of glucose, such as the mRNA abundance of LXRα, FBPase and SREBP1c. Altogether, these results support the presence of a functional glucosensing mechanism based on LXR in rainbow trout hypothalamus, thus giving further support to the results obtained after intraperitoneal (i.p.) treatments (Otero-Rodiño et al., 2015). In hindbrain, in our previous i.p. study (Otero-Rodiño et al., 2015), we did not obtain consistent information regarding the possible presence of a glucosensing system based on LXR. However, in the present *in vitro* experiment, we observed changes in several parameters that would be in agreement with its presence, such as a decrease in FBPase activity and mRNA abundance when glucose levels in the medium were high. Furthermore, geranyl-geranyl pyrophosphate also inhibited the response to glucose of mRNA abundance of SREBP1c and PPARγ. As a whole, we suggest from the present results at least a partial response to glucose of the glucosensing system based on LXR in the hindbrain.

In the glucosensing mechanism dependent on mitochondrial activity, glucose sensing is negatively regulated by raised UCP2 activity (Kong et al., 2010; Beall et al., 2012). In our previous *in vivo* study (Otero-Rodiño et al., 2015), we obtained evidence supporting the presence of this mechanism in the hypothalamus. However, here we observed very few changes in the parameters assessed. We observed an increase in UCP2 mRNA abundance under conditions of high glucose levels in the medium, which concurs with the expected results based on the mammalian model (Diano and Horvath, 2012). The decrease observed in CPT1 activity was expected in the low glucose concentration (2 mmol l^{-1}) but not in the high glucose concentration (8 mmol l^{-1}). However, no changes were observed in the remaining parameters. This lack of clear evidence was also noted when assessing the effects of two well-known inhibitors of mitochondrial activity: genipin and trolox. In the presence of either of these inhibitors, no changes were noted in the parameters assessed compared with the group treated with glucose alone. In general, these changes do not fully support the existence in rainbow trout hypothalamus of a glucosensing mechanism based on mitochondrial activity. Thus, the results obtained when assessing these parameters *in vivo* (Otero-Rodiño et al., 2015) might be due to an indirect effect mediated by changes in the levels of metabolites or hormones. In contrast, in the hindbrain, there were several changes in the parameters assessed in response to the presence of high glucose concentration in the medium, including increased mRNA abundance of CPT1d, COX4 and UCP2a as well as decreased activity of HOAD. With the exception of the decreased activity of HOAD, the changes in the remaining parameters are those expected for a mitochondrial glucosensing mechanism (Diano and Horvath, 2012), suggesting its presence in the hindbrain. These changes are also comparable with the increased CPT1 mRNA abundance observed in liver of rainbow trout fed a carbohydrate-enriched diet (Craig and Moon, 2013). Furthermore, when we assessed the response to glucose of these parameters in the presence of specific inhibitors of mitochondrial activity, such as genipin and trolox, we observed a clear inhibitory effect on the mRNA abundance of HOAD, CPT1d, COX4 and UCP2a. As a whole, these results provide partial support for the presence of a glucosensor system based on mitochondrial activity in trout hindbrain in contrast with the results previously observed *in vivo* (Otero-Rodiño et al., 2015).

We also assessed the response of parameters associated with the sweet taste receptor-dependent glucosensing mechanism. In the hypothalamus, exposure to enhanced glucose resulted in a decrease in the mRNA abundance of T1R3, a response similar than that observed *in vivo* (Otero-Rodiño et al., 2015). The response of the other markers assessed, T1R2 and Gnat3, to low glucose concentration in the medium was also similar to that previously observed under *in vivo* conditions, and is in agreement with the increase observed in mammalian brain under low glucose levels (Shin et al., 2010). These changes further support the differential response of these parameters compared with the mammalian model, where in the hypothalamus the increase in glucose concentration elicits a decrease in the mRNA abundance of T1R2 but not of T1R3 and Gnat3 (Ren et al., 2009). These changes support the presence and functioning of this mechanism in the hypothalamus. However, the addition to the medium of an inhibitor of T1R3 (bezafibrate) failed to induce any inhibition in the parameters assessed. In the hindbrain, the evidence obtained was contradictory as we observed an increase in the mRNA abundance of T1R2 and T1R3 under both low and high glucose concentrations. This is reasonable in the case of low glucose concentrations (in agreement with the results observed in the hypothalamus) but not in the case of high glucose levels as the increased glucose in this system should elicit an inhibition of the expression of the sweet taste receptor proteins. Furthermore, the presence of bezafibrate clearly inhibited T1R3 mRNA abundance.

The mRNA abundance of SGLT-1 can directly respond to increased levels of glucose. In the present study, the increased levels of glucose in the medium increased the mRNA abundance of SGLT1 in the hindbrain but not in the hypothalamus, i.e. results similar to those obtained in our previous *in vivo* study (Otero-Rodiño et al., 2015). This response is different from that characterized in mammals, where increased glucose levels also

resulted in increased SGLT-1 expression in the hypothalamus (González et al., 2009). However, we observed a similar increase in both tissues when glucose levels were low in the incubation medium, which is not logical for a glucosensing mechanism. The presence of a SGLT-1 inhibitor such as phloridzin clearly induced a decrease in the mRNA abundance of SGLT-1 in both tissues, thus supporting the functioning of this system in both tissues. These results clearly support the presence of this mechanism in the hindbrain but not so clearly in the hypothalamus.

In summary, our previous *in vivo* studies provided evidence for the existence in the rainbow trout hypothalamus and hindbrain of glucosensor mechanisms independent on GK. The parameters related to these mechanisms respond to changes in glucose levels with changes similar (with exceptions) to those previously demonstrated in mammals (Otero-Rodiño et al., 2015). In the present study, we assessed *in vitro* the responses of the same tissues to increased glucose concentration in the medium, and in some cases the responses observed were consistent with a glucosensing capacity. The incubation of tissues in the presence of glucose and specific inhibitors/antagonists provides further support for the hypothesis that critical components of the different glucosensing mechanisms are functioning in rainbow trout hypothalamus and hindbrain. Considering the three available experiments together (*in vivo* hypoglycaemic and hyperglycaemic treatments, *in vitro* incubation with increased glucose concentration, and *in vitro* incubation with inhibitors/antagonists), we conclude the existence of three different categories in the responses of the mechanisms assessed. The first category includes those cases where the results obtained were coincident among the three available studies, thus providing strong evidence for the presence and functioning of these mechanisms, such as those based on LXR in the hypothalamus and SGLT-1 in the hindbrain. The second category includes those mechanisms for which evidence obtained was not so strong, as not all experiments provided the same results, though the evidence partially supports the presence of a glucosensing mechanism. These include the sweet taste receptor in the hypothalamus or mitochondrial activity in the hindbrain. Finally, a third category includes those systems where we did not obtain concordant results when comparing *in vivo* and *in vitro* experiments, such as sweet taste receptor and LXR in the hindbrain, and mitochondrial activity and SGLT-1 in the hypothalamus. In future studies, the localization of the precise neurons in which the main components of these mechanisms are present is therefore essential. It is also important to assess the possible intracellular pathways shared among mechanisms as well as the precise physiological role (food intake control and/or counter-regulatory mechanisms) in which these glucosensing mechanisms are likely to be involved in rainbow trout brain.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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