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RESEARCH ARTICLE

In vitro insulin treatment reverses changes elicited by nutrients in cellular metabolic processes that regulate food intake in fish

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ABSTRACT

This research assessed the direct effects of insulin on nutrient-sensing mechanisms in the brain of rainbow trout (*Oncorhynchus mykiss*) using an *in vitro* approach. Cultured hypothalamus and hindbrain were exposed to 1 µmol l^{-1} insulin for 3 h, and signals involved in appetite regulation and nutrient-sensing mechanisms were measured. Additionally, the involvement of the phosphatidylinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway in the actions of insulin was studied by using the inhibitor wortmannin. Treatment with insulin alone did not elicit many changes in the appetite regulators and nutrient-sensing-related genes and enzymes tested in the hypothalamus and hindbrain. However, we found that, when insulin and nutrients were added together, insulin reversed most of the effects exerted by nutrients alone, suggesting that insulin changes responsiveness to nutrients at the central level. Effects reversed by insulin included expression levels of genes related to the sensing of both glucose (*slc2a2*, *slc5a1*, *gck*, *pck1*, *pklr*, *g6pcb*, *gys1*, *tas1r3* and *nr1h3* in the hindbrain, and *slc2a2*, *pklr* and *pck1* in the hypothalamus) and fatty acid (*cd36* in the hindbrain, and *cd36* and *acly* in the hypothalamus). Nutrient-induced changes in the activity of Acly and Cpt-1 in the hindbrain and of Pepck, Acly, Fas and Hoad in the hypothalamus were also reversed by insulin. Most of the insulin effects disappeared in the presence of wortmannin, suggesting the PI3K/Akt pathway is a mediator of the effects of insulin reported here. This study adds new information to our knowledge of the mechanisms regulating nutrient sensing in fish.

KEY WORDS: Insulin, Nutrient sensing, Hypothalamus, Hindbrain, PI3K/Akt, Fish

INTRODUCTION

Several brain regions, especially the hypothalamus and the hindbrain, are able to integrate endocrine and metabolic information to produce key factors that either stimulate or inhibit food intake (Delgado et al., 2017; Marty et al., 2007). These areas possess specific mechanisms able to sense changes in the levels of nutrients, particularly glucose, fatty acids and amino acids, as demonstrated in mammals (Efeyan et al., 2015) and fish (Conde-Sieira and Soengas, 2017; Soengas, 2014). The activation of nutrient-sensing systems results in changes in the expression of brain appetite-regulating neuropeptides, mainly increased expression of the anorexigenic peptides pro-opio melanocortin (POMC) and cocaine- and amphetamine-related transcript (CART), and decreased production of the orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), ultimately leading to decreased food intake (Blouet and Schwartz, 2010; Soengas et al., 2018). Activation of nutrient-sensing systems leads to the activation of mammalian target of rapamycin (mTOR) and the inhibition of AMP-activated protein kinase (AMPK), which in turn modulate the transcription factors forkhead box protein O1 (FOXO1), cAMP response element binding protein (CREB) and brain homeobox transcription factor (BSX). Such transcription factors produce changes in the abundance of neuropeptides, as demonstrated in mammals (Blanco Martínez de Morentin et al., 2011; Diéguez et al., 2011; Gao et al., 2013) and fish (Conde-Sieira et al., 2018; Dai et al., 2014; Librán-Pérez et al., 2015b; Otero-Rodiño et al., 2017).

Peripheral hormones are involved in the regulation of food intake through specific receptors located in central areas such as the hypothalamus and hindbrain (Blouet and Schwartz, 2010). Besides a direct action, these peripheral hormones modulate nutrient sensing through mechanisms that are not well understood (Blouet and Schwartz, 2010). One of the main hormones influencing food intake regulation in mammals is insulin (Schwartz et al., 2000). At least in humans, insulin circulates in proportion to body fat and thus serves to inform the central nervous system (CNS) about energy stores (Porte et al., 2005). Insulin penetrates the blood–brain barrier (Gray et al., 2014) and binds to receptors located in brain regions including the hypothalamus and hindbrain (Arble and Sandoval, 2013). The presence of insulin receptors in the brain of fish, including rainbow trout (Caruso et al., 2010) and other species (Cruz et al., 2010; Ma et al., 2017), has been previously reported. Additionally, central actions of insulin could relate to its synthesis in brain, as demonstrated in several fish species (Caruso and Sheridan, 2011; Hrytsenko et al., 2008; Ma et al., 2017; Papasani et al., 2006). The role of insulin on appetite in mammals is characterized by a dose-dependent reduction in food intake and body mass when administered directly into the CNS (Woods et al., 2006), although both food intake and body mass were reported to increase after intraperitonal (i.p.) infusion of insulin (Larue-Achagiotis and Le Magnen, 1985). In fish, the effects of insulin treatment on food intake are contradictory. Administration of i.p. insulin in rainbow trout resulted in either inhibition (Librán-Pérez et al., 2015a) or activation (Conde-Sieira et al., 2010b; Polakof et al., 2008) of food intake, whereas intracerebroventricular (i.c.v.) treatment did not affect food intake in catfish (Silverstein and Plisetskaya, 2000) but caused a decrease in rainbow trout (Soengas and Aldegunde, 2004). The anorectic effects of insulin are in agreement with increased anorexigenic potential elicited by i.p. insulin treatment, as suggested by decreased *npy* mRNA abundance in rainbow trout (Librán-Pérez et al., 2015a) and increased *cartpt* in rainbow trout (Librán-Pérez

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et al., 2015a) and catfish (Subhedar et al., 2011). The role of insulin in the regulation of food intake is also supported by its decreased mRNA expression in the brain and endocrine pancreas (Brockmann body) of food-deprived rainbow trout (Caruso and Sheridan, 2012), and the increased food intake levels in insulin receptor knockout zebrafish (Yang et al., 2018).

Previous studies demonstrated that i.p. treatment with insulin may modulate central mechanisms sensing glucose (Conde-Sieira et al., 2010a,b; Polakof et al., 2007a, 2008) and fatty acids (Librán-Pérez et al., 2015a) in fish. However, contradictory results have been observed, which can be attributed to the fact that changes in circulatory levels of metabolites (such as glucose or fatty acid) occur in response to insulin treatment, and that these changes in metabolite levels are known to induce changes in food intake (Conde-Sieira and Soengas, 2017; Soengas et al., 2018). The present study aimed to gather a deeper understanding on the putative role of insulin in regulating glucose- and fatty acid-sensing systems in the hypothalamus and hindbrain of the fish model rainbow trout using an *in vitro* approach. An anorectic effect for insulin in fish after i.c.v. administration was described in some studies (Caruso and Sheridan, 2012; Soengas and Aldegunde, 2004). Similarly, raised levels of nutrients in fish brain resulted in anorectic effects through the activation of nutrient-sensing systems (Conde-Sieira and Soengas, 2017; Soengas et al., 2018). Based on these findings, we hypothesized that the effects of insulin on nutrient-sensing mechanisms would synergize with those of nutrients. We also aimed to characterize whether insulin modulates the integrative pathways likely involved in mediating changes in nutrient systems with changes in the expression of appetite regulators. Finally, we aimed to determine whether the phosphatidylinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway is involved in central insulin actions in fish, in a way comparable to that known in mammalian hypothalamus (Roh et al., 2016), using the selective inhibitor wortmannin.

MATERIALS AND METHODS

Animals

Immature, diploid, female rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792); mean±s.e.m. body mass: 120±20 g; age: ∼6 months], were obtained from a local commercial supplier (Keet's Fish Farm, Grandora, SK, Canada). Fish were housed in 700 l aquaria with filtered fresh water at 13±1°C and continuous aeration, and maintained under a 12 h light:12 h dark photoperiod (lights on at 07:00 h). Food from a commercial pellet diet specifically designed for salmonids (Ewos Pacific 3 mm, Fish Farm Supply Co, Surrey, BC, Canada) was offered daily at 11:00 h until visual apparent satiety. All studies adhered to the Canadian Council of Animal Care guidelines, and were approved by the Animal Research Ethics Board of the University of Saskatchewan (protocol number 2012-0082).

Reagents

D-Glucose was obtained from Thermo Fisher Scientific (Ottawa, ON, Canada), and oleate, bovine insulin and wortmannin were purchased from Sigma-Aldrich (Oakville, ON, Canada). Bovine insulin has been used repeatedly to understand the actions of this hormone in teleosts in both *in vivo* and *in vitro* studies (Ottolenghi et al., 1982; Plagnes-Juan et al., 2008; Polakof et al., 2010a,b, 2011; Van Raaij et al., 1995). Among the two isoforms of insulin identified in rainbow trout (Caruso and Sheridan, 2011), bovine insulin shares 53% identity with insulin 1 (GenBank accession number ABN69072.1) and 51% identity with insulin 2 (GenBank accession number ABN69073.1). Most of the observed variability is, however, in the C-peptide region of the preproinsulin (Caruso et al., 2008), which is removed during processing, resulting in a mature insulin peptide with a similar tertiary structure between rainbow trout and mammals (61.9% for insulin chain A and 79.3% for insulin chain B) (Caruso and Sheridan, 2011). Moreover, rainbow trout insulin receptors share the basic structural features of mammalian receptors (Caruso et al., 2010). Wortmannin has also been successfully used in fish models to block insulin actions (Paul et al., 2009). Stock solutions of all reagents were prepared in ultrapure water at a concentration of 24 mmol l^{-1} glucose, 100 mmol l^{-1} oleate, 30 µmol l^{-1} insulin and 600 µmol l^{-1} wortmannin. Then, all stock solutions were diluted in Hanks' Balanced Salt Solution (HBSS, WISENT Inc., St-Bruno, QC, Canada) supplemented with 50 U ml^{-1} penicillin and 50 µg_ml^{-1} streptomycin sulfate (referred to as basal medium) to reach the required experimental concentrations just before use. The oleate working solution was supplemented with 17 mmol l^{-1} saline–hydroxypropyl-β-cyclodextrin (HPB) to facilitate oleate dissolution. No effects of HPB alone were observed in previous similar experiments (Velasco et al., 2016b).

Experimental design

Tissue culture was performed as previously described in rainbow trout (Polakof et al., 2007b) with slight modifications. Every morning of an experiment, sterile 24-well culture plates were first loaded with 600 µl of modified Hanks' medium (92.56 mmol l^{-1} NaCl, 3.63 mmol l^{-1} KCl, 2.81 mmol l^{-1} $NaHCO_3$, 0.85 mmol l^{-1} $CaCl_2$, 0.55 mmol l^{-1} $MgSO_4$, 0.4 mmol l^{-1} KH_2PO_4, 0.23 mmol l^{-1} Na_2HPO_4, 7.5 mmol l^{-1} Hepes, 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin sulfate, pH 7.0; referred to as basal medium) containing the different treatments: (i) control (no treatment), (ii) glucose, (iii) oleate, (iv) insulin, (v) glucose+insulin, (vi) oleate+insulin, (vii) wortmannin, (viii) wortmannin+glucose, (ix) wortmannin+oleate, (x) wortmannin+insulin, (xi) wortmannin+glucose+insulin, or (xii) wortmannin+oleate+insulin. Concentrations were chosen based on previous studies in fish (Aguilar et al., 2011; Paul et al., 2009; Polakof et al., 2007b; Sánchez-Gurmaches et al., 2012; Velasco et al., 2017): 8 mmol l^{-1} glucose, 500 µmol l^{-1} oleate, 1 µmol l^{-1} insulin and 10 µmol l^{-1} wortmannin. The number of wells per treatment was 10 for the assessment of enzymatic activity, 6 for mRNA abundance and 5 for quantification of protein levels. Because of a limitation in the number of available fish, only treatments i–vi were evaluated for enzymatic activity and western blot. Once plates were prepared, we proceeded with the fish sampling. Thus, fish were dip-netted from the tank, anesthetized using tricaine methanesulfonate (MS-222; 0.2 g l^{-1}; Syndel Laboratories, Nanaimo, BC, Canada) and euthanized by spinal dissection. The hypothalamus (without the preoptic area) and hindbrain of each fish were quickly removed, rinsed with modified Hanks' medium, sliced on chilled Petri dishes, and placed in a chilled Petri dish containing 100 ml of modified Hanks' medium per g of tissue that was continuously gassed with 0.5% CO_2/99.5% O_2. Subsequently, tissues (hypothalamus: ∼20 mg; hindbrain: ∼40 mg) were placed in the previously prepared 24-well culture plates (each well containing 20 mg of hypothalamus or 40 mg of hindbrain from one fish). Plates were incubated at 19°C for 3 h. Culture time was chosen based on previous studies from our research group (Aguilar et al., 2011). At the end of the culture time, samples were collected, quickly frozen in liquid nitrogen and stored at −80°C until further analysis.

Assessment of enzyme activity

Samples used to assess enzyme activity were homogenized by ultrasonic disruption with 9 volumes of ice-cold buffer consisting of

50 mmol l^{-1} Tris (pH 7.6), 5 mmol l^{-1} EDTA, 2 mmol l^{-1} 1,4-dithiothreitol and a protease inhibitor cocktail (P8340, Sigma-Aldrich). The homogenates were centrifuged (10,000 ***g***) and the supernatant used immediately for determining the activity of glucokinase (Gck; EC 2.7.1.2), pyruvate kinase (Pk; EC 2.7.1.40), phosphoenolpyruvate carboxykinase (Pepck; EC 4.1.1.32), glycogen synthase (Gsase; EC 2.4.1.11), ATP citrate lyase (Acly; EC 4.1.3.8), fatty acid synthase (Fas; EC 2.3.1.85), carnitine palmitoyltransferase 1 (Cpt-1; EC 2.3.1.21) and 3-hydroxyacyl-CoA dehydrogenase (Hoad; EC 1.1.1.35), as previously described and validated for rainbow trout (Conde-Sieira et al., 2010a; Librán-Pérez et al., 2013b; Sangiao-Alvarellos et al., 2005; Velasco et al., 2016b). Briefly, enzymatic activity was determined in 96-well plates loaded with 10–50 µl homogenate (containing 1–5 mg tissue) and 160–270 µl reaction buffer (omitting the substrate in control wells). Composition of the reaction buffers used for the enzymes tested is detailed in the references cited above. Once plates were loaded, reactions were allowed to proceed at 37°C for pre-established times (3–25 min). Reaction rates of enzymes were determined by the decrease in absorbance of NADH at 340 nm (in the cases of Pk, Pepck, Gsase, Acly, Fas and Hoad), the increase in absorbance of NADPH at 340 nm (Gck), or the increase of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)-CoA complex at 412 nm (Cpt-1). All measurements were carried out in a SpectraMax 190 microplate reader (Molecular Devices, San Jose, CA, USA). Enzyme activity is expressed per mg protein, which was assayed by Bradford assay (Bio-Rad, Mississauga, ON, Canada).

Quantification of mRNA abundance

Total RNA was isolated using PureZOL™ RNA Isolation Reagent (Bio-Rad). RNA purity was validated by optical density (OD) absorption ratio ($A_{260/280}$) using a NanoDrop 2000c (Thermo Fisher Scientific, Vantaa, Finland). Then, an aliquot of 1 µg of total RNA was reverse transcribed into cDNA in a 20 µl reaction volume using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed using SensiFAST SYBR No-ROX Kit (FroggaBio, Toronto, ON, Canada), to measure the expression of mRNAs involved in: (i) appetite regulation (*npy*, *agrp1*, *pomca1*, *cartpt*); (ii) glucose sensing and metabolism: *slc2a2* (encoding Glut2), *slc5a1* (encoding Sglt-1), *gck*, *pklr* (encoding Pk), *g6pcb* (encoding glucose 6-phosphatase, G6pase), *gys1* (encoding Gsase), *pck1* (encoding Pepck), *guanine nucleotide-binding protein G(t) subunit alpha transducing 3* (*gnat3*), *taste 1 receptor member 3* (*tas1r3*), *liver X receptor* (*lxr*); (iii) fatty acid sensing and metabolism: *fatty acid translocase* (*cd36*), *acly*, *fasn* (encoding Fas), *cpt1c* (encoding Cpt-1), *lipoprotein lipase* (*lpl*); (iv) mitochondrial activity: *uncoupling protein 2a* (*ucp2a*), (v) ATP-dependent K^+ channel: *kcnj11* (encoding inward-rectifier K channel pore type 6, Kir6x) and *abbcc8* (encoding sulfonylurea receptor 1, Sur-1); and (vi) intracellular signaling and transcription factors: *peroxisome proliferator-activated receptor type alpha and gamma* (*ppara*, *pparg*), *srebf* (encoding sterol regulatory element-binding protein type 1c, Srebp1c), *mtor*, *creb1*, *foxo1* and *bsx*. The specific primer sequences used for target genes and reference genes (*β-actin* and *elongation factor 1α*) are shown in Table 1 and were obtained from IDT (Toronto, ON, Canada). All primers used in this study were previously validated in rainbow trout (Otero-Rodiño et al., 2015; Velasco et al., 2016a). Genes were amplified in duplicated RT-qPCR runs using a 96-well plate loaded with 1 µl of cDNA and 500 nmol l^{-1} of each forward and reverse primer in a final volume of 10 µl. Water instead of cDNA and RNA samples (without reverse transcriptase) was run for each reaction as a negative control. RT-qPCR cycling conditions consisted of an initial step of 95°C for 3 min, and 35 cycles of 95°C for 10 s with a specific annealing and extension temperature (Table 1) for 30 s. A melting curve was systematically monitored (temperature gradient at 0.5°C/5 s from 65 to 95°C) at the end of each run to confirm specificity of the amplification reaction. All runs were performed using a CFX Connect Real-Time System (Bio-Rad). The 2-ΔΔCt method (Livak and Schmittgen, 2001) was used to determine relative mRNA expression.

Analysis of protein levels by western blot

Tissue samples (n=5 fish) were homogenized in T-PER tissue protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA), and proteins were extracted according to the manufacturer's instructions and quantified by Bradford assay (Bio-Rad). Western blot protocol was performed as previously described (Blanco et al., 2017). The samples (containing 20 µg protein) were prepared in 1× Laemmli buffer containing 0.2% 2-mercaptoethanol (Bio-Rad) and boiled at 95°C for 10 min. Then, the whole sample volume was electrophoresed in 8–16% Mini-PROTEAN® TGX™ precast protein gel (Bio-Rad). Precision plus protein™ Dual Color Standards (Bio-Rad) was used as a molecular weight marker. Following electrophoresis, proteins were transferred to a 0.2 µm pore-size nitrocellulose membrane (Bio-Rad) using the Trans-Blot® Turbo™ transfer system (Bio-Rad), and membrane was blocked in 1× RapidBlock™ solution (aMReSCO). Then, membranes were incubated overnight with specific primary antibody (obtained from Cell Signaling Technology, Danvers, MA, USA, unless otherwise specified): 1:1000 anti-phospho Akt (Ser473) (cat. no. 4060), 1:1000 anti-carboxyl terminal Akt (cat. no. 9272), 1:250 anti-phospho FoxO1 (Thr-24) (cat. no. 9464), 1:250 anti-FoxO1 (L27) (cat. no. 9454), 1:500 anti-phospho CREB (Ser-133) (cat. no. 9198), 1:500 anti-CREB (48h2) (cat. no. 9197), 1:250 anti-phospho AMPKα (Thr-172) (cat. no. 2531), 1:250 anti-AMPKα (cat. no. 2532), 1:500 anti-phospho-mTOR (Ser-2448) (cat. no. 5536) and 1:1000 anti-vinculin (Abcam, Toronto, ON, Canada; cat. no. ab91459). All these antibodies cross-react with rainbow trout proteins of interest and were previously used in trout studies (Kamalam et al., 2012; Velasco et al., 2016b). Membranes were washed then incubated with goat anti-rabbit IgG (H+L) HRP conjugate (Bio-Rad) diluted 1:2000. For protein visualization, membranes were incubated in Clarity™ Western ECL substrate (Bio-Rad) and imaged using ChemiDoc™ MP imaging system (Bio-Rad) with chemiluminescence detection. Protein bands were quantified by densitometry using Image Lab software.

Statistical analysis

Statistical differences in enzymatic activity, mRNA expression or protein levels among experimental groups i–vi were assessed using two-way ANOVA followed by Holm–Šidák multiple comparison test, after data were checked for normality and homogeneity of variance. Data that failed one of these requirements were log-transformed and re-checked. Nutrient (none, glucose, oleate) and insulin (absence, presence) treatments were the main factors, whereas nutrient×insulin was the first-order interaction. For studies using wortmannin, statistical differences in mRNA abundance among groups were determined by three-way ANOVA followed by Holm–Šidák (for equal variance) or Dunnett's C (for unequal variance) multiple comparison test. Nutrient (none, glucose, oleate), insulin (absence, presence) and wortmannin

Journal of Experimental Biology (2020) 223, jeb213454. doi:10.1242/jeb.213454

Table 1. Primers used for quantifying gene expression by RT-qPCR in this study

Gene	Database	Accession number	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Annealing temperature (°C)
abcc8	Sigenae	tcce0019d.e.20_3.1.s.om.8	CGAGGACTGGCCCCAGCA	GACTTTCCACTTCCTGTGCGTCC	62
actb	GenBank	NM_ 001124235.1	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59
acly	GenBank	CA349411.1	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	60
agrp1	GenBank	CR376289	ACCAGCAGTCCTGTCTGGGTAA	AGTAGCAGATGGAGCCGAACA	60
bsx	GenBank	MG310161	CATCCAGAGTTACCCGGCAAG	TTTTCACCTGGGTTTCCGAGA	60
cartpt	GenBank	NM_001124627	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	60
cd36	DFCI	AY606034.1	CAAGTCAGCGACAAACCAGA	ACTTCTGAGCCTCCACAGGA	62
cpt1c	GenBank	AJ619768	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	59
creb1	GenBank	MG310160	CGGATACCAGTTGGAGGAGGA	AGCAGCAGCACTCGTTTAGGC	60
eefla1	GenBank	AF498320	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59
fasn	Sigenae	tcab0001c.e.065.1.s.om.8	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	59
foxo1	GenBank	MG310159	AACTCCCACAGCCACAGCAAT	CGATGTCCTGTTCCAGGAAGG	60
g6pcb	Sigenae	cay0019b.d.18_3.1.s.om.8.1-1693	CTCAGTGGCGACAGAAAGG	TACACAGCAGCATCCAGAGC	55
gck	GenBank	AF053331	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCCTTTGGTCCAG	60
gnat3	Sigenae	CU073912	GCAAGACGTGCTGAGGACCA	ATGGCGGTGACTCCCTCAAA	60
gys1	GenBank	BT073381.1	CGTGGTGAGAGGAAGGAACTGAGC	CCGTTGAGACCGTGGAGACA	59
kcnj11	Sigenae	CA346261.1.s.om.8:1:773:1	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	60
lpl	GenBank	AJ224693	TAATTGGCTGCAGAAAACAC	CGTCAGCAAACTCAAAGGT	59
mtor	GenBank	EU179853	ATGGTTCGATCACTGGTCATCA	TCCACTCTTGCCACAGAGAC	60
npy	GenBank	NM_001124266	CTCGTCTGGACCTTTATATGC	GTTCATCATATCTGGACTGTG	58
nr1h3	GenBank	FJ470291	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	62
pck1	GenBank	AF246149	GTTGGTGCTAAAGGGCACAC	CCCGTCTTCTGATAAGTCCAA	59
pklr	GenBank	AF246146	CCATCGTCGCGGTAACAAGA	ACATAGGAAAGGCCAGGGGC	59
pomca1	Tigr	TC86162	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	60
ppara	GenBank	AY494835	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	55
pparg	DFCI	CA345564	GACGGCGGGTCAGTACTTTA	ATGCTCTTGGCGAACTCTGT	60
slc2a2	GenBank	AF321816	GTGGAGAAGGAGGCGCAAGT	GCCACCGACACCATGGTAAA	59
slc5a1	GenBank	AY210436	GGGCTGAACATCTACCTTGCT	CTCATAACCTCCCACCTCATTG	59
srebp1c	GenBank	CA048941.1	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	60
ucp2a	GenBank	DQ295324	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	57

abcc8, sulfonylurea receptor 1; *actb*, β-actin; *acly*, ATP-citrate lyase; *agrp1*, agouti-related peptide 1; *bsx*, brain homeobox transcription factor; *cartpt*, cocaine- and amphetamine-related transcript; *cd36*, fatty acid translocase; *cpt1c*, carnitine palmitoyl transferase 1 type c; *creb1*, cAMP response element binding protein; *eefla1*, elongation factor 1α; *fasn*, fatty acid synthetase; *foxo1*, forkhead boxO1; *g6pcb*, glucose 6-phosphatase; *gck*, glucokinase; *gnat3*, guanine nucleotide-binding protein G(t) subunit alpha transducing 3; *gys1*, glycogen synthase; *kcnj11*, inward-rectifier K channel pore type 6.x-like; *lpl*, lipoprotein lipase; *mtor*, mechanistic target of rapamycin; *npy*, neuropeptide Y; *nr1h3*, liver X receptor; *pck1*, phosphoenolpyruvate carboxykinase; *pklr*, pyruvate-kinase; *pomca1*, pro-opiomelanocortin A1; *ppara*, peroxisome proliferator-activated receptor type α; *pparg*, peroxisome proliferator-activated receptor type γ; *slc2a2*, glucose facilitative transporter 2; *slc5a1*, sodium-glucose linked transporter 1; *srebf1*, sterol regulatory element-binding protein 1c; *ucp2a*, mitochondrial uncoupling protein 2a.

(absence, presence) were the main factors. Nutrient×insulin, nutrient×wortmannin and insulin×wortmannin were the first-order interactions. Nutrient×insulin×wortmannin was the second-order interaction. Significance was assigned when $P<0.05$. All analyses were carried out using SigmaPlot version 12.0 (Systat Software Inc., San Jose, CA, USA) and GraphPad Prism version 8.1.1 (GraphPad Software Inc., San Diego, CA, USA) statistical packages.

RESULTS

Abundance of mRNAs involved in glucose and fatty acid sensing is modulated by insulin in the rainbow trout hypothalamus and hindbrain

As can be seen in Tables S1 and S2, treatment of the hypothalamus and hindbrain with insulin had a significant effect on the expression of several genes involved in glucose and fatty acid sensing. This is demonstrated by the considerable number of genes for which the two-way ANOVA detected significant differences in mRNA abundance when comparing experimental groups lacking insulin versus those treated with the hormone. Altered genes were related to glucose sensing (*gck*, *pklr*), fatty acid sensing (*fasn*), transcription factors (*bsx*, *creb1*, *pparg*) and neuropeptides (*npy*) in the hypothalamus (Table S1), and to glucose sensing (*g6pcb*, *gck*, *nr1h3*, *slc2a2*), fatty acid sensing (*acly*, *lpl*), K_{ATP} (*abcc8*, *kcnj11*) and neuropeptides (*agrp1*, *pomca1*) in the hindbrain (Table S2). The specific changes in the levels of these mRNAs in response to insulin are shown in Table 2 (hypothalamus) and Table 3 (hindbrain) (as indicated by asterisks). Please refer also to Tables 2 and 3 for the effects of glucose and oleate exposure of cultured rainbow trout hypothalamus and hindbrain on the mRNA expression of target genes.

Insulin decreases the activity of some key glucose- and fatty acid-sensing enzymes in the rainbow trout hypothalamus and hindbrain

The effects of exposure of cultured rainbow trout hypothalamus and hindbrain to glucose and oleate, in the absence or presence of insulin, on the activity of key enzymes implicated in glucose and fatty acid sensing are shown in Fig. 1. *P*-values obtained after two-way ANOVA of assessed parameters are shown in Tables S1 and S2 for the hypothalamus and hindbrain, respectively. In the absence of insulin, treatment with glucose and oleate led to a decrease in the hypothalamic activity of Gck, Pepck and Acly (Fig. 1A,C,E). Activity of Gsase, Fas and Hoad in the hypothalamus was also significantly downregulated by oleate exposure, but not by glucose exposure (Fig. 1D,F,H). In the presence of insulin, there was a significant increase in hypothalamic Gck and Hoad activity in the

Journal of Experimental Biology (2020) 223, jeb213454. doi:10.1242/jeb.213454

Table 2. *In vitro* effects of exposure of rainbow trout hypothalamus to glucose, oleate and insulin on mRNA abundance of key genes involved in appetite regulation, glucose and fatty acid sensing, mitochondrial activity and intracellular signaling and transcription factors

		Absence of insulin			Presence of insulin		
Gene		Control	Glucose	Oleate	Control	Glucose	Oleate
Glucose sensing	*fbpase*	1.11±0.25	0.72±0.10	–	0.97±0.09	0.90±0.08	–
	g6pcb	1.03±0.11	2.83±0.35	–	2.22±0.39	1.93±0.38	–
	gck	1.00±0.05^{a}	1.43±0.12^{a}	2.04±0.20^{b}	1.01±0.11	1.14±0.16	1.25±0.18*
	gnat3	1.01±0.07	1.56±0.15	1.92±0.29	1.49±0.19	1.28±0.08	0.99±0.05
	gys1	1.01±0.08	1.33±0.12	–	0.96±0.18	1.05±0.15	–
	nr1h3	1.00±0.01^{a}	1.24±0.12^{a}	2.43±0.38^{b}	1.65±0.25	1.04±0.11	1.03±0.11
	pck1	1.00±0.07^{a}	1.93±0.07^{b}	0.99±0.27^{a}	1.01±0.10	1.41±0.13	1.06±0.18
	pklr	1.20±0.12^{a}	3.40±1.54^{b}	5.80±0.72^{b}	2.56±0.62	4.39±1.40	4.41±0.05
	slc2a2	1.02±0.09^{a}	3.25±0.97^{b}	1.94±0.19^{c}	3.61±0.58^{a}	1.52±0.11^{b}	0.76±0.10^{c}
	slc5a1	1.11±0.24	5.09±1.60	–	2.51±0.35	1.69±0.15	–
	tas1r3	1.06±0.21	1.63±0.02	–	2.79±0.65	1.26±0.27	–
Fatty acid sensing	*acly*	1.01±0.09^{a}	–	3.78±0.41^{b}	1.58±0.02	–	2.29±0.36
	cd36	1.04±0.17^{a}	–	2.87±0.42^{b}	1.55±0.33	–	2.62±0.30
	cpt1c	1.00±0.02	1.11±0.19	1.14±0.23	0.81±0.05	0.86±0.11	1.04±0.12
	fasn	1.00±0.02	0.82±0.13	0.85±0.27	0.98±0.09	1.26±0.20	1.25±0.08
	lpl	1.35±0.49	–	1.75±0.29	1.57±0.38	–	1.26±0.10
K_{ATP}	*abcc8*	1.00±0.07	0.72±0.03	1.17±0.22	1.07±0.10	0.97±0.05	0.69±0.10
	kcnj11	1.02±0.11	0.65±0.10	0.97±0.18	1.02±0.12	0.83±0.10	1.10±0.16
Mitochondrial activity	*ucp2a*	1.02±0.10	1.03±0.13	1.13±0.07	0.86±0.09	1.15±0.17	0.84±0.19
Cell signaling	*mtor*	1.01±0.06^{a}	1.85±0.24^{b}	3.00±0.25^{c}	1.96±0.41	1.57±0.09	1.94±0.12
Transcription factors	*bsx*	1.00±0.10^{a,b}	0.85±0.12^{a}	1.22±0.21^{b}	0.18±0.04^{a,*}	0.72±0.04^{b}	0.98±0.20^{c,*}
	creb1	1.01±0.08^{a}	0.89±0.06^{b}	1.51±0.17^{c}	0.95±0.19^{a}	0.61±0.08^{b}	0.97±0.06^{a,*}
	foxo1	1.00±0.02^{a}	2.33±0.22^{a,b}	3.58±0.43^{b}	2.29±0.45	2.38±0.15	2.77±0.67
	ppara	1.04±0.13^{a}	1.21±0.18^{a}	3.53±0.84^{b}	1.51±0.16	1.69±0.32	1.67±0.17
	pparg	1.00±0.06	2.05±0.44	2.42±0.16	1.53±0.23	0.83±0.08*	1.22±0.27*
	srebf	1.00±0.04	0.92±0.14	1.08±0.25	0.64±0.13	1.22±0.19	0.95±0.20
Neuropeptides	*agrp1*	0.74±0.13^{a}	4.14±0.58^{b}	2.34±0.30^{c}	2.45±0.37	1.70±0.23	1.91±0.51
	cartpt	1.03±0.13	0.95±0.02	0.97±0.15	0.61±0.24	0.80±0.10	0.93±0.12
	npy	1.11±0.30^{a}	0.42±0.27^{b}	0.12±0.05^{c}	0.07±0.02*	0.23±0.08	0.31±0.13
	pomca1	1.00±0.02^{a}	3.91±0.93^{b}	2.05±0.06^{a}	1.97±0.43^{a}	1.84±0.50^{a}	3.79±1.06^{b}

Samples of hypothalamus (6 fish per group) were placed in 24-well culture plates (each well containing tissue from one fish) and incubated with culture media alone (control) or containing 8 mmol l^{-1} glucose or 500 µmol l^{-1} oleate, in the absence or presence of 1 µmol l^{-1} insulin, for 3 h. mRNA abundance is shown as means+s.e.m. (n=6) with reference to the control group in the absence of insulin (results were previously normalized by *actb* and *eef1a1* mRNA levels, which did not show changes among groups). Different letters denote statistical differences among control, glucose and oleate groups in the presence or absence of insulin; asterisks indicate a significant effect of insulin within a particular nutrient; as assessed by two-way ANOVA and Holm–Šidák multiple comparison test (P<0.05). Refer to Table 2 for P-values obtained in the statistical analysis. This experiment was carried out once.

glucose-treated group compared with the no-nutrient (control) group (Fig. 1A,H), and a significant decrease in Pepck activity in the oleate-treated group (Fig. 1C). Treatment with insulin had a significant effect on the activity of Gsase, Acly and Hoad when no nutrient was added to the culture media (Fig. 1D,E,H), and on the activity of Gsase in the presence of glucose (Fig. 1D). In the hindbrain, glucose and oleate alone significantly downregulated the activity of Gsase, Acly and Cpt-1 (Fig. 1L,M,O). Additionally, glucose increased Pepck activity, while oleate stimulated Fas activity, when they were added to the media together with insulin (Fig. 1K,N). Treatment with insulin resulted in changes in the activity of Gck, Gsase, Acly, Cpt-1 and Hoad in the hindbrain (Fig. 1I,L,M,O,P); specifically, activity of these enzymes was lower in the presence of insulin compared with values obtained in the absence of the hormone. Such a decrease in activity occurred in the control only (Gck and Acly), in the presence of glucose only (Gck and Hoad), in the presence of oleate only (Acly, Cpt-1 and Hoad), or under the three nutrient conditions assessed (Gsase).

Insulin affects the phosphorylation status of key signaling proteins and transcription factors in the rainbow trout hypothalamus and hindbrain

Figs 2 and 3 show the phosphorylation status of several signaling proteins and transcription factors known to be involved in glucose and fatty acid metabolism in the hypothalamus and hindbrain in response to treatment with glucose, oleate and insulin. P-values obtained after two-way ANOVA of assessed parameters are shown in Table S1 (hypothalamus) and Table S2 (hindbrain). In the absence of insulin, exposure of the hypothalamus to oleate led to a significant increase in the phosphorylation status of Akt (Fig. 2A), and a significant decrease in the phosphorylation status of Ampkα (Fig. 2D). Major results obtained in the rainbow trout hypothalamus upon exposure to insulin alone revealed that the phosphorylation status of Akt (Fig. 2A) and mTOR (Fig. 2E) increased, while the phosphorylation of Ampkα (Fig. 2D) decreased. We also detected a significant insulin-dependent decrease in the phosphorylation status of Akt (Fig. 2A) and Ampkα (Fig. 2D) in the hypothalamus in the presence of oleate and glucose, respectively. In the hindbrain, insulin produced a significant increase in the phosphorylation status of Akt when added alone to the culture media (Fig. 3A), while it led to a significant decrease in the phosphorylation status of Foxo1 when added together with oleate (Fig. 3B). There were no other major changes in the levels of proteins assessed in response to glucose, oleate or insulin in the hindbrain.

Wortmannin reverses insulin-evoked changes in gene expression in the rainbow trout hypothalamus

The effects of wortmannin on insulin-induced changes in the expression of genes involved in glucose and lipid metabolism in the

Journal of Experimental Biology (2020) 223, jeb213454. doi:10.1242/jeb.213454

Table 3. *In vitro* effects of exposure of rainbow trout hindbrain to glucose, oleate and insulin on mRNA expression of key genes involved in appetite regulation, glucose and fatty acid sensing, mitochondrial activity, and intracellular signaling and transcription factors

		Absence of Insulin			Presence of Insulin		
Gene		Control	Glucose	Oleate	Control	Glucose	Oleate
Glucose sensing	*fbpase*	1.02±0.13	2.58±0.51	–	2.82±0.75	1.81±0.22	–
	g6pcb	1.31±0.41^{a}	3.66±0.83^{b}	–	1.62±0.28	1.51±0.21*	–
	gck	0.66±0.03^{a}	2.42±0.27^{b}	1.84±0.16^{c}	2.99±0.20^{a,*}	2.06±0.09^{b}	2.57±0.17^{a,b,*}
	gnat3	1.14±0.26^{a}	2.32±0.27^{b}	1.53±0.19^{a}	1.69±0.19	1.82±0.14	1.47±0.16
	gys1	0.96±0.25^{a}	3.09±0.60^{b}	–	1.79±0.28	1.65±0.20	–
	nr1h3	1.17±0.19^{a}	2.84±0.61^{b}	1.95±0.27^{a}	1.37±0.11	1.74±0.32	0.93±0.15*
	pck1	1.31±0.46^{a}	3.79±0.30^{b}	1.96±0.37^{a}	2.42±0.26	2.25±0.35	2.86±0.46
	pklr	1.36±0.47^{a}	3.97±0.94^{b}	1.44±0.21^{a}	3.99±0.67^{a}	1.84±0.34^{b}	1.76±0.15^{b}
	slc2a2	1.02±0.15^{a}	3.42±1.09^{b}	4.55±0.19^{b}	4.71±0.60^{a,*}	4.57±0.61^{a}	2.01±0.61^{b,*}
	slc5a1	0.92±0.28	3.09±0.69	2.11±0.04	2.31±0.47	2.14±0.49	1.44±0.15
	tas1r3	1.22±0.40^{a}	4.06±0.87^{b}	–	2.27±0.19	2.49±0.49	–
Fatty acid sensing	*acly*	1.78±0.28	–	2.32±0.14	1.80±0.23	–	1.23±0.20*
	cd36	1.09±0.25^{a}	–	2.49±0.46^{b}	2.25±0.10	–	2.46±0.34
	cpt1c	1.09±0.28	1.32±0.23	0.94±0.24	1.34±0.14	1.42±0.12	1.18±0.15
	fasn	1.01±0.08^{a}	1.77±0.19^{b}	1.18±0.02^{a,b}	1.43±0.19	1.37±0.29	0.83±017
	lpl	1.05±0.17	–	1.10±0.07	0.72±0.03	–	0.65±0.09*
K_{ATP}	*abcc8*	1.40±0.18	2.12±0.44	1.88±0.15	1.50±0.25	1.14±0.09*	1.31±0.08
	kcnj11	1.06±0.16^{a}	1.91±0.11^{b}	0.59±0.09^{c}	1.46±0.13	1.47±0.08*	1.79±0.21*
Mitochondrial activity	*ucp2a*	1.09±0.24^{a}	2.59±0.29^{b}	1.98±0.31^{a}	1.47±0.24	2.77±0.52	1.77±0.25
Cell signaling	*mtor*	1.18±0.10^{a}	2.72±0.20^{b}	1.77±0.12^{a}	1.75±0.17	2.00±0.40	1.17±0.12
Transcription factors	*bsx*	1.13±0.38	4.92±0.65	3.76±0.40	4.49±0.86	3.10±0.56	2.33±0.15
	creb1	1.16±0.29	2.52±0.30	1.79±0.32	3.89±0.86	1.86±0.26	1.03±0.20
	foxo1	1.26±0.45	3.01±0.11	3.27±0.34	2.81±0.27	1.60±0.43	3.00±0.84
	ppara	1.04±0.18	2.86±0.64	3.33±0.78	2.45±0.38	1.91±0.23	1.29±0.16
	pparg	1.26±0.37^{a,b}	2.47±0.42^{a}	1.27±0.08^{b}	1.66±0.14	1.60±0.23	0.97±0.06
	srebf	1.20±0.37^{a}	3.90±0.83^{b}	1.88±0.24^{a}	2.19±0.46	1.88±0.25	1.70±0.28
Neuropeptides	*agrp1*	1.05±0.18	1.31±0.22	0.74±0.21	1.03±0.08^{a}	0.59±0.06^{a,b,*}	0.53±0.12^{b}
	cartpt	1.00±0.07	2.40±0.46	2.07±0.34	1.67±0.30	1.68±0.38	1.08±0.19
	npy	1.06±0.28^{a}	0.18±0.06^{b}	0.05±0.03^{b}	0.20±0.11	0.20±0.13	0.57±0.06
	pomca1	1.04±0.17^{a}	2.64±0.66^{b}	1.46±0.19^{a}	1.40±0.05	1.40±0.17*	0.60±0.06*

Samples of hindbrain (6 fish per group) were placed in 24-well culture plates (each well containing tissue from one fish) and incubated with culture media alone (control) or containing 8 mmol l^{-1} glucose or 500 µmol l^{-1} oleate, in the absence or presence of 1 µmol l^{-1} insulin, for 3 h. mRNA abundance is shown as means+s.e.m. (n=6) with reference to the control group in the absence of insulin (results were previously normalized by *actb* and *eef1a1* mRNA levels, which did not show changes among groups). Different letters denote statistical differences among control, glucose and oleate groups in the presence or absence of insulin; asterisks indicate a significant effect of insulin within a particular nutrient; as assessed by two-way ANOVA and Holm–Šidák multiple comparison test (P<0.05). Refer to Table 3 for P-values obtained in the statistical analysis. This experiment was carried out once.

rainbow trout hypothalamus and hindbrain are shown in Figs 4 and 5, respectively. P-values obtained after three-way ANOVA of assessed parameters are shown in Table 4. Only those genes affected by insulin treatment alone (i.e. those genes showing a significant P-value corresponding to Factor=Insulin in Tables S1 and S2) were studied in this section. In the absence of wortmannin, insulin treatment downregulated hypothalamic levels of *bsx* (in the no-nutrient, control group), *creb1* (oleate group), *pparg* (glucose and oleate groups) and *npy* (control group) (Fig. 4). In the hindbrain, a significant increase in the levels of *gck* (in the control group), *slc2a2* (control group) and *kcnj11* (oleate group), and a significant decrease in the expression of *g6pcb* (glucose group), *slc2a2* (oleate group), *abcc8* (glucose group) and *agrp1* (glucose group) were detected upon exposure to insulin (Fig. 5). In the hypothalamus, when wortmannin was added to the culture media, many of the insulin-induced changes in gene expression were reversed, resulting in mRNA abundance comparable to that of tissues not treated with either insulin or wortmannin (Fig. 4D,F,G). The exceptions were *gck* and *creb1*, whose mRNA abundance did not significantly change in the presence of insulin in response to wortmannin treatment (Fig. 4A,E). In the hindbrain, wortmannin was able to reverse, at least partially, the increase in *gck* and *slc2a2* mRNA abundance evoked by insulin in the absence of nutrients (Fig. 5A,C). Incubation with wortmannin also cancelled most of the insulin-evoked changes in mRNA expression in the simultaneous presence of nutrients, as observed for *slc2a2*, *kcnj11*, *agrp1* and *pomca* (Fig. 5C,H–J).

DISCUSSION

Effects of glucose or oleate treatment alone

The exposure of rainbow trout hypothalamus and hindbrain to glucose or oleate induced significant changes in parameters related to glucose- and fatty acid-sensing mechanisms. This is indicated by the observed decrease in the enzymatic activity of Pepck, Acly, Fas and Hoad, an increased abundance of *slc2a2*, *slc5a1*, *gck*, *pklr*, *g6pcb*, *gys1*, *gnat3*, *cd36*, *ppara* and *pparg* mRNA, and a decrease in the expression of *abcc8* and *kcnj11* mRNA. Most of these changes were comparable to those previously reported in the brain of the same species after treatment with comparable nutrient conditions (Conde-Sieira and Soengas, 2017; Librán-Pérez et al., 2013a; Otero-Rodiño et al., 2016; Velasco et al., 2017), thus validating our experimental design. The validity of the experimental design is also supported by the changes in cellular signaling and transcription factors elicited by glucose or fatty acids, which were comparable in general to those observed in previous reports (Conde-Sieira et al., 2018; Otero-Rodiño et al., 2017; Velasco et al., 2017). In the hypothalamus, these included an increase in the mRNA abundance of *mtor* and a decrease in *creb1* after glucose treatment,

Journal of Experimental Biology (2020) 223, jeb213454. doi:10.1242/jeb.213454

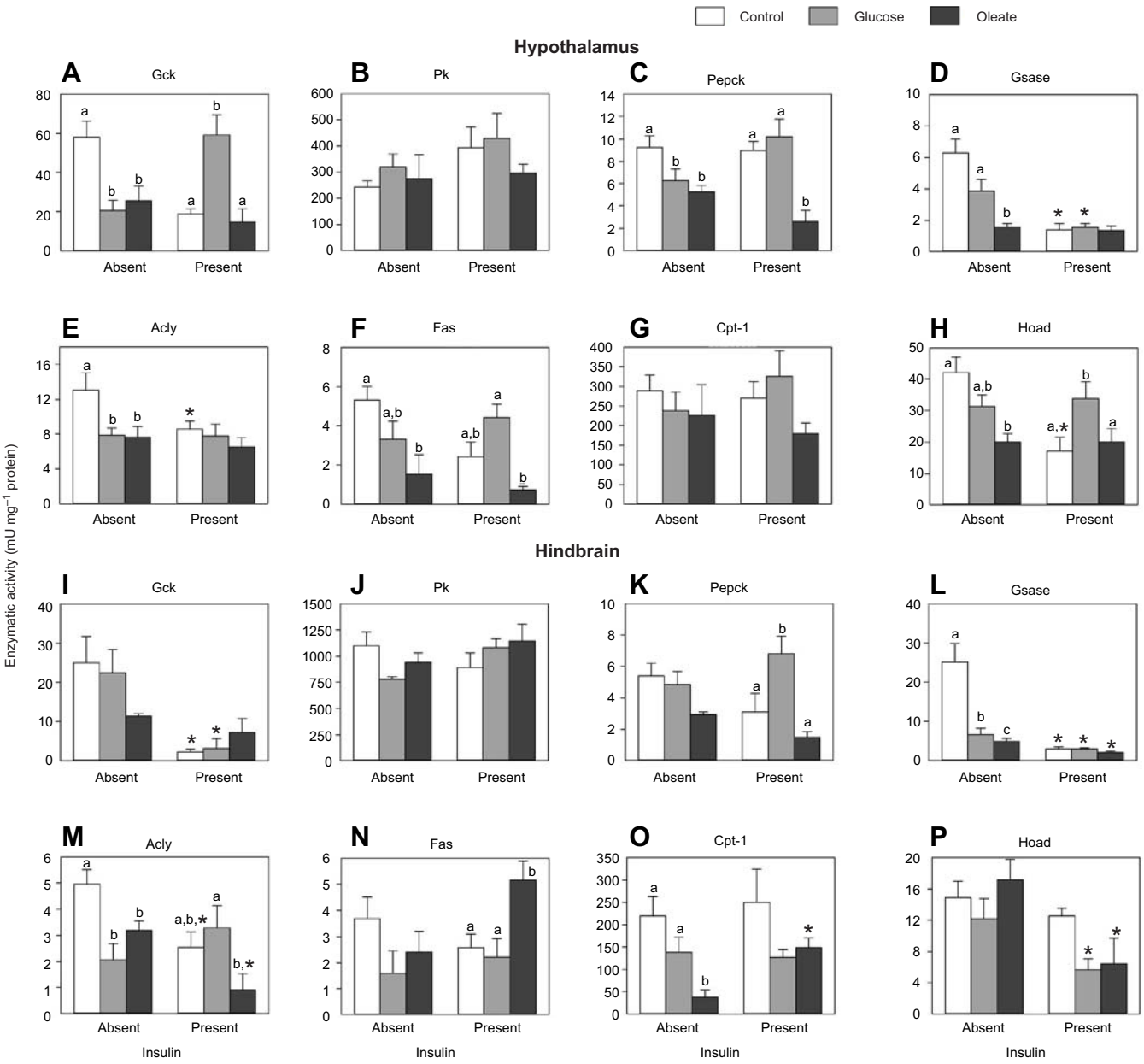

Fig. 1. *In vitro* effects of exposure of rainbow trout hypothalamus and hindbrain to glucose, oleate and insulin on the activity of key enzymes involved in glucose and fatty acid sensing. Samples of hypothalamus (A–H) and hindbrain (I–P) (10 fish per group) were placed in 24-well culture plates (each well containing tissue from one fish) and incubated with culture media alone (control) or containing 8 mmol l^{-1} glucose or 500 µmol l^{-1} oleate, in the absence or presence of 1 µmol l^{-1} insulin, for 3 h. (A,I) Gck, (B,J) Pk, (C,K) Pepck, (D,L) Gsase, (E,M) Acly, (F,N) Fas, (G,O) Cpt-1, (H,P) Hoad; for abbreviations, see Materials and Methods. Data are means+s.e.m. (n=10 samples). Different letters denote statistical differences among control, glucose and oleate groups in the presence or absence of insulin; asterisks indicate a significant effect of insulin within a particular nutrient; as assessed by two-way ANOVA and Holm–Šidák multiple comparison test (P<0.05). Refer to Table 2 (for hypothalamus) and Table 3 (for hindbrain) for P-values obtained in the statistical analysis. This experiment was carried out once.

whereas oleate treatment induced a rise in the phosphorylation status of Akt, and the mRNA abundance of *mtor* and *foxo1*. In the hindbrain, glucose treatment enhanced the mRNA abundance of *mtor* and *fox01*, whereas oleate induced a rise in *fox01* mRNA. Finally, our results showed that nutrient exposure modulates the hypothalamic and hindbrain expression of key neuropeptide mRNAs involved in the control of food intake, as demonstrated by decreased *npy* but increased *pomca1* and *cartpt* mRNA upon *in vitro* treatment with glucose or oleate. These observations agree with prior evidence obtained in rainbow trout brain exposed to glucose (Conde-Sieira et al., 2010b) or oleate (Librán-Pérez et al., 2013a; Velasco et al., 2016a), again supporting the experimental design. The increased anorexigenic potential is also in agreement with decreased food intake observed *in vivo* in the same species when subjected to raised levels of glucose (Polakof et al., 2008) or oleate (Librán-Pérez et al., 2012, 2014).

Effects of insulin treatment alone

In mammals, it is well known that insulin modulates central nutrient-sensing systems (Blouet and Schwartz, 2010). In fish, however, the insulin-dependent modulation of nutrient-sensing systems in the brain is poorly understood, and results obtained so far

Journal of Experimental Biology (2020) 223, jeb213454. doi:10.1242/jeb.213454

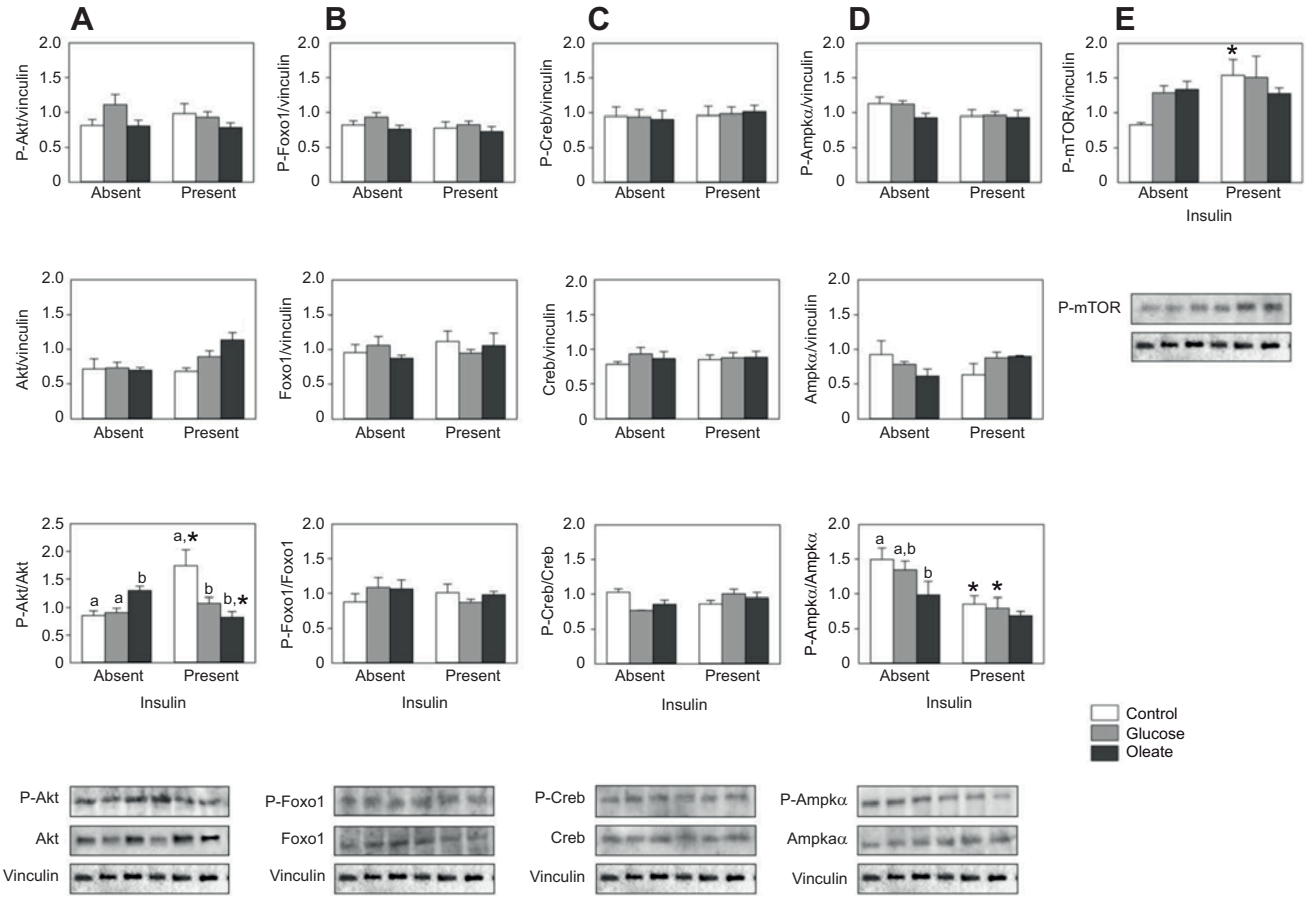

Fig. 2. *In vitro* effects of exposure of rainbow trout hypothalamus to glucose, oleate and insulin on levels and phosphorylation status of Akt, Foxo1, Creb, Ampkα and mTOR. Samples of hypothalamus (5 fish per group) were placed in 24-well culture plates (each well containing tissue from one fish) and incubated with culture media alone (control) or containing 8 mmol l^{-1} glucose or 500 µmol l^{-1} oleate, in the absence or presence of 1 µmol l^{-1} insulin, for 3 h. Western blots were performed on *n*=5 individual samples per treatment, with 20 µg of total protein loaded per lane, and representative blots are shown below the graphs. Except for mTOR, graphs represent the amount of phosphorylated protein (top), the total amount of target protein (middle) and the ratio between the phosphorylated protein and the total amount of target protein (bottom). (A) Akt, (B) Foxo1, (C) Creb, (D) Ampkα, (E) mTor. Different letters denote statistical differences among control, glucose and oleate groups in the presence or absence of insulin; asterisks indicate a significant effect of insulin within a particular nutrient; as assessed by two-way ANOVA and Holm–Šidák multiple comparison test (P<0.05). Refer to Table S1 for *P*-values obtained in the statistical analysis. This experiment was carried out once.

are controversial (Librán-Pérez et al., 2015a; Polakof et al., 2008). This is likely due to the fact that such changes are caused by alterations in metabolite levels induced by insulin treatment, rather than by insulin treatment per se. In the present study, the *in vitro* treatment with insulin alone, in the absence of nutrients, induced few changes in the parameters assessed in the hypothalamus and hindbrain. The most relevant insulin-induced change observed was the rise in the phosphorylation status of Akt in both the hypothalamus and hindbrain. A similar rise in Akt has been observed after insulin treatment in goldfish brain cells (Ma et al., 2017), but this is the first time this has been observed in brain areas such as the hypothalamus and hindbrain. Our results also demonstrated that insulin exerts a direct effect on the abundance of some intracellular signaling molecules and transcription factors in the rainbow trout hypothalamus, as indicated by the rise in mTOR protein levels and the decrease in the phosphorylation status of Ampkα and in the mRNA abundance of *npy* and *bsx*. These changes agree with those expected in this brain location under conditions resulting in decreased food intake (Delgado et al., 2017). Thus, under such conditions, decreased Ampk and/or increased mTOR would cause a decrease in the transcription factor Bsx, resulting in decreased anorectic potential and reduced food intake (Conde-Sieira et al., 2018; Otero-Rodiño et al., 2017; Velasco et al., 2017), in a similar way to that occurring in mammals (Blanco Martínez de Morentin et al., 2011; Diéguez et al., 2011; Gao et al., 2013). However, it is interesting to note that, in contrast to mammals, where four neuropeptides (NPY, AgRP, POMC and CART) were affected by insulin treatment (Schwartz et al., 2000), here we only observed changes in the abundance of *npy* mRNA. As the hypothalamus integrates endocrine information involved in the regulation of food intake, the changes in NPY in this region provide additional evidence for an important role for this peptide in energy balance. It is also noteworthy that these responses did not occur in the hindbrain. The hindbrain would be more related to the integration of metabolic information for the regulation of energy expenditure (Marty et al., 2007; Roh et al., 2016). Additional studies are required to determine region-specific changes in target genes in response to insulin.

As for nutrient-sensing systems, the impact of insulin treatment alone was practically non-existent in the hypothalamus and minimal in the hindbrain. We had observed a comparable lack of response in parameters related to fatty acid-sensing systems in the hypothalamus of the same species, although after i.p. administration of oleate or octanoate (Librán-Pérez et al.,

Journal of Experimental Biology (2020) 223, jeb213454. doi:10.1242/jeb.213454

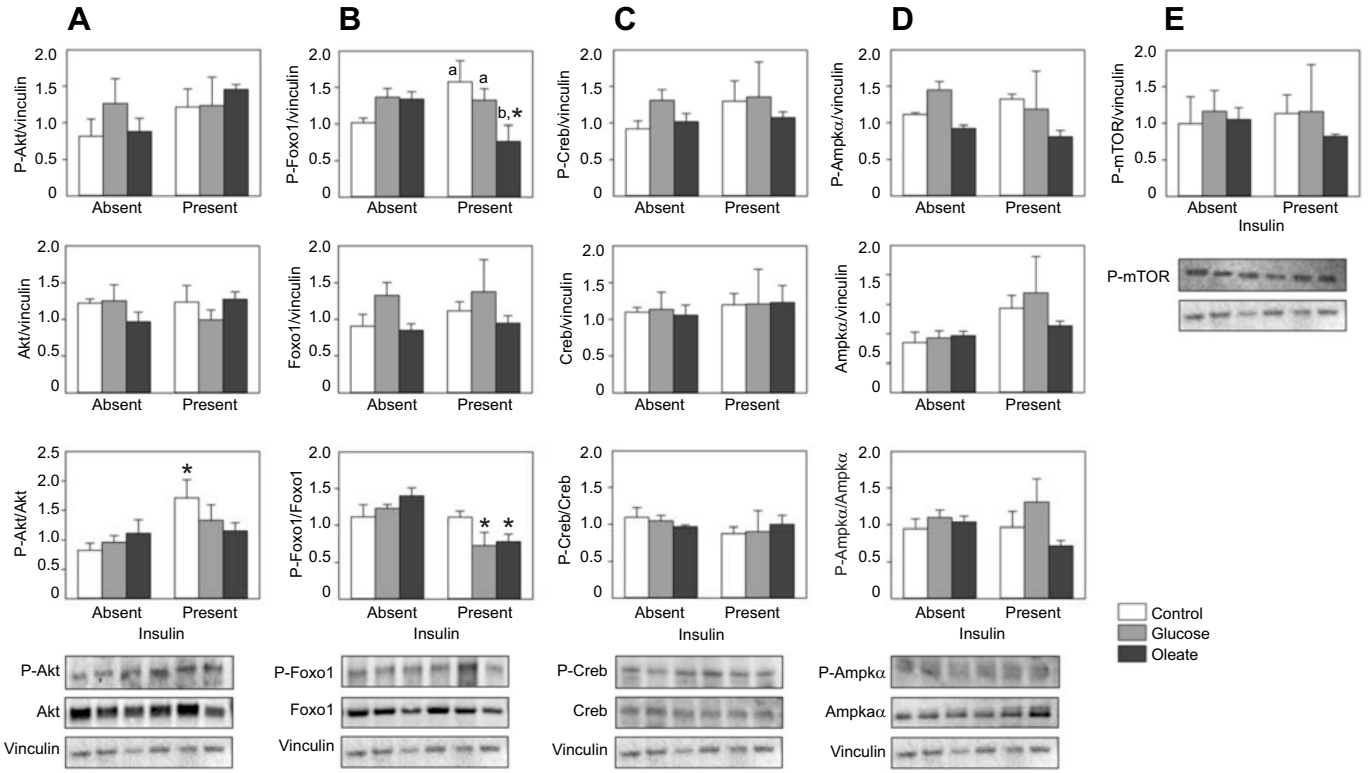

Fig. 3. *In vitro* effects of exposure of rainbow trout hindbrain to glucose, oleate and insulin on levels and phosphorylation status of Akt, Foxo1, Creb, Ampkα and mTOR. Samples of hindbrain (5 fish per group) were placed in 24-well culture plates (each well containing tissue from one fish) and incubated with culture media alone (control) or containing 8 mmol l^{-1} glucose or 500 µmol l^{-1} oleate, in the absence or presence of 1 µmol l^{-1} insulin, for 3 h. Western blots were performed on *n*=5 individual samples per treatment, with 20 µg of total protein loaded per lane, and representative blots are shown below the graphs. Except for mTOR, graphs represent the amount of phosphorylated protein (top), the total amount of target protein (middle) and the ratio between the phosphorylated protein and the total amount of target protein (bottom). (A) Akt, (B) Foxo1, (C) Creb, (D) Ampkα, (E) mTor. Different letters denote statistical differences among control, glucose and oleate groups in the presence or absence of insulin; asterisks indicate a significant effect of insulin within a particular nutrient; as assessed by two-way ANOVA and Holm–Šidák multiple comparison test (P<0.05). Refer to Table S2 for *P*-values obtained in the statistical analysis. This experiment was carried out once.

2015a). In the mammalian hypothalamus, *in vitro* insulin treatment increased the mRNA abundance of *fas* and *srebf* (Kim et al., 2007), which was not observed in rainbow trout in the present study. Available literature on fish has reported that insulin treatment increased the phosphorylation ratios of MEK, PI3K and Akt in goldfish brain cells (Ma et al., 2017), but no studies are available on the *in vitro* impact of insulin on parameters involved in nutrient sensing in brain regions such as the hypothalamus and hindbrain. In the periphery, *in vitro* studies in rainbow trout reported decreased lipolysis and increased lipogenesis in hepatocytes (Cowley and Sheridan, 1993; Harmon et al., 1993), myocytes (Albalat et al., 2005; Sánchez-Gurmaches et al., 2010) and adipocytes (Cruz-Garcia et al., 2012) upon treatment with insulin. While no prior information is available regarding the impact of insulin treatment alone on nutrient-sensing systems in the hindbrain, the results here obtained are comparable to those of hypothalamus. As a whole, it seems that the putative effect of insulin on nutrient-sensing systems in the rainbow trout brain is minor (if any) in the absence of the nutrient stimulus. Therefore, it can be concluded that changes observed in central nutrient-sensing systems after *in vivo* insulin administration in previous reports (Conde-Sieira et al., 2010a,b; Librán-Pérez et al., 2015a; Polakof et al., 2007a, 2008) are likely the result of an indirect effect of insulin through changes in circulating levels of metabolites or its interaction with other hormonal systems.

Insulin modulates nutrient sensing and neuropeptide integration in the presence of nutrients

Considering the anorectic nature of insulin in fish described in some studies after i.c.v. administration (Caruso and Sheridan, 2012; Soengas and Aldegunde, 2004), as well as the anorectic effects of raised levels of nutrients in fish brain detected through the activation of nutrient-sensing systems (Conde-Sieira and Soengas, 2017; Soengas et al., 2018), we expected *a priori* that the effects of insulin would synergize with those of nutrients in both the hypothalamus and hindbrain. However, this was not the case as in many instances the effects of raised levels of nutrients disappeared when insulin was present simultaneously. This interaction between nutrient and insulin treatments was more effective in the hindbrain than in the hypothalamus, and, in general, was more evident for glucose-sensing systems. Thus, the parameters related to glucose-sensing mechanisms that were activated by glucose treatment alone displayed lack of response (and in some cases even a contrary response) when insulin was present in the medium simultaneously. Such a response affected the mRNA abundance of *slc2a2*, *slc5a1*, *gck*, *pck1*, *pklr*, *g6pcb*, *gys1*, *tas1r3* and *nr1h3* in the hindbrain, and the activity of Pepck and mRNA abundance of *slc2a2*, *pklr* and *pck1* in the hypothalamus. No synergistic effect was noticed for any of the parameters assessed in either the hindbrain or the hypothalamus. As for fatty acid sensing, the activation of fatty acid-sensing mechanisms elicited by the presence of oleate was, in general

Journal of Experimental Biology (2020) 223, jeb213454. doi:10.1242/jeb.213454

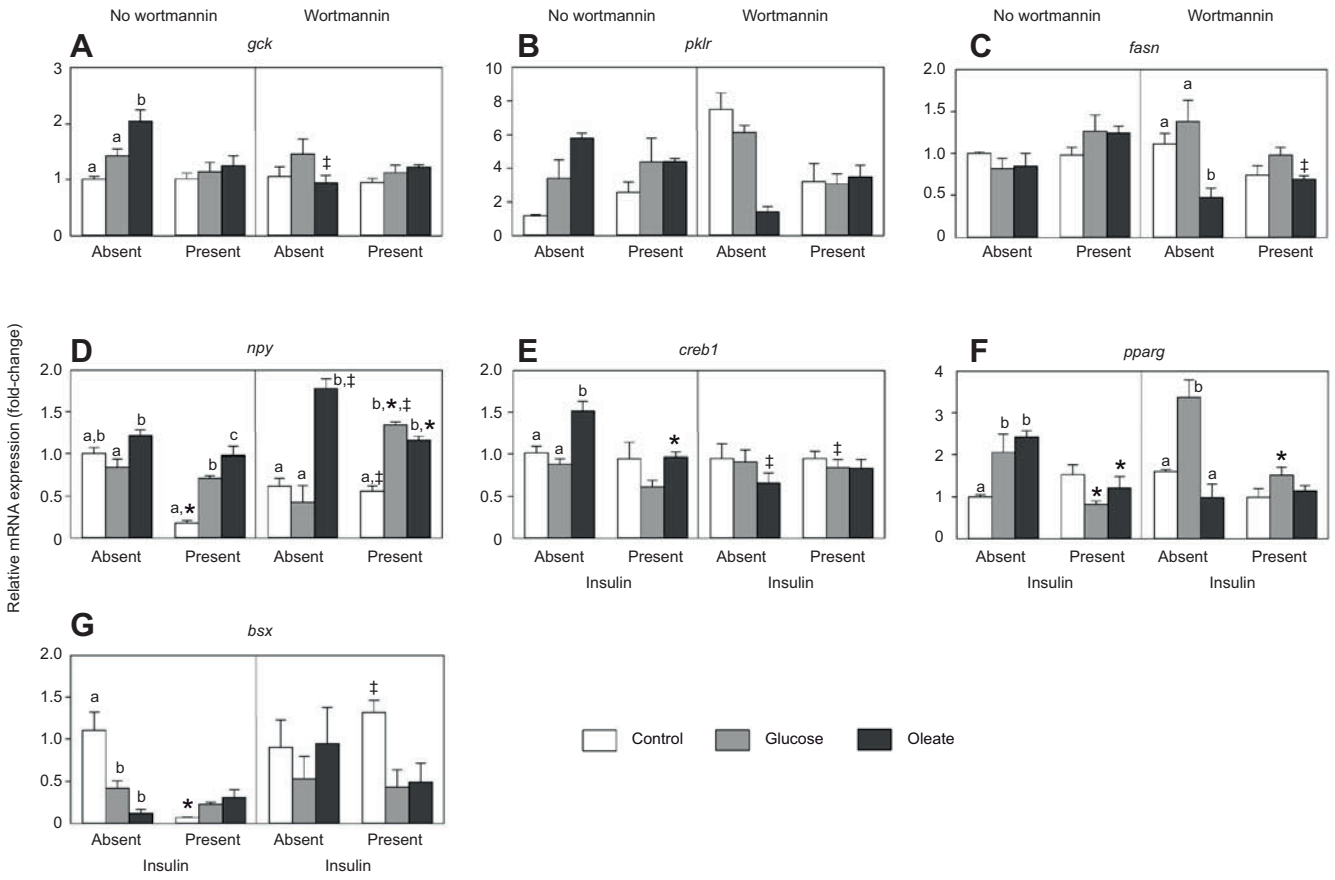

Fig. 4. *In vitro* assessment of effects of wortmannin on insulin-induced changes in expression of genes involved in glucose and lipid metabolism in rainbow trout hypothalamus. Samples of hypothalamus (6 fish per group) were placed in 24-well culture plates (each well containing tissue from one fish) and were incubated for 3 h with culture media alone (control) or containing 8 mmol l^{-1} glucose or 500 µmol l^{-1} oleate, in the absence or presence of 1 µmol l^{-1} insulin, as well as in the absence or presence of 10 µmol l^{-1} wortmannin. mRNA abundance is shown as mean+s.e.m. (n=6) fold-change with reference to the control group in the absence of insulin (results were previously normalized by *actb* and *eef1a1* mRNA levels, which did not show changes among groups). (A) *gck*, (B) *pklr*, (C) *fasn*, (D) *npy*, (E) *creb1*, (F) *pparg*, (G) *bsx*; for abbreviations, see Materials and Methods. Different letters denote statistical differences among control, glucose and oleate groups in each set of data (absence or presence of insulin, together with absence or presence of wortmannin); asterisks indicate a significant effect of insulin within a particular nutrient in the absence or presence of wortmannin; and double daggers indicate a significant effect of wortmannin within a particular nutrient in the absence or presence of insulin; as assessed by three-way ANOVA followed by Holm–Šidák (for equal variance) or Dunnett's *C* (for unequal variance) multiple comparison test (P<0.05). Refer to Table 4 for *P*-values obtained in the statistical analysis. This experiment was carried out once.

terms, again counteracted by the simultaneous presence of insulin. In the hindbrain, these included Acly and Cpt-1 activity as well as the mRNA abundance of *cd36*. In the hypothalamus, these included Acly, Fas and Hoad activity and mRNA abundance of *cd36* and *acly*. It is important to mention that no synergistic effects were noticed for fatty acid sensing either, and that the number of parameters affected was lower compared with the number involved in glucose-sensing mechanisms. The different actions observed for insulin in the hypothalamus versus the hindbrain may rely on a differential distribution of insulin receptors in these two tissues or on the fact that a different receptor subtype is mediating insulin actions in each location. Four distinct insulin receptors have been identified in rainbow trout, which are differentially expressed in the brain (Caruso et al., 2010). While no study is available on the distribution of the four receptor subtypes within the specific areas of the rainbow trout brain, the fact that they show a differential expression level in the brain opens the possibility of different expression levels in the hypothalamus and hindbrain, which could explain the tissue-specific actions observed here. It is interesting that in goldfish, higher levels of insulin mRNA occur in the hypothalamus compared with the hindbrain (Ma et al., 2017).

Apart from nutrient-sensing systems, our results show that cell signaling mechanisms and neuropeptide expression that displayed changes in response to the presence of glucose or oleate alone were altered by the simultaneous presence of insulin. In general terms, cell signaling mechanisms were observed to respond in a way similar to that described for the sensing systems, i.e. the effect of insulin cancelled the response induced by nutrients alone. In both the hypothalamus and hindbrain, the counteractive effects of insulin occurred in the presence of both glucose (mRNA abundance of *mtor* and *bsx* in hypothalamus, and abundance of *mtor* and *foxo1* in hindbrain) and oleate (phosphorylation status of Akt and mRNA abundance of *creb1* and *foxo1* in hypothalamus, and mRNA abundance of *foxo1* in hindbrain). As for the neuropeptides, the response of their mRNA abundance to the presence of nutrients changed in the presence of insulin such that the nutrient-elicited changes in the neuropeptides indicative of anorexigenic potential were cancelled by the additional presence of insulin. This effect was evident in the hypothalamus in the presence of glucose or oleate for the mRNA abundance of *npy* and *pomca1*, and also for *agrp1*, although in this last case the effects observed for nutrient treatment alone were contrary to those expected. In the hindbrain, insulin

Journal of Experimental Biology (2020) 223, jeb213454. doi:10.1242/jeb.213454

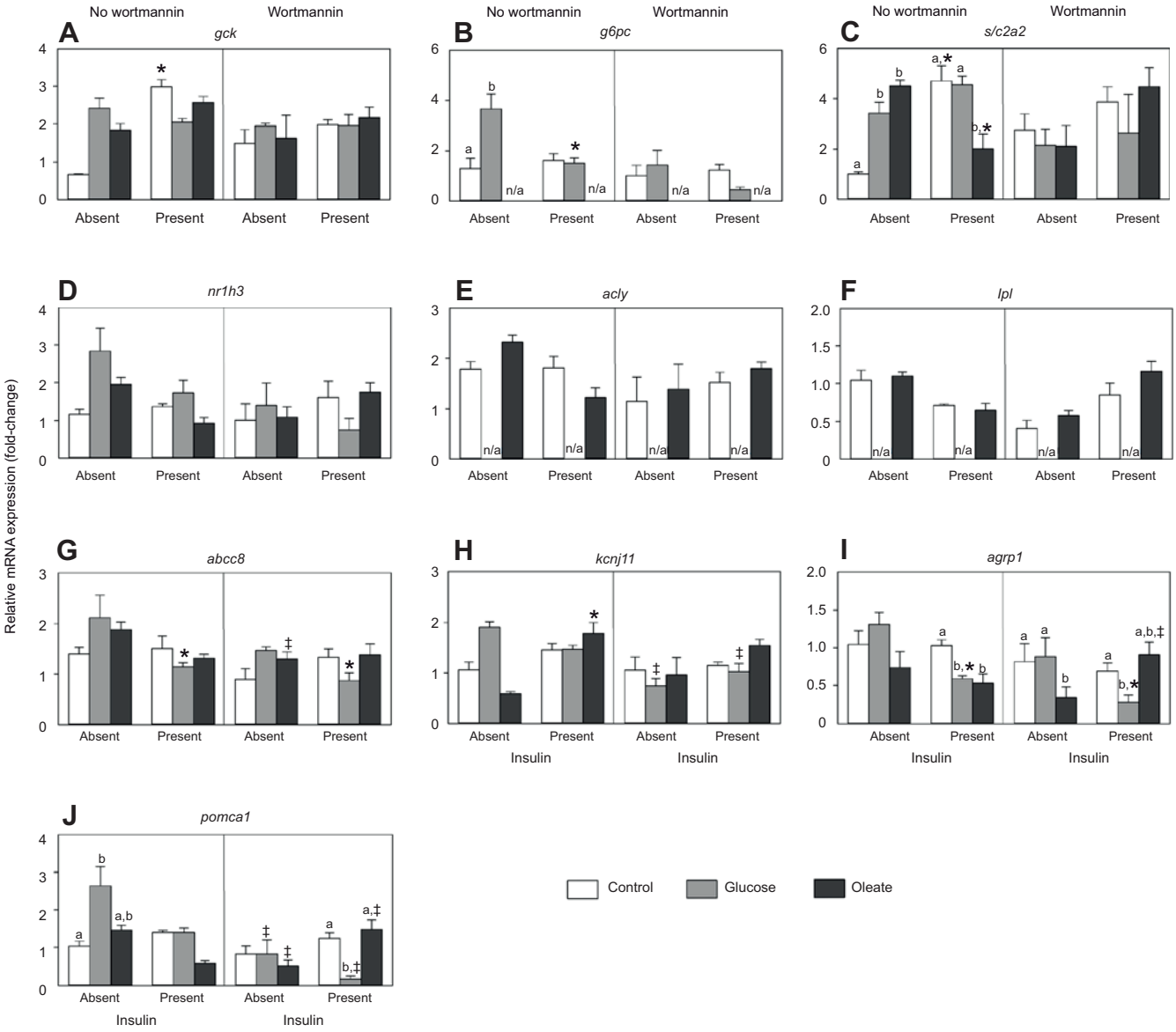

Fig. 5. *In vitro* assessment of effects of wortmannin on insulin-induced changes in expression of genes involved in glucose and lipid metabolism in rainbow trout hindbrain. Samples of hypothalamus (6 fish per group) were placed in 24-well culture plates (each well containing tissue from one fish) and incubated for 3 h with culture media alone (control) or containing 8 mmol l^{-1} glucose or 500 µmol l^{-1} oleate, in the absence or presence of 1 µmol l^{-1} insulin, as well as in the absence or presence of 10 µmol l^{-1} wortmannin. mRNA abundance is shown as mean+s.e.m. (n=6) fold-change with reference to the control group in the absence of insulin (results were previously normalized by *actb* and *eef1a1* mRNA levels, which did not show changes among groups). (A) *gck*, (B) *g6pc*, (C) *slc2a2*, (D) *nr1h3*, (E) *acly*, (F) *lpl*, (G) *abcc8*, (H) *kcnj11*, (I) *agrp1*, (J) *pomca1*. Different letters denote statistical differences among control, glucose and oleate groups in each set of data (absence or presence of insulin, together with absence or presence of wortmannin); asterisks indicate a significant effect of insulin within a particular nutrient in the absence or presence of wortmannin; and double daggers indicate a significant effect of wortmannin within a particular nutrient in the absence or presence of insulin; as assessed by three-way ANOVA followed by Holm–Šidák (for equal variance) or Dunnett's *C* (for unequal variance) multiple comparison test (P<0.05). Refer to Table 4 for P-values obtained in the statistical analysis. This experiment was carried out once.

counteracted the effect of glucose on the mRNA abundance of *npy*, *pomca1* and *cartpt*, and the effect of oleate on the mRNA abundance of *npy* and *cartpt*.

As a whole, the responses that mimic an anorectic state in the rainbow trout hypothalamus and hindbrain under exposure to nutrients alone or exposure to insulin alone changed dramatically when both nutrients and insulin were provided simultaneously. The effect of insulin was the cancellation of the changes elicited by nutrients alone. It is possible that the presence of nutrients resulted in a shift in the mechanism and/or priority of insulin action. It is clear that the integration of the metabolic and endocrine information within the hypothalamus and hindbrain is complex, and that the mechanisms downstream of insulin receptors (Caruso and Sheridan, 2011) and nutrient-sensing mechanisms (Conde-Sieira and Soengas, 2017; Soengas et al., 2018) display complicated interactions among them. Further studies are required to unravel such complexities in the roles and mechanisms of action of insulin in fish.

Putative role of PI3K/Akt pathway on the effects of insulin

Insulin signaling typically involves the activation of the PI3K/Akt intracellular pathway (Cheng et al., 2010). No available studies in

Journal of Experimental Biology (2020) 223, jeb213454. doi:10.1242/jeb.213454

Table 4. *P*-values obtained after three-way ANOVA of parameters assessed in rainbow trout hypothalamus and hindbrain after incubation in culture media alone (control) or containing 8 mmol l^{-1} glucose or 500 µmol l^{-1} oleate, in the absence or presence of 1 µmol l^{-1} insulin for 3 h

	Nutrient	Insulin	Wortmannin	Nutrient×Insulin	Nutrient×Wortmannin	Insulin×Wortmannin	Nutrient×Insulin×Wortmannin
Hypothalamus							
bsx	<0.001	0.002	0.002	<0.001	0.005	<0.001	<0.001
creb1	0.059	0.058	0.056	0.591	0.001	0.016	0.121
fasn	0.008	0.274	0.024	0.009	0.003	0.034	0.189
gck	0.014	0.097	0.055	0.566	0.074	0.080	0.042
npy	0.005	0.086	0.001	0.768	0.330	0.199	0.002
pklr	0.129	0.735	0.458	0.142	<0.001	0.068	<0.001
pparg	0.004	<0.001	0.538	0.002	<0.001	0.653	0.005
Hindbrain							
abcc8	0.427	0.032	0.004	0.002	0.755	0.054	0.825
acly	0.643	0.886	0.098	0.255	0.474	0.023	0.259
agrp1	0.080	0.056	0.024	0.002	0.264	0.168	0.149
g6pcb	0.085	0.020	<0.001	0.002	0.020	0.320	0.250
gck	0.252	<0.001	0.183	0.001	0.839	0.098	0.025
kir6x	0.741	<0.001	0.010	0.003	0.009	0.974	0.084
lpl	0.159	0.455	0.125	0.973	0.142	<0.001	0.436
nr1h3	0.778	0.722	0.017	0.098	0.065	0.113	0.209
ppomca1	0.090	0.308	<0.001	<0.001	<0.001	0.127	<0.001
slc2a2	0.051	0.002	0.187	0.001	0.231	0.351	<0.001

Incubations were carried out in the absence or presence of 10 µmol l^{-1} wortmannin. Nutrient (none, glucose, oleate), insulin (absence, presence) and wortmannin (absence, presence) were the main factors. Nutrient×insulin, nutrient×wortmannin and insulin×wortmannin were first-order interactions. Nutrient×insulin×wortmannin was a second-order interaction.

fish have assessed the mechanisms involved in insulin modulation of nutrient sensing in the brain. Results from this study suggest that the PI3K/Akt signaling pathway mediates (at least in part) the actions of insulin alone in the rainbow trout hypothalamus and hindbrain. This is evidenced by the fact that changes in mRNA abundance induced by incubation with insulin alone were in some cases cancelled by preincubation with the selective inhibitor wortmannin, as observed in the hypothalamus (*npy*, *bsx*) and hindbrain (*slc2a2* and *gck*). Further studies are needed to identify whether other pathways (besides PI3K/Akt) are mediating insulin actions on the metabolic regulation of food intake in the rainbow hypothalamus and hindbrain.

The *post hoc* tests carried out after three-way ANOVA analysis revealed significant interaction of wortmannin treatment with the response of tissues to nutrient and glucose for several parameters. In the hypothalamus, the modulatory effect of insulin on the response of parameters to nutrients disappeared in some cases in the presence of wortmannin, as observed for *npy*, *gck*, *pklr*, *fasn*, *pparg*, *creb1* and *bsx*. In the hindbrain, these include neuropeptides such as *agrp1* and *pomca1*, parameters related to glucose sensing such as *slc2a2*, *gck*, *g6pcb* or *nr1h3*, parameters related to the K_{ATP} channel such as *kcnj11* and *abcc8*, and a couple of parameters related to fatty acid sensing – *acly* and *lpl*. In general, the effects induced by insulin treatment in the response of parameters to glucose or oleate disappeared in the presence of wortmannin, suggesting that the PI3K/Akt pathway is involved in mediating not only direct actions of insulin on nutrient-sensing systems but also the effects of insulin in the interaction with nutrients.

Conclusions

To obtain information regarding the ability of insulin to directly modulate the regulation of food intake occurring in the central areas of fish brain, we incubated rainbow trout hypothalamus and hindbrain in the presence of insulin alone or in combination with nutrients (glucose or oleate) known to activate nutrient sensors involved in the control of food intake in the same species (Conde-Sieira and Soengas, 2017). The validation of the experimental design comes from results obtained after incubation of tissues with nutrients in the absence of insulin. Insulin treatment alone had little effect on nutrient-sensing mechanisms in the hypothalamus and hindbrain in the absence of nutrients, allowing us to suggest that results previously obtained in fish regarding *in vivo* insulin administration might relate to the indirect effect of insulin on circulating levels of metabolites. In the hypothalamus, but not in the hindbrain, insulin treatment alone (in the absence of nutrients) induced changes in cellular signaling mechanisms comparable to those known in the mammalian model, which supports the anorectic role of this hormone. In the presence of nutrients, insulin elicited changes in parameters related to nutrient sensing, cellular signaling, transcription factors and neuropeptide mRNA abundance in both the hypothalamus and hindbrain, such that it reversed most of the effects elicited by nutrients alone. In general, these interactive effects were more important in the hindbrain, allowing us to suggest a differential specificity of insulin effects between the two brain areas. The effects of insulin in the interaction with nutrients appear to be dependent on the PI3K/Akt pathway, as most of the responses disappeared in the presence of the selective inhibitor wortmannin. In summary, we provided new information regarding the direct impact of insulin on central mechanisms involved in the regulation of food intake in rainbow trout. Such information describes few effects for insulin in the absence of nutrients, but important interactions in the presence of nutrients, especially in the hindbrain. This underlines the complexity of the insulin mechanism of action in fish. Future lines of investigation should focus on characterizing the intracellular pathways involved in mediating central actions of insulin in the regulation of food intake, which might help us to understand the complex interactions between this hormone and the responses elicited by nutrients.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.M.B., J.L.S., S.U.; Methodology: A.M.B., S.U.; Investigation: A.M.B., J.I.B.; Resources: S.U.; Data curation: A.M.B.; Writing - original draft: A.M.B., J.I.B., J.L.S.; Writing - review & editing: A.M.B., J.L.S., S.U.; Supervision: J.L.S., S.U.; Project administration: A.M.B., J.L.S., S.U.; Funding acquisition: S.U.

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Supplementary information
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