The Journal of Experimental Biology 211, 1075-1086
Published by The Company of Biologists 2008
doi:10.1242/jeb.014050

Involvement of lactate in glucose metabolism and glucosensing function in selected tissues of rainbow trout

Sergio Polakof and José L. Soengas*

Laboratorio de Fisioloxía Animal, Departamento de Bioloxía Funcional e Ciencias da Saúde, Facultade de Bioloxía, Universidade de Vigo, 36310 Vigo, Spain

*Author for correspondence (e-mail: jsoengas@uvigo.es)

Accepted 24 January 2008

SUMMARY

The aim of this study was to obtain evidence in rainbow trout for a role of lactate in glucose homeostasis as well as in the function of glucosensing tissues. In a first set of experiments, trout were injected, either (1) intraperitoneally (*N*=8) with 5 ml kg^{-1} of Cortland saline alone (control) or saline containing L-(+)-lactate (22.5 mg kg^{-1} or 45 mg kg^{-1}), oxamate (22.5 mg kg^{-1}) or D-glucose (500 mg kg^{-1}), or (2) intracerebroventricularly (*N*=11) with 1 μl 100 g^{-1} body mass of Cortland saline alone (control) or containing D-glucose (400 μg μl^{-1}) or L-(+)-lactate (400 μg μl^{-1}), with samples being obtained 6 h after treatment. In a second set of experiments, hypothalamus, hindbrain and Brockmann bodies were incubated *in vitro* for 1 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} L-(+)-lactate alone (control) or with 50 mmol l^{-1} oxamate, 1 mmol l^{-1} DIDS, 1 mmol l^{-1} dichloroacetate, 10 mmol l^{-1} 2-deoxy-D-glucose, 1 mmol l^{-1} α-cyano-4-hydroxy cinnamate or 10 mmol l^{-1} D-glucose. The response of parameters assessed (metabolite levels, enzyme activities and glucokinase expression) in tissues provided evidence for (1) a role for lactate in the regulation of glucose homeostasis through changes not only in brain regions but also in liver energy metabolism, which are further reflected in changes in plasma levels of metabolites; (2) the possible presence in trout brain of an astrocyte–neuron lactate shuttle similar to that found in mammals; and (3) the lack of capacity of lactate to mimic *in vitro* (but not *in vivo*) glucose effects in fish glucosensing regions.

Key words: rainbow trout, glucosensor, lactate, glucose homeostasis, hypothalamus, hindbrain, Brockmann bodies.

INTRODUCTION

The mammalian brain keenly depends on glucose for energy, and therefore mammals have redundant systems to control glucose production. An increase in circulating glucose inhibits glucose production in the liver, but this negative feedback is impaired in type 2 diabetes (Matschinsky et al., 2006). A primary increase in hypothalamic glucose levels lowers blood glucose through inhibition of glucose production. This process requires conversion of glucose to lactate followed by stimulation of pyruvate metabolism, which leads to activation of ATP-sensitive potassium channels (Lam et al., 2005). Moreover, lactate and ketones can serve as alternate energy sources or signalling molecules to glucose once it is transported into neurons by monocarboxylate transporter (MCT) (Kang et al., 2004). Glucose is the major source of lactate formed within the brain, which can be generated in large amounts under certain conditions. Large transmembrane fluxes of lactate are characteristic of cultured neurons and, specially, astrocytes, and lactate production and export rates are high (Dienel and Hertz, 2001). The transfer of glucose-derived lactate from astrocytes to neurons is referred to as the astrocyte–neuron lactate shuttle (ANLS) hypothesis (Pellerin and Magistretti, 1994). Briefly, blood-borne glucose, which is the major energy substrate for the brain, enters the brain parenchyma *via* glucose transporter 1 (GLUT1; also known as SLC2A1) located on endothelial cells forming capillaries. It is then available to both neurons and astrocytes *via* specific glucose transporters (GLUT3 and GLUT1). In astrocytes, glucose is stored as glycogen. Owing to basal conversion of glucose into lactate through glycolysis and its release into the extracellular space, especially by astrocytes, an extracellular lactate pool is maintained. Lactate will leave astrocytes by a specific transporter, monocarboxylate transporter 1 (MCT1). Meanwhile, in addition to glucose, activated neurons will take up lactate from the extracellular pool *via* their own specific monocarboxylate transporter, MCT2. Lactate will be converted to pyruvate, favoured by the preferential expression of lactate dehydrogenase 1 (LDH1) isoform in neurons, before entering the tricarboxylic acid cycle. Use of lactate by neurons, in addition to glucose, could have multiple purposes as it increases redox potential by providing cytoplasmic NADH and it can be used to generate ATP as well as enter into glutamate synthesis (Pellerin, 2003).

There have been no studies about ANLS in fish, but there is evidence for the ability of the brain to use lactate as a fuel in the absence of glucose (Soengas et al., 1998). Moreover, in a recent study in rainbow trout (Polakof et al., 2007a) we observed that the addition of lactate to the incubation medium decreased glucokinase (GK; also known as hexokinase IV) activity as the glucose concentration in the medium increased in hypothalamus and hindbrain but not in Brockmann bodies [BB; a distinct grouping of pancreatic endocrine cells near to the gall bladder (Youson et al., 2006)]. Those results allowed us to hypothesize that lactate may have a similar metabolic role in brain as that described in mammals (Marty et al., 2007).

Lactate is known to reduce glucose sensitivity in glucose-inhibited neurons of suckling rats (Song and Routh, 2006), and brain of suckling rats can use lactate as fuel (Song and Routh, 2006) in a way similar to that of fish brain (Soengas et al., 1998). We have

demonstrated in previous studies the existence of glucosensing mechanisms in hypothalamus, hindbrain and BB of rainbow trout (Soengas et al., 2006; Polakof et al., 2007a; Polakof et al., 2007b). In hypothalamus and hindbrain incubated *in vitro*, lactate treatment elicited a decrease in GK activity (Polakof et al., 2007a), which may also support a similar capacity for lactate in glucosensing brain regions of fish. Therefore, we hypothesized that lactate, under high glucose concentration, induces an inhibitory effect on the glucosensing machinery, whereas under low glucose concentrations lactate would be used as an alternative fuel, thus maintaining the potential of glucosensing (Polakof et al., 2007b). Moreover, if the BB does not use lactate as a fuel in a way similar to the mammalian pancreas (Kang et al., 2004), no effects of lactate would be expected in BB compared with brain regions.

Therefore, we hypothesize the existence of central effects of glucose and lactate on systemic glucose homeostasis in rainbow trout, in a way that activation of neuronal pyruvate flux is required for hypothalamic glucose sensing as well as for control of blood glucose levels and liver glucose metabolism, therefore supporting the existence of ANLS in fish. Therefore, we have treated fish intracerebroventricularly with lactate or glucose to assess how glucose metabolism responds to increased levels of metabolites within the brain. To distinguish these effects from those induced by peripheral increases of metabolites, we have also carried out intraperitoneal treatments with lactate. Moreover, we also aimed to elucidate whether or not, in fish, lactate is able to induce similar responses to those induced by glucose in glucosensing tissues such as hypothalamus, hindbrain and BB. Thus, those regions were incubated *in vitro* with increased concentrations of lactate in the presence of different agents such as (1) oxamate, an inhibitor of LDH (Wong et al., 1997), (2) α-cyano-4-hydroxy cinnamate (4-CIN) and 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), inhibitors of lactate transport through MCT (Cassady et al., 2001), (3) dichloroacetate (DCA), a stimulator of pyruvate dehydrogenase (Itoh et al., 2003), (4) 2-deoxy-D-glucose (2-DG), an inhibitor of glucose use, and (5) glucose.

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (Soutorredondo, Spain). Fish were maintained for 1 month in 100 l tanks under laboratory conditions and a natural photoperiod in dechlorinated tap water at 14°C. Fish mass was 78±2 g. Fish were fed once daily (09.00 h) to satiety with commercial dry fish pellets (Dibaq-Diprotg SA, Segovia, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat and 11.5% ash; 20.2 MJ kg^{-1} of feed). The experiments described comply with the Guidelines of the European Union Council (86/609/EU), and of the Spanish Government (RD 1201/2005) for the use of animals in research.

In vivo experiments

Intraperitoneal injections

Following 1 month acclimation period, fish were randomly assigned to 100 l experimental tanks, and each tank was randomly assigned to one of four experimental treatments. Fish were lightly anaesthetized with MS-222 (50 mg l^{-1}) buffered to pH 7.4 with sodium bicarbonate, weighed and given an intraperitoneal (IP) injection of 5 ml kg^{-1} body mass of Cortland saline alone (control, *N*=8) or containing different treatments: L-(+)-lactate (22.5 mg kg^{-1} body mass; *N*=8), L-(+)-lactate (45 mg kg^{-1} body mass; *N*=8) or sodium oxamate (22.5 mg kg^{-1} body mass; *N*=8). Concentrations were selected based on studies carried out in mammals addressing the role of lactate in energy metabolism (Cassady et al., 2001; Lam et al., 2005). Sampling was carried out 6 h after injection since this time was observed in previous experiments to elicit changes in the glucosensing system in rainbow trout (Polakof et al., 2007a). Fish were fasted for 24 h before injection to ensure basal hormone levels were achieved.

Intracerebroventricular injections

Fish were randomly assigned to 100 l experimental tanks, and each tank was randomly assigned to one of three experimental treatments. Intracerebroventricular (ICV) injections were administered by following co-ordinates verified for accurate placement into the third ventricle with Methylene Blue dye and histological examination of brain tissues as described previously (Sangiao-Alvarellos et al., 2003; Sangiao-Alvarellos et al., 2004). Briefly, prior injection, the fish were anaesthetized as above and placed on a Plexiglas board with Velcro© straps adjusted to hold them in place. A 29.5 gauge needle attached through a polyethylene cannula to a 10 μl Hamilton syringe was aligned with the sixth preorbital bone at the rear of the eye socket, and from this point the syringe was moved through the space in the frontal bone into the third ventricle. Lack of injury to the ventricle lining was demonstrated by microscopic evaluation of the lining following histological processing of the brain. The plunger of the syringe was slowly depressed to dispense 1 μl 100 g^{-1} body mass of Cortland saline alone (control, *N*=11) or containing L-(+)-lactate (400 μg μl^{-1}; *N*=11) or D-glucose (400 μg μl^{-1}; *N*=11). The mass of the fish used in the experiments was very homogeneous resulting in a homogeneous ICV volume. After injection, fish were returned to their tanks and allowed to recover. After 6 h fish were removed from the holding tanks and sampled. Fish were fasted for 24 h before injection to ensure basal hormone levels were achieved.

After IP or ICV injections, fish were removed from replicate holding tanks, anaesthetized as above and weighed. Blood was obtained by caudal puncture with ammonium-heparinized syringes. Plasma was obtained after centrifugation of blood (1 min at 10 000 ***g***) and divided into two aliquots. One aliquot was immediately frozen in liquid nitrogen for the assessment of plasma protein while the second aliquot was deproteinized (6% perchloric acid) and neutralized (1 mol l^{-1} potassium bicarbonate) before freezing in liquid nitrogen and further storage at –80°C until assayed. The liver and BB were removed, frozen in liquid nitrogen, and stored at –80°C until assayed. The brain was removed, placed on a chilled Petri dish and the hypothalamus and hindbrain obtained as described previously (Soengas et al., 2006), frozen in liquid nitrogen and stored at –80°C until assayed.

In vitro experiments

In vitro experiments were carried out as described before (Polakof et al., 2007a). Every morning of an experiment, fish were dipnetted from the tank, anaesthetized with MS-222 (50 mg l^{-1}) buffered to pH 7.4 with sodium bicarbonate, decapitated and weighed. The hypothalamus and hindbrain were removed and dissected as described previously (Soengas et al., 2006; Polakof et al., 2007a; Polakof et al., 2007b); BB were also dissected and cleaned of surrounding vessels, bile ducts, and connective tissue. Tissues were rinsed with 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 i.u. ml^{-1} penicillin and 50 mg ml^{-1} streptomycin sulphate, pH 7.0; referred to a basal

medium), sliced in chilled Petri dishes, placed in a chilled Petri dish containing 100 ml of modified Hanks' medium g^{-1} tissue, and gassed with 0.5% CO_2/99.5% O_2. To ensure adequate mass, tissues were combined from different fish resulting in pools of three hypothalami, three to four hindbrains, and three BB. The tissue was finely minced and mixed and then placed in 48-well culture plates with 100 ml of modified Hanks' medium g^{-1} tissue.

Preliminary experiments indicated that the optimal incubation period was 1 h (data not shown) at 15°C. In a second preliminary experiment, tissues were incubated at 15°C for 1 h with modified Hanks' medium containing 0, 0.5, 1, 2, 4 and 8 mmol l^{-1} lactate (previously neutralized) to determine the linear range of response in parameters linked to changes in lactate concentration. Good linearity was observed at concentrations from 2 to 8 mmol l^{-1} lactate (data not shown) and these were selected for further experiments.

All subsequent experiments used freshly obtained tissues incubated in 48-well culture plates at 15°C for 1 h with 100 ml g^{-1} modified Hanks' medium that was gassed with 0.5% CO_2/99.5% O_2. Control wells contained medium with 2, 4 or 8 mmol l^{-1} L-(+)-lactate (previously neutralized). Treated wells contained medium at the same lactate concentration and one of the selected agents related to lactate metabolism. These included (final concentration): an inhibitor of lactate dehydrogenase (50 mmol l^{-1} sodium oxamate), an inhibitor of pyruvate dehydrogenase complex (1 mmol l^{-1} sodium dichloroacetate; DCA), inhibitors of the monocarboxylic acid transporter [1 mmol l^{-1} α-cyano-4-hydroxy cinnamate (4-CIN) and 1 mmol l^{-1} 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS)], an inhibitor of glucose utilization (10 mmol l^{-1} 2-deoxy-D-glucose) and D-glucose (10 mmol l^{-1}). All reagents were dissolved in modified Hanks' medium, except for DIDS (0.5% dimethylsulphoxide; DMSO) and 4-CIN (0.5% ethanol). The concentrations of DIDS and 4-CIN were selected based on those previously used in fish (Soengas and Moon, 1995) and mammals (Philis et al., 2001) whereas for the other agents, concentrations were selected based on experiments carried out in mammals (Wong et al., 1997; Wender et al., 2000; Cassady et al., 2001; Itoh et al., 2003; Lam et al., 2005). No effects on the parameters assessed were observed for the vehicle alone (data not shown). After 1 h incubation, tissues were quickly removed, filtered, rinsed with modified Hanks' medium, frozen in liquid nitrogen, and stored at –80°C until assay.

For each experiment, one set of 21 tissue pools was assessed (seven treatments and three lactate concentrations) for enzyme activities (GK and LDH), whereas a separate set of 21 tissue pools was used for the assay of tissue metabolites (lactate, glycogen and glucose levels). The number of independent experiments carried out for enzyme activities was three (N=3) for treatments and ten (N=10) for controls, whereas a similar number of experiments was carried out to assess tissue metabolites.

Assessment of metabolite levels and enzyme activities

Plasma glucose and lactate levels were determined enzymatically using commercial kits (Spinreact, Barcelona, Spain) adapted to a microplate format. Plasma total α-amino acids were assessed colorimetrically using the ninhydrin method of Moore (Moore, 1968); alanine was used to develop a standard curve.

Samples used to assess metabolite levels in tissues were homogenized immediately by ultrasonic disruption in 7.5 vols of ice-cooled 6% perchloric acid, and neutralized (using 1 mol l^{-1} potassium bicarbonate). The homogenate was centrifuged, and the supernatant used to assay metabolites. Glycogen levels were assessed using the method of Keppler and Decker (Keppler and Decker, 1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercial kit (Biomérieux, Madrid, Spain). Glucose 6-phosphate levels were estimated by decreases in absorbance of NADH at 340 nm after incubation of sample with (in mmol l^{-1}) 0.1 Na_2HPO_4 and 0.1 NaH_2PO_4 (pH 7.0), 0.2 $NADP^+$, and excess glucose 6-phosphate dehydrogenase. Di-hydroxiacetone phosphate (DHAP) levels were assessed by decreases in absorbance of NADH at 340 nm after incubation of sample with (in mmol l^{-1}) 50 imidazole (pH 7.6), 0.2 NADH, and excess α-glycerophosphate dehydrogenase. Lactate and total α-amino acids were assessed as described above for plasma samples.

Samples for enzyme activities in tissues were homogenized by ultrasonic disruption with 9 vols 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 (Sigma Chemical Co., St Louis, MO, USA; P-2714). The homogenate was centrifuged and the supernatant used immediately for enzyme assays. Enzyme activities were determined using a microplate reader SPECTRAFluor (Tecan, Grödig, Austria) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of supernatant (15 μl) at a pre-established protein concentration, omitting the substrate in control wells (final volume 265–295 μl), and allowing the reactions to proceed at 20°C for pre-established times (3–10 min). Enzyme activities are expressed per mg protein. Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method (Smith, 1985) with bovine serum albumin (Sigma) as standard. Enzyme activities were assessed at maximum rates determined by preliminary tests to determine optimal substrate concentrations. Fructose 1,6-bisphosphatase, glucose 6-phosphatase (G6Pase), glycogen synthase (GSase), glycogen phosphorylase, LDH-R (LDH reductase), LDH-O (LDH oxidase), Low K_m hexokinase I (HK), GK and pyruvate kinase (PK) activities were estimated as described previously (Soengas et al., 2006; Polakof et al., 2007b).

RT–PCR analysis of glucokinase (glucokinase gene) expression

Total RNA was extracted from frozen brains using TRIzol reagent as recommended by the manufacturer (Sigma). The quality and quantity of the isolated RNA was assessed spectrophotometrically. Total RNA (2 μg) was reverse transcribed into first-strand cDNA when primed with random primers (Amersham Biosciences, Barcelona, Spain) using M-MLV reverse transcriptase for 1 h at 37°C by methods recommended by the manufacturer (Promega, Madison, WI, USA). The expression of single isoforms of glucokinase (GK; via GK gene expression) were assessed. GK cDNA was PCR-amplified using specific primers developed for rainbow trout by Panserat et al. (Panserat et al., 2000): 5′-GATGTTGGTGAAGGTGGGG-3′ (forward) and 5′-TTCAGTAGGATGCCCTTGTC-3′ (reverse); amplification with these primers resulted in a 250 bp product. The housekeeping gene used to assess the relative cDNA levels of GK was rainbow trout 18s, which was amplified by PCR using specific primers (Kusakabe et al., 2006): 5′-TCAAGAACGAAAGTCGGAGG-3′ (forward) and 5′-GGACATCTAAGGGCATCACA-3′ (reverse). The PCR reactions were carried out using a PTC-200 Peltier thermal cycler (MJ Research Inc, Waltham, MA, USA) in a final volume of 20 μl, containing cDNA template (8 μl for GK, and 1 μl for 18s), 1× buffer (50 mmol l^{-1} KCl, 20 mmol l^{-1} Tris–HCl and 0.1% Triton X-100), 0.2 mmol l^{-1} dNTPs, 1.5 mmol l^{-1}

Table 1. Metabolite levels and enzyme activities in liver of rainbow trout sampled 6 h after intracerebroventricular injection into the third ventricle of 1 μl 100 g^{-1} body mass of Cortland saline alone (control) or containing D-glucose (400 μg μl^{-1}) or L-(+)-lactate (400 μg μl^{-1})

Parameter	ICV Treatment		
	Control	Glucose	Lactate
Metabolite levels			
Glucose (μmol g^{-1} wet mass)	4.73±0.56[a]	2.73±0.29[b]	3.68±0.42[a,b]
Glycogen (μmol g^{-1} wet mass)	85.9±23.9[a]	13.6±4.75[b]	100.5±26.2[a]
Lactate (μmol g^{-1} wet mass)	3.07±0.21[a,b]	3.89±0.24[b]	2.91±0.20[a]
α-Amino acid (μmol g^{-1} wet mass)	31.2±1.18[a]	24.5±1.38[b]	22.2±1.10[b]
DHAP (nmol g^{-1} wet mass)	27.6±5.56[a]	56.9±9.20[b]	29.5±4.72[a]
GAP (nmol g^{-1} wet mass)	17.4±2.68	24.7±2.87	34.2±4.93
Glucose 6-phosphate (μmol g^{-1} wet mass)	1.18±0.14[a]	0.65±0.08[b]	2.20±0.36[c]
Enzyme activities			
GK (mi.u. mg^{-1} protein)	9.83±0.70[a,b]	9.52±0.98[a]	7.95±0.42[b]
Low K_m HK (mi.u. mg^{-1} protein)	0.37±0.03	0.35±0.04	0.29±0.04
LDH-O (i.u. mg^{-1} protein)	1.02±0.08[a]	0.94±0.05[a]	0.70±0.09[b]
LDH-R (i.u. mg^{-1} protein)	33.4±1.00[a]	27.9±1.96[b]	36.1±1.41[a]
PK (i.u. mg^{-1} protein)	0.75±0.04[a]	0.65±0.05[a]	0.39±0.02[b]
FBPase (i.u. mg^{-1} protein)	0.25±0.02	0.22±0.02	0.27±0.02
G6Pase (mi.u. mg^{-1} protein)	4.53±0.31[a]	3.19±0.32[b]	3.33±0.32[b]
GSase			
Total activity (mi.u. mg^{-1} protein)	352±34.8[a]	400±36.8[a]	234±15.5[b]
% GSase[a]	13.9±1.59[a]	22.7±2.05[b]	26.7±3.09[b]
GPase			
Total activity (mi.u. mg^{-1} protein)	63.2±6.97	124±18.4	109±14.2
% GPase[a]	48.4±7.13	49.7±2.37	55.7±5.81

ICV treatment, intracerebroventricular injection; DHAP, di-hydroxiacetone phosphate; GAP, glyceraldehyde 3-phosphate; GK, glucokinase; HK, hexokinase 1; LDH-O, lactate dehydrogenase oxidase; LDH-R, lactate dehydrogenase reductase; PK, pyruvate kinase; FBPase, fructose 1,6-bisphosphatase; G6Pase, glucose 6-phosphatase; GSase, glycogen synthase; GPase, glycogen phosphorylase.

Each value is the mean ± s.e.m. of N=8 fish per group. When necessary (ratios) values were log transformed prior to statistical analysis. Different letters indicate significant differences among treated groups (one-way ANOVA, P<0.05).

$MgCl_2$, 2 pmol of each primer (forward and reverse), and 1 i.u. of Taq polymerase (Ecogen, Barcelona, Spain). The optimal number of cycles for amplification was established (reactions were terminated in the logarithmic phase of the PCR reaction). Amplification of cDNA was achieved with an initial denaturation at 94°C followed by 35 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s) and extension (72°C for 30 s); a final extension period of 10 min occurred prior to termination. Negative controls without reverse transcriptase or cDNAs were performed to ensure observed bands were not a result of contamination. The PCR products were subjected to electrophoresis in 1.5% agarose gel. The size of PCR reaction products was established by comparison with a 50 bp DNA step ladder (Promega). Semi-quantification of PCR products was performed by densitometric analysis of the bands of interest using the gel electrophoresis documentation and analysis system EDAS 290 (Kodak, Rochester, NY, USA) of images captured from UV transiluminated ethidium-bromide-stained gels. Results are shown as arbitrary units and represent the ratio (%) between GK and 18s expression. To ensure the bands of interest were in fact trout GK, each band was gel-purified using the GFX PCR DNA and gel band purification kit (Amersham Biosciences) and cloned using Pgem-T Vector Systems II (Promega). White colonies were amplified by PCR using primers T7 and M13 (flanking the insert) and sequenced in both directions using the dRhodamine terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The reactions were run on an Applied Biosystems automated sequencer model ABI PRISM 310. The resulting sequences were compared with known sequences in GenBank using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/).

Statistics

Data are presented as means ± s.e.m. Comparisons among groups were performed using SigmaStat (SPSS Inc., Chicago, IL, USA) by one-way ANOVA (*in vivo* experiments) or two-way ANOVA (*in vitro* experiments, with lactate concentration and treatment as main factors). When necessary (ratios), data were log transformed to fulfil the conditions of the analysis of variance. *Post-hoc* comparisons were made using a Student–Newman–Keuls test, and differences were considered statistically significant at P<0.05.

RESULTS

In vivo experiments

In liver (Table 1), ICV treatment with glucose decreased glucose, glycogen, α-amino acid, glucose 6-phosphate levels, and LDH-R and G6Pase activities, whereas increases were noticed in DHAP levels and total GSase activity. ICV treatment with lactate decreased α-amino acid levels and LDH-O, PK, G6Pase and total GSase activities but increased glycogen and glucose 6-phosphate levels and the percentage GSase *a* activity.

In plasma, glucose levels (Fig. 1A) decreased after ICV lactate treatment but increased when lactate was administered in IP treatment. Plasma lactate levels (Fig. 1B) increased when fish were IP injected with lactate or oxamate, whereas a decrease was observed when the treatment was administered by ICV injection. Plasma α-amino acids levels (Fig. 1C) showed a decrease when glucose was administered by ICV injection.

GK gene expression increased in hypothalamus (Fig. 2B) of trout after ICV glucose treatment. In BB of trout given ICV injection of glucose, the GK gene expression was higher than in the control group (Fig. 2D). No significant changes in GK gene expression were noticed in liver (Fig. 2A) or hindbrain (Fig. 2C).

Glucose levels (Fig. 3A) increased in hypothalamus after ICV glucose injections. By contrast, glucose levels were lower than controls in BB after IP oxamate injection. Lactate increased glucose levels in hypothalamus when this metabolite was administered both by IP (L45) and ICV injection. Glycogen levels (Fig. 3B) increased in hindbrain and hypothalamus but not in BB after IP oxamate injection. Glycogen levels increased after IP lactate treatment in hypothalamus and BB. α-Amino acid levels (Fig. 3C) increased in the hypothalamus (L22.5 or 45) after IP lactate injections, and in hindbrain (L400) after ICV injection. In hindbrain α-amino acid levels increased after IP lactate treatment.

GK activity (Fig. 4A) increased in all tissues studied after ICV injections of glucose or lactate (L400) except after lactate treatment in BB. In hindbrain, L45 or oxamate treatments increased GK activity, whereas in hypothalamus GK activity increased after IP

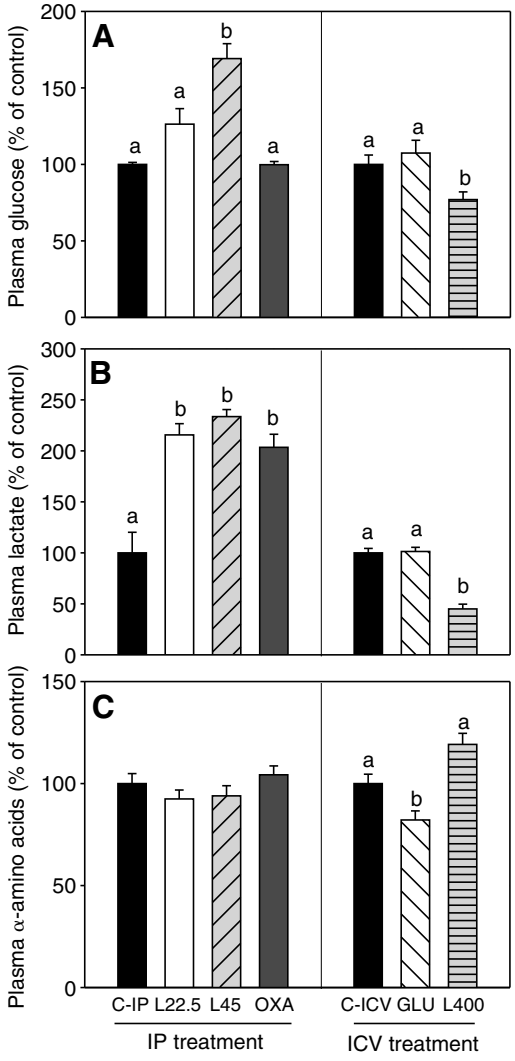

Fig. 1. Glucose (A), lactate (B) and α-amino acid (C) levels in plasma of rainbow trout sampled 6 h after intraperitoneal injection (IP treatment) of 5 ml kg^{-1} of Cortland saline alone (control, C) or saline containing L-(+)-lactate (L22.5, 22.5 mg kg^{-1} or L45, 45 mg kg^{-1}), sodium oxamate (OXA, 22.5 mg kg^{-1}) or D-glucose (GLU, 500 mg kg^{-1}), or 6 h after intracerebroventricular injection (ICV treatment) into the third ventricle of 1 μl 100 g^{-1} body mass of Cortland saline alone (control, C) or containing D-glucose (GLU, 400 μg μl^{-1}) or L-(+)-lactate (L400, 400 μg μl^{-1}). Results are shown as percentages of control values (control=100%). Each value is the mean ± s.e.m. of N=11 (IP treatment) or N=8 (ICV treatment) fish per group. Different letters indicate significant differences among treated groups within each treatment (one-way ANOVA, P<0.05).

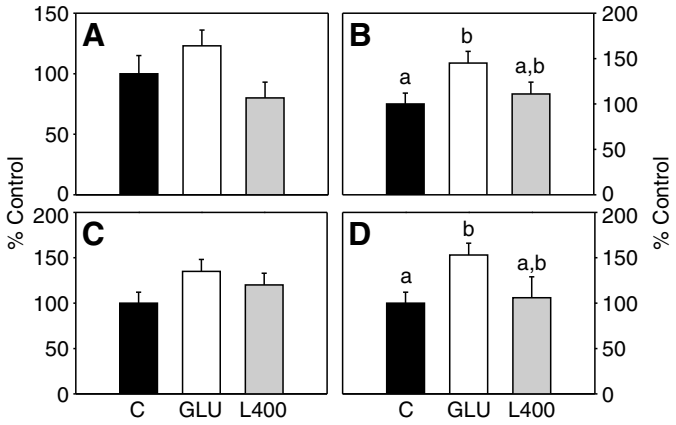

Fig. 2. Relative abundance of glucokinase (GK) mRNA expression in liver (A), hypothalamus (B), hindbrain (C) and Brockmann bodies (D) of rainbow trout sampled 6 h after intracerebroventricular injection into the third ventricle of 1 μl 100 g^{-1} body mass of Cortland saline alone (control, C) or containing D-glucose (GLU, 400 μg μl^{-1}) or L-(+)-lactate (L400, 400 μg μl^{-1}). Results are shown as percentages of control values (control=100%). Relative abundance of RT–PCR products is presented in arbitrary units as mean ± s.e.m. (N=4) between GK and 18s expression. Different letters indicate significant differences among treated groups within each treatment (one-way ANOVA, P<0.05).

lactate treatment. By contrast, both IP lactate doses (L22.5 or 45) increased GK activity in BB. Low-K_m HK activity (Fig. 4B) did not show major changes in hypothalamus or hindbrain, except for a decrease in hindbrain after oxamate treatment. By contrast, low K_m HK activity decreased in BB with IP injection of lactate (L45) and ICV administration of glucose. In the same way, a decrease in low K_m HK activity in BB was noticed after IP lactate treatment. PK activity (data not shown) only increased in hypothalamus when trout received an IP injection of lactate (L22.5) and in hindbrain under glucose or lactate treatment.

Lactate levels (Fig. 5A) decreased after IP lactate treatment in hypothalamus and hindbrain. LDH-O activity (Fig. 5B) increased in hypothalamus and hindbrain after ICV lactate treatment, but decreased in hindbrain (L45) when lactate was administered by IP injection. The activity also decreased in hypothalamus after ICV injection of glucose. LDH-R activity (Fig. 5C) decreased in hindbrain with the three IP treatments. A similar trend was found in hypothalamus, with a decrease after an IP injection of oxamate and a slight decrease after lactate treatments. LDH-R activity decreased in hypothalamus after glucose or lactate treatments.

In vitro experiments

Hypothalamus

In hypothalamus, no changes were observed in glucose levels (Fig. 6A) in control tissues. No changes were found either after oxamate or 4-CIN treatments. However, higher glucose levels than controls were detected after D-glucose and 2-DG treatments. By contrast, a lower glucose concentration was found in hypothalamus incubated with DCA. Lower levels than controls were observed after treatment with DIDS at 8 mmol l^{-1} lactate. Glycogen levels (Fig. 6B) did not show any changes in control tissues, whereas lower values than controls were found after oxamate, 4-CIN or DIDS treatments at 4 mmol l^{-1} lactate. Lower glycogen levels than controls were observed after incubation with DCA at 2 and 8 mmol l^{-1} lactate; but threefold higher levels were detected at 4 mmol l^{-1} lactate. Higher glycogen levels than controls were detected after glucose treatment at 2 and 4 mmol l^{-1} lactate, but almost undetectable levels were found at 8 mmol l^{-1} lactate after 2DG incubations. A clear positive correlation was found between lactate concentration in the medium and lactate levels (Fig. 6C). Higher lactate levels than controls were found after glucose treatments at 2 and 4 mmol l^{-1} lactate whereas no changes were found in the other treatments. No changes in GK activity (Fig. 6D) were detected in the control group and lower activities than controls were observed after DIDS and 4-CIN (all lactate concentrations), 2-DG (2 and 8 mmol l^{-1} lactate), DCA (2 mmol l^{-1} lactate) or oxamate (4 and 8 mmol l^{-1} lactate) treatments. However, higher GK activities than controls were found when glucose was added to the medium at 2 and 4 mmol l^{-1} lactate

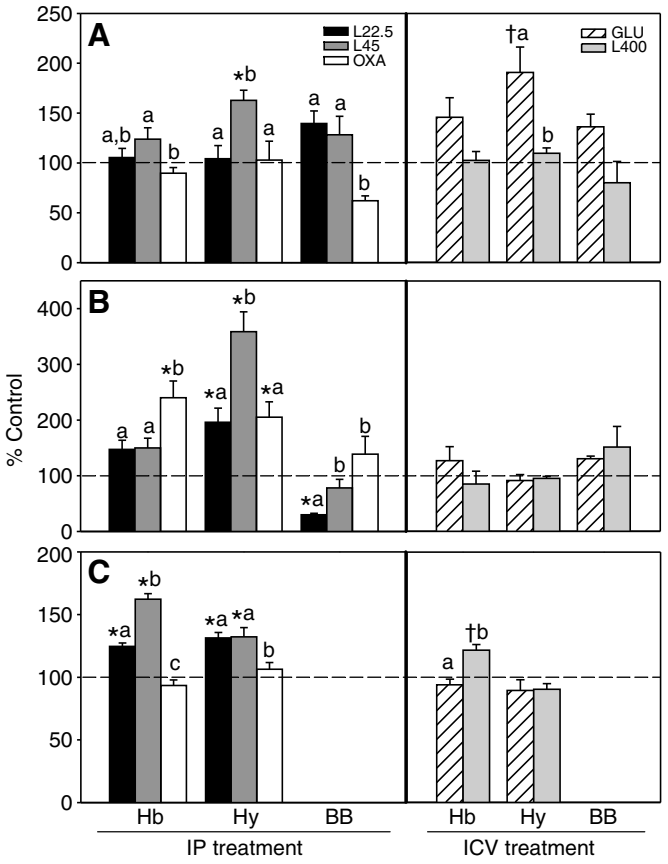

Fig. 3. Glucose (A), glycogen (B) and α-amino acid (C) levels in hindbrain (Hb), hypothalamus (Hy), and Brockmann bodies (BB) of rainbow trout sampled 6 h after intraperitoneal injection (IP treatment) of 5 ml kg^{-1} of Cortland saline alone (control, C) or saline containing L-(+)-lactate (L22.5, 22.5 mg kg^{-1} or L45, 45 mg kg^{-1}), sodium oxamate (OXA, 22.5 mg kg^{-1}) or D-glucose (GLU, 500 mg kg^{-1}), or 6 h after intracerebroventricular injection (ICV treatment) into the third ventricle of 1 μl 100 g^{-1} body mass of Cortland saline alone (control, C) or containing D-glucose (GLU, 400 μg μl^{-1}) or L-(+)-lactate (L400, 400 μg μl^{-1}). Results are shown as percentages of control values (control=100%, dotted line). Each value is the mean ± s.e.m. of *N*=11 (IP treatment) or *N*=8 (ICV treatment) fish per group. Different letters indicate significant differences among treated groups within each tissue (one-way ANOVA, $P<0.05$). *Significantly different from control (dotted line) in IP treatments ($P<0.05$); †significantly different from control (dotted line) in ICV treatments ($P<0.05$).

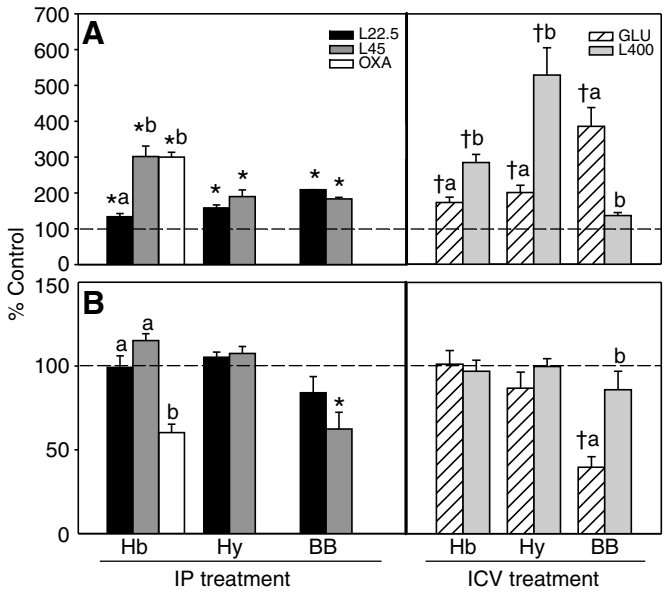

Fig. 4. Activities of glucokinase (A), and low K_m hexokinase (B) in hindbrain (Hb), hypothalamus (Hy) and Brockmann bodies (BB) of rainbow trout sampled 6 h after intraperitoneal injection (IP treatment) of 5 ml kg^{-1} of Cortland saline alone (control, C) or saline containing L-(+)-lactate (L22.5, 22.5 mg kg^{-1} or L45, 45 mg kg^{-1}), sodium oxamate (OXA, 22.5 mg kg^{-1}) or D-glucose (GLU, 500 mg kg^{-1}), or 6 h after intracerebroventricular injection (ICV treatment) into the third ventricle of 1 μl 100 g^{-1} body mass of Cortland saline alone (control, C) or containing D-glucose (GLU, 400 μg μl^{-1}) or L-(+)-lactate (L400, 400 μg μl^{-1}). Results are shown as percentages of control values (control=100%, dotted line). Each value is the mean ± s.e.m. of *N*=11 (IP treatment) or *N*=8 (ICV treatment) fish per group. Different letters indicate significant differences among treated groups within each tissue (one-way ANOVA, $P<0.05$). *Significantly different from control (dotted line) in IP treatments ($P<0.05$); †significantly different from control (dotted line) in ICV treatments ($P<0.05$).

concentration. LDH-R activity in control samples was lower at 8 mmol l^{-1} than at 2 or 4 mmol l^{-1} lactate whereas lower activities than controls were observed after oxamate, DIDS (higher values at 8 mmol l^{-1} lactate) or DCA treatments.

Hindbrain

In hindbrain, no changes were observed in glucose levels in controls (Fig. 7A) or after oxamate, DIDS or 4-CIN incubations whereas higher values than controls were noticed after 2-DG, glucose or DCA treatments. Glycogen levels in control group (Fig. 7B) were higher at 2 mmol l^{-1} than at 4 or 8 mmol l^{-1} lactate whereas no differences among lactate concentrations were found with oxamate, DIDS, DCA or 4-CIN treatments. However, a peak in glycogen levels was observed at 4 mmol l^{-1} lactate in glucose incubations. Except with DCA treatment (higher values than controls) all treatments showed lower glycogen levels than controls at 2 mmol l^{-1} lactate and higher at 4 and 8 mmol l^{-1} lactate. A positive correlation between lactate levels and lactate concentrations in the media (Fig. 7C) was found in control treatments. Oxamate in the medium produced an increase in hindbrain lactate levels at 4 and 8 mmol l^{-1} lactate, whereas lower values than controls were found after DIDS or DCA treatments at 2 mmol l^{-1} lactate. By contrast, at 8 mmol l^{-1} lactate, DCA treatment enhanced tissue lactate levels. GK activity in controls (Fig. 7D) showed a peak at 4 mmol l^{-1} lactate and lower activities at 8 mmol l^{-1} lactate. After DIDS, DCA or 2-DG treatments, GK activities showed lowest values at 4 mmol l^{-1} lactate. GK activity was not affected by glucose in the medium at 2 or 4 mmol l^{-1} lactate, but was at very low values at 8 mmol l^{-1} lactate. No changes were observed in LDH-R activity (Fig. 7E) in control samples, although lower activities than controls were found with all treatments.

Brockmann bodies

In BB, no changes were observed in glucose levels (Fig. 8A) in control samples. No significant differences were found compared with controls under oxamate, DIDS, DCA or 4-CIN treatments. However, higher glucose levels than controls were obtained in tissues incubated with either glucose or 2-DG. No differences were found in glycogen levels (Fig. 8B) in controls. A positive correlation with lactate in the medium was observed in tissues incubated with oxamate. In addition, glycogen levels were higher than controls in tissues incubated with 2-DG (all lactate concentrations), DIDS (4 and 8 mmol l^{-1} lactate), glucose or 4-CIN (both at 8 mmol l^{-1} lactate) treatments. A positive correlation between lactate concentration in

the medium and lactate levels (Fig. 8C) was found in control tissues. Lactate levels were lower than controls after glucose (at 2 and 4 mmol l^{-1} lactate), DIDS (2 mmol l^{-1} lactate), DCA (2 and 8 mmol l^{-1} lactate) or DIDS (2 mmol l^{-1} lactate) treatments. By contrast, a peak in lactate levels was evident (maximum at 4 mmol l^{-1} lactate) when 2-DG was added to the medium. A negative correlation with lactate in the medium was found in GK activity (Fig. 8D) in control tissues whereas a positive correlation was found when glucose was added to this medium. However, no GK activity was detectable after 2-DG (all lactate concentrations), DCA or 4-CIN (at 8 mmol l^{-1} lactate) or DIDS (4 and 8 mmol l^{-1} lactate) treatments. When oxamate was added to the culture medium higher GK activity than controls was observed at 8 mmol l^{-1} lactate.

DISCUSSION

Involvement of lactate in glucose homeostasis and brain function

In mammals, according to the ANLS hypothesis (Pellerin and Magistretti, 1994), neurons utilize lactate produced by astrocytes to provide energy (Brown et al., 2001). In fish, there are many pieces of evidence that indirectly suggest the existence of the ANLS, such as the fact that rainbow trout brain can oxidize lactate in the absence of glucose at almost the same rates as glucose (Soengas et al., 1998) and recent evidences obtained *in vitro* in rainbow trout hypothalamus and hindbrain demonstrating that the presence of lactate in the culture media induces changes in glucose metabolism in those tissues (Polakof et al., 2007a). Moreover, mammalian neurons have developed mechanisms for sensing glucose, designed to protect them from hypoglycemic injury (Routh, 2002). In this way, moderate increases in extracellular hypothalamic glucose levels are sufficient to lower blood glucose levels through a robust inhibition of liver glucose production (Lam et al., 2005). There is no evidence to date in fish literature regarding such a counteregulatory mechanism.

In the present study, ICV infusion of glucose elicited in hypothalamus and hindbrain increased glucose levels and GK activity (increasing glucosensing capacity) addressing a local response of those brain regions to increased glucose levels, in a way similar to that previously observed after IP infusion and *in vitro* experiments (Polakof et al., 2007a; Polakof et al., 2007b). Furthermore, the increase in cytosolic glucose levels after ICV treatment is similar to that observed in mammals (Lam et al., 2005). Lactate IP treatment increased both glucose and lactate levels in plasma, but only the increase in glucose but not lactate levels was blocked by oxamate. The effect on plasma glucose levels suggests an enhancement of glucose production in liver through glycogenolysis and/or gluconeogenesis. In fact, both pathways were activated in the present experiment (data not shown). When glucose treatment was ICV, no changes were noticed in plasma glucose levels in contrast to the decreased blood glucose levels occurring in mammals after ICV glucose infusion (Lam et al., 2005). However, this counteregulatory response was observed after lactate injection, resulting in decreased glucose and lactate levels in plasma in agreement with that observed in mammals (Lam et al., 2005).

Glucose levels clearly increased after IP lactate treatment in hypothalamus whereas oxamate generally blocked those responses. This suggests that lactate is being converted into glucose. Glycogen levels clearly increased in hypothalamus after IP lactate treatment with no major changes found after ICV lactate treatment. Again this effect was blocked by oxamate, and was not apparent in BB. Together with the results observed for glucose levels, these results suggest that lactate is being used in brain regions as a glycogenic substrate in a way similar to that suggested in mammalian brain

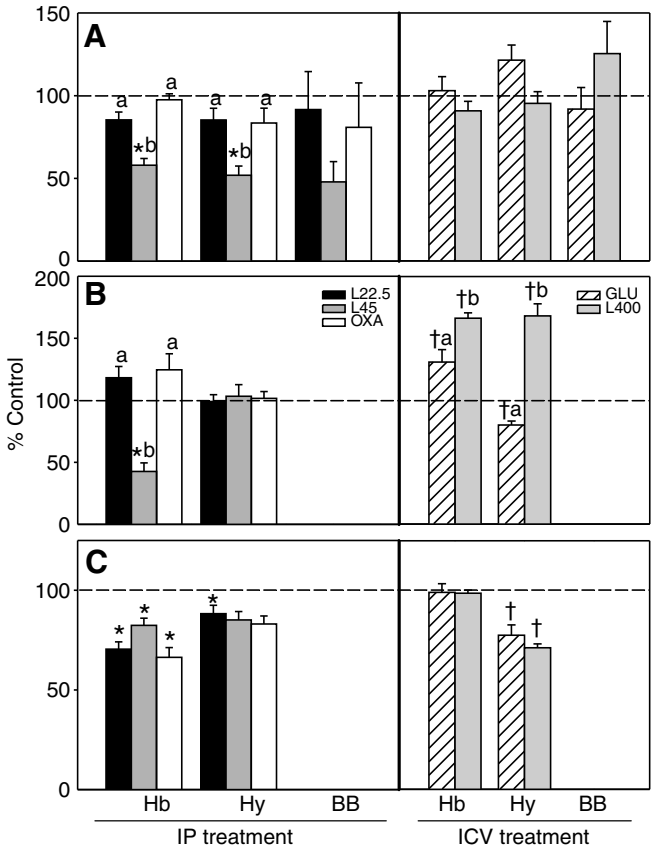

Fig. 5. Lactate (A) levels, lactate dehydrogenase oxidase (B) and lactate dehydrogenase reductase (C) activities in hindbrain (Hb), hypothalamus (Hy) and Brockmann bodies (BB) of rainbow trout sampled 6 h after intraperitoneal injection (IP treatment) of 5 ml kg^{-1} of Cortland saline alone (control, C) or saline containing L-(+)-lactate (L22.5, 22.5 mg kg^{-1} or L45, 45 mg kg^{-1}), sodium oxamate (OXA, 22.5 mg kg^{-1}) or D-glucose (GLU, 500 mg kg^{-1}), or 6 h after intracerebroventricular injection (ICV treatment) into the third ventricle of 1 μl 100 g^{-1} body mass of Cortland saline alone (control, C) or containing D-glucose (GLU, 400 μg μl^{-1}) or L-(+)-lactate (L400, 400 μg μl^{-1}). Results are shown as percentages of control values (control=100%, dotted line). Each value is the mean ± s.e.m. of N=11 (IP treatment) or N=8 (ICV treatment) fish per group. Different letters indicate significant differences among treated groups within each tissue (one-way ANOVA, P<0.05). *Significantly different from control (dotted line) in IP treatments (P<0.05); †significantly different from control (dotted line) in ICV treatments (P<0.05).

according to the ANLS hypothesis (Pellerin and Magistretti, 1994). After ICV injections of lactate, we found an increase in GK activity in hypothalamus and hindbrain reflecting increased glucosensing capacity that was similar to that observed under hyperglycemic conditions *in vivo* (Polakof et al., 2007b) in agreement with increased GK expression in mammalian hindbrain after lactate infusion (Vavaiya and Briski, 2007). It is possible that neurons in both fish brain regions interpret this lactate increase as a rise in glucose levels, activating the glucosensing system and starting a counteregulatory response. Moreover, other findings of the present study were similar to those in mammals (Routh, 2002), pointing to the existence of an ANLS in fish, because: (1) IP lactate treatment induced a rise in plasma glucose and a subsequent increase in brain glycogen levels (hypothalamus and hindbrain); (2) ICV lactate treatment decreased plasma glucose, accompanied in brain regions by decreased glycogen levels and increased lactate levels and LDH-O activity; (3) IP lactate treatment induced increased LDH-O activity

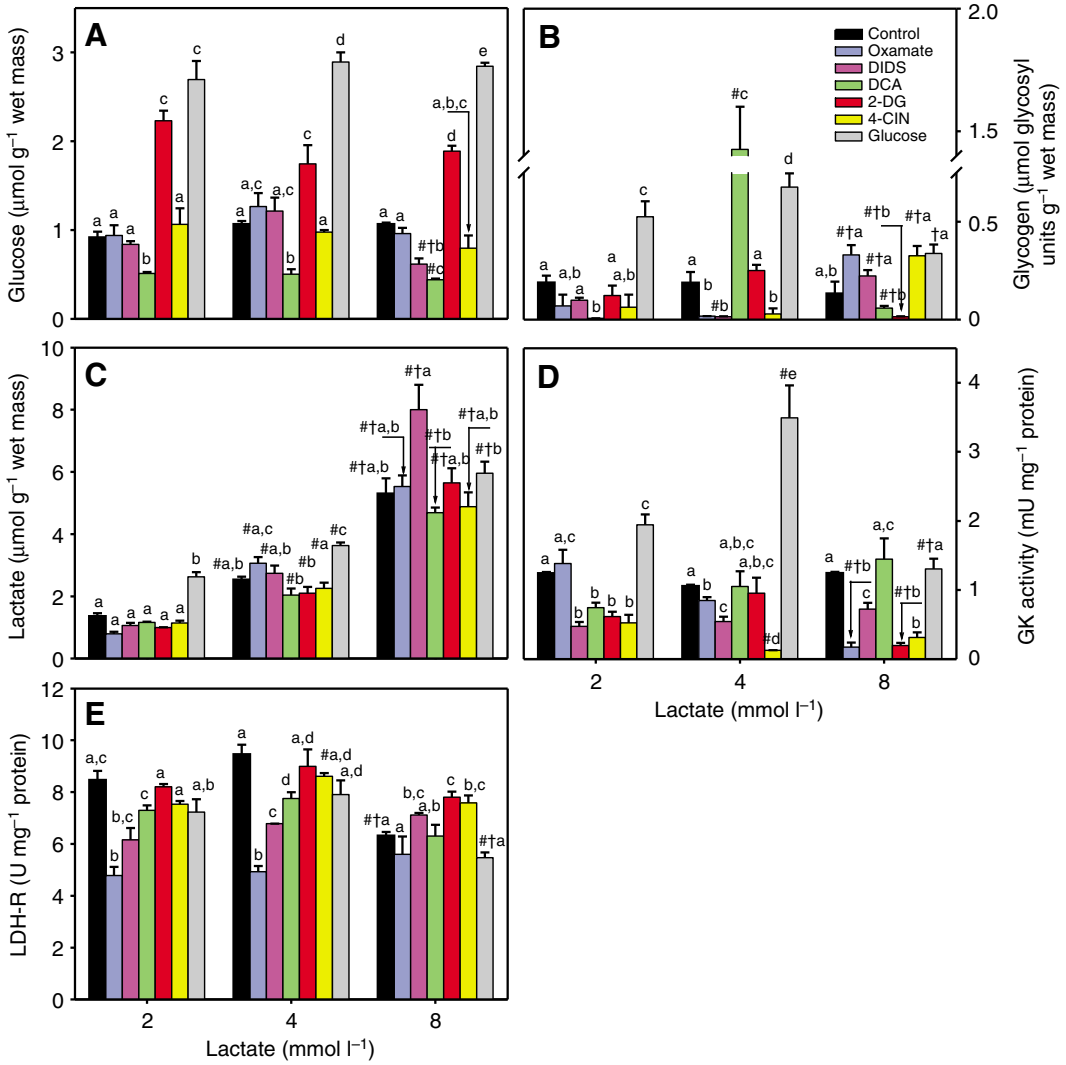

Fig. 6. Glucose (A), glycogen (B) and lactate (C) levels, and glucokinase (D) and lactate dehydrogenase reductase (E) activities in hypothalamus of rainbow trout incubated *in vitro* for 1 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} L-(+)-lactate alone (control) or with 50 mmol l^{-1} sodium oxamate, 1 mmol l^{-1} 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), 1 mmol l^{-1} sodium dichloroacetate (DCA), 10 mmol l^{-1} 2-deoxy-D-glucose (2-DG), 1 mmol l^{-1} α-cyano-4-hydroxy cinnamate (4-CIN) or 10 mmol l^{-1} D-glucose. Each value is the mean ± s.e.m. of ten (control) or three (treatments) independent experiments carried out with pools of tissues from three to four different fish (21 pools per experiment: seven treatments times three glucose concentrations). Different letters indicate significant differences (P<0.05) among treatments within each lactate concentration. #Significantly different from groups incubated with 2 mmol l^{-1} lactate within the same treatment (P<0.05); †significantly different from groups incubated with 4 mmol l^{-1} lactate within the same treatment (P<0.05).

in brain; and (4) the increase in lactate levels in the *in vitro* experiments when glucose (but not 2-DG) is added to the medium. However, *in vitro* assays showed that an elevation in lactate levels (from 2 to 8 mmol l^{-1}) in control conditions (no glucose) did not stimulate GK activity and did not raise glucose levels, which is in contrast to the ANLS hypothesis (Routh, 2002; Marty et al., 2007).

ICV treatment with glucose or lactate also elicited changes in liver function, reflecting a functional connection between detection of fuels within brain and production/release of fuels from liver, in a way similar to that proposed in mammals (Marty et al., 2007). Thus, ICV treatment with glucose induced enhanced glycogenolysis, and a fall of amino acid levels accompanied by decreased glucose 6-phosphate levels and increased capacity for glycolysis (DHAP and glyceraldehyde 3-phosphate levels). It seems that the presence of glucose in brain produces a signal indicative that no production and release of glucose from liver is needed to sustain plasma glucose levels. This is reflected by the fact that G6Pase activity actually decreased after glucose treatment and therefore less glucose is being exported into the plasma. When lactate was supplied by ICV infusion, changes were observed in liver metabolism that were different in some cases from those detected after glucose treatment such as the absence of changes in glycogen levels, the increase in glucose 6-phosphate levels, and the decrease in LDH-O activity. However, lactate treatment produced an inhibition of glucose release into the plasma through a fall in G6Pase activity as well as a decrease in amino acid levels, and glycolytic potential decreased. Altogether, it seems that ICV lactate treatment is causing a decrease in glucose release from liver and at the same time is inducing, in liver, a lower capacity for oxidation of lactate, based on the decrease observed in LDH-O activity. Therefore, ICV lactate treatment is inducing some sort of metabolic depression in liver by inhibiting catabolic pathways (PK and LDH-O activities), reflecting the energy signal (rise in lactate levels interpreted as a rise in glucose) coming from the brain. However, other metabolic pathways, such as lipid or amino acid metabolism, may be not affected by treatment. These results are comparable to those observed in mammals after similar ICV treatment (Lam et al., 2005), although in mammals glucose and lactate elicited quantitatively the same response, which is not the case in rainbow trout. Interestingly, changes in amino acid levels were similar, increasing in all brain regions after lactate treatment, whereas oxamate did produce the converse effect. This is probably indicative of lactate is being used as carbon skeletons for synthesis of amino acids within the brain (probably in astrocytes).

In BB, ICV treatment with glucose elicited a glucosensing response characterized by an increase in GK and a decrease in low K_m HK activities. This may suggest an increase in plasma insulin levels as part of the system trying to counteregulate the supposed increase in plasma glucose levels elicited by ICV treatment.

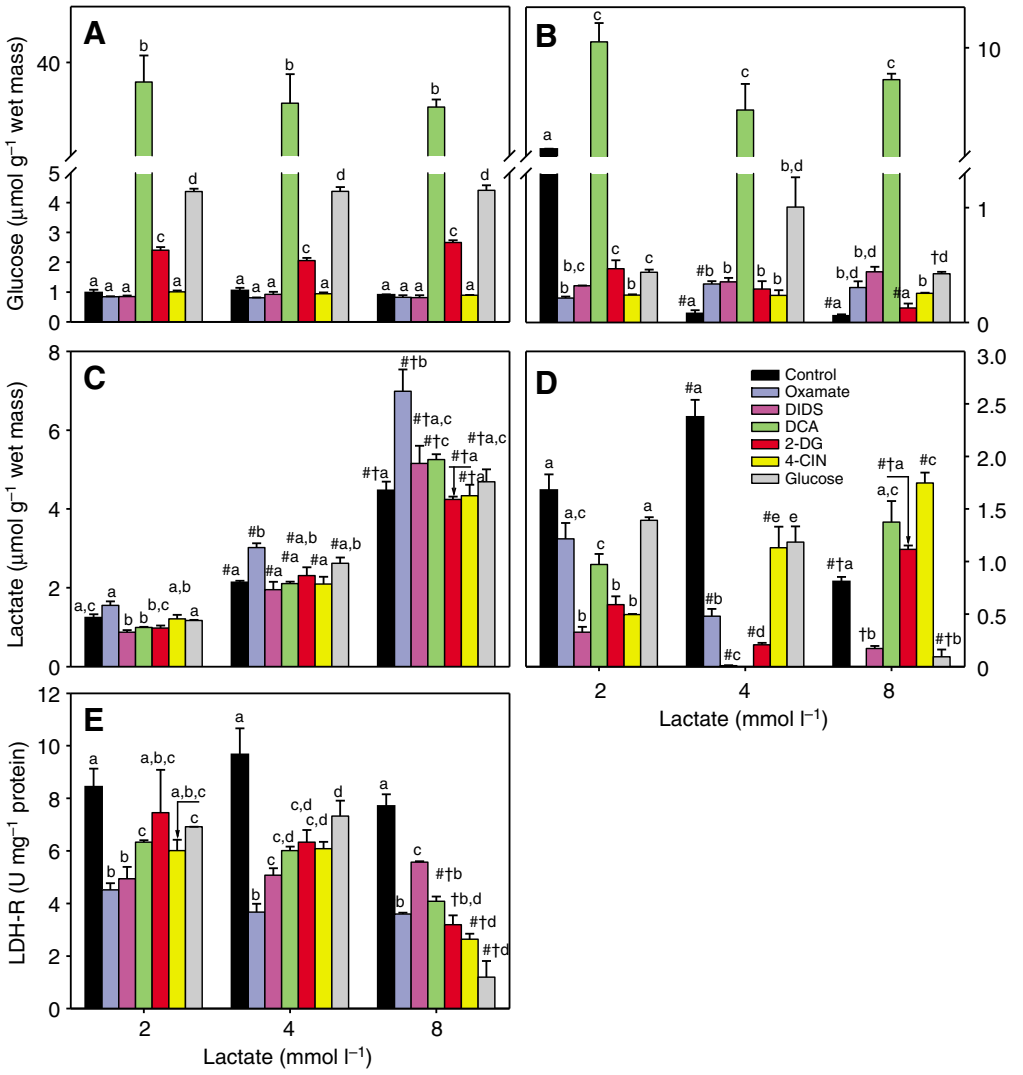

Fig. 7. Glucose (A), glycogen (B) and lactate (C) levels, and glucokinase (D) and lactate dehydrogenase reductase (E) activities in hindbrain of rainbow trout incubated *in vitro* for 1 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} L-(+)-lactate alone (control) or with 50 mmol l^{-1} sodium oxamate, 1 mmol l^{-1} 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), 1 mmol l^{-1} sodium dichloroacetate (DCA), 10 mmol l^{-1} 2-deoxy-D-glucose (2-DG), 1 mmol l^{-1} α-cyano-4-hydroxy cinnamate (4-CIN) or 10 mmol l^{-1} D-glucose. Each value is the mean ± s.e.m. of ten (control) or three (treatments) independent experiments carried out with pools of tissues from three to four different fish (21 pools per experiment: seven treatments times three glucose concentrations). Different letters indicate significant differences (P<0.05) among treatments within each lactate concentration. #Significantly different from groups incubated with 2 mmol l^{-1} lactate at the same treatment (P<0.05); †significantly different from groups incubated with 4 mmol l^{-1} lactate at the same treatment (P<0.05).

GK activity showed dramatic increases after lactate treatment in all regions assessed (except BB after ICV treatment). In IP treatments, lactate is probably being converted into glucose by the liver, resulting in higher plasma levels of glucose, and, as a result, an increase of glucose availability. A higher GK activity and expression in brain and BB under hyperglycemic conditions (but not in low K_m HK activity) is in agreement with previous experiments in rainbow trout (Polakof et al., 2007b). In ICV treatments, the increased GK activity produced by lactate could be due to increased glucose production *in situ*. However, ICV lactate treatment did not alter GK expression in any of the tissues assessed, in contrast to the increased GK expression observed in mammalian hindbrain (Vavaiya and Briski, 2007), suggesting that changes in activity were due to alosteric/covalent modification rather than to changes in enzyme expression. However, since gene expression assessed by conventional RT–PCR is not strictly quantitative, the mRNA expression data reported in Fig. 2 must be interpreted cautiously. In any case, glucose or lactate ICV treatments did produce the same effect on GK activity thus resulting in the same metabolic signal.

Lactate levels decreased in brain regions assessed after IP but not ICV treatments with lactate, which was not observed after oxamate treatment. Since plasma lactate levels also increased simultaneously, a higher entry of lactate in tissues through MCT could result in increased lactate levels. However, a higher portion of increased lactate levels in plasma is probably not directed towards the brain but to other tissues. Milligan and Girard (Milligan and Girard, 1993) described that the increased lactate levels observed in plasma of rainbow trout after exercise are mostly directed to white muscle and liver, which in the case of white muscle is logical considering the size of the tissue.

These results suggest that lactate metabolism in brain can participate in the regulation of glucose homeostasis through changes not only in brain regions but also in liver energy metabolism that are further reflected in changes in plasma levels of metabolites. By contrast, this connection is not apparently present in BB. This is in agreement with the finding that lactate does not induce insulin release in the mammalian pancreas (Ishihara et al., 1999; Kang et al., 2004) since in pancreatic cells LDH activity is very low (similar to that observed in BB in the present study). Furthermore, several findings also provide evidence for the presence in trout brain of an ANLS similar to that proposed in mammals, supporting previous data obtained in rainbow trout brain of the use of lactate as fuel in the absence of glucose (Soengas et al., 1998).

Evidence for a role of lactate in the activity of glucosensing tissues

Glucosensing neurons in mammals may sense substrates other than glucose, such as lactate (Himmi et al., 2001; Vavaiya and Briski,

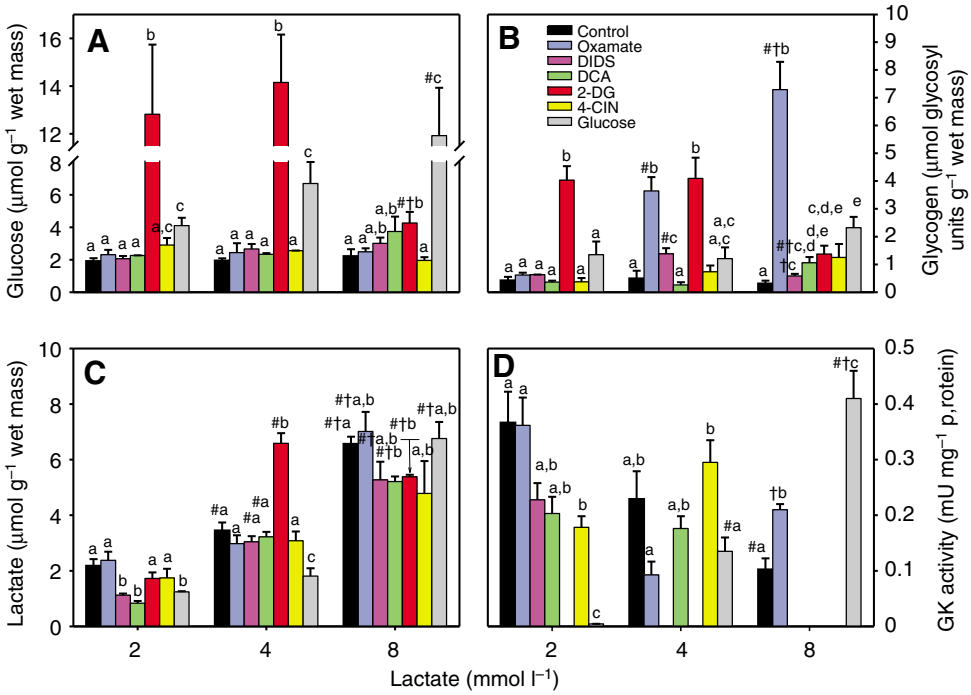

Fig. 8. Glucose (A), glycogen (B) and lactate (C) levels, and glucokinase (D) activities in Brockmann bodies of rainbow trout incubated *in vitro* for 1 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} L-(+)-lactate alone (control) or with 50 mmol l^{-1} sodium oxamate, 1 mmol l^{-1} 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), 1 mmol l^{-1} sodium dichloroacetate (DCA), 10 mmol l^{-1} 2-deoxy-D-glucose (2-DG), 1 mmol l^{-1} α-cyano-4-hydroxy cinnamate (4-CIN) or 10 mmol l^{-1} D-glucose. Each value is the mean ± s.e.m. of ten (control) or three (treatments) independent experiments carried out with pools of tissues from three to four different fish (21 pools per experiment: seven treatments times three glucose concentrations). Different letters indicate significant differences (P<0.05) among treatments within each lactate concentration; #significantly different from groups incubated with 2 mmol l^{-1} lactate at the same treatment (P<0.05); †significantly different from groups incubated with 4 mmol l^{-1} lactate at the same treatment (P<0.05).

2007). Moreover, in mammalian glucosensing systems glycolytic intermediates such as lactate mimic glucose effects (Yang et al., 1999; Schuit et al., 2001), and potentially use astrocyte-derived lactate as an alternate regulator of firing rate (Borg et al., 2003; Lam et al., 2005; Vavaiya and Briski, 2007). This is different from β-cells that are unable to use lactate to increase insulin secretion (Kang et al., 2004).

Since, in previous studies (Polakof et al., 2007a), we hypothesized that lactate could be also considered as a metabolic signal in brain glucosensing systems, we aimed to assess the role of lactate as a metabolic signal in glucosensing tissues such as hypothalamus, hindbrain and BB. In control fish, incubation with increased concentrations of lactate resulted in increased lactate levels in all brain regions assessed, as well as in BB, suggesting a fast transport of lactate. Blockade of neuronal lactate uptake by 4-CIN in mammals resulted in dramatic increases in extracellular lactate levels (Philis et al., 2001). By contrast, in the present study, the presence of 4-CIN did not alter levels of lactate in the brain regions assessed. A similar lack of action occurred with another MCT blocker, DIDS, suggesting either the absence of MCT in brain regions and/or the presence of different isoforms of MCT (Poole and Halestrap, 1993). Strikingly, the presence of oxamate in the media resulted in lactate levels higher than those of controls only in hindbrain but not in the other regions assessed. Treatment with glucose did not modify the trend observed in controls, although levels were higher than in controls in hypothalamus but lower in BB. The presence of an inhibitor of glucose use, such as 2-DG, despite minor changes does not generally alter lactate levels in any tissue assessed. The presence of DCA did not significantly alter lactate levels in tissues, except for the decrease observed in hindbrain and BB at low lactate concentrations, which is in agreement with the situation in mammals where DCA induced in brain decreased lactate levels (Itoh et al., 2003).

The presence of glucose in the culture media did not alter lactate levels and LDH activity (except in hypothalamus). This is comparable to the situation in mammals where glucose concentration did not alter utilization of lactate, even under hyperglycemia (Bliss and Sapolsky, 2001). The trend displayed by lactate levels in controls was different from that of LDH activity, which did not increase with lactate concentration in any tissue. Oxamate generally inhibited LDH activity in all tissues assessed, in agreement with its role in mammals (Wong et al., 1997). Strikingly, considering the lack of effect of oxamate treatment on lactate levels (see above), DIDS treatment decreased LDH activity in all tissues assessed, whereas 4-CIN was effective only in hindbrain. Therefore, in the tissues assessed, the presence of increased lactate concentrations in the media did not significantly alter LDH activity. This raises another question of whether or not parameters related to glucosensing capacity (mainly glucose levels and GK activity) can be activated by lactate, mimicking the effects of glucose, as described in mammals (Yang et al., 1999; Schuit et al., 2001).

Glucose and glycogen levels in controls were not affected by lactate concentration in the media in any tissue assessed, confirming that lactate is not being used as a gluconeogenic substrate, in agreement with the low gluconeogenic capacity already suggested for those tissues (Soengas et al., 2006). The presence of glucose in the media elevated both glucose and glycogen levels compared with controls in all tissues assessed. The presence of glucose *per se* is therefore increasing glucose levels and the levels of other related metabolites (i.e. glycogen) irrespective of lactate concentration. The different agents tested elicited very different responses on glucose and glycogen levels that can be summarized as increased levels of glycogen after oxamate, DIDS, 4-CIN, 2-DG and DCA treatments (the latter only in hindbrain) and no major changes in glucose levels, except for the increase after 2-DG treatment, in all tissues. The increased glycogenic capacity is probably the result of a blockade of lactate transport and oxidation in neurons. Under those conditions, the only pathway available for lactate use would be synthesis of glucose 6-phosphate through gluconeogenesis, to store as glycogen. In a similar way, the inhibition of glucose use by 2-DG clearly resulted in increased glucose levels that cannot be further metabolized.

GK activity in controls did not differ significantly at the different lactate concentrations assessed, except in hindbrain in which there

was increased activity at 4 mmol l^{-1} lactate. It is interesting that in mammals increased lactate concentration has been shown to inhibit glucose utilization in brain regions under normo- and hypo- but not hyperglycemic conditions (Bliss and Sapolsky, 2001) in a comparable situation to that herein reported. DIDS or 4-CIN treatment decreased GK activity at all concentrations of lactate assessed in all tissues, which is again striking considering that those molecules did not affect lactate levels (see above). Since GK is not inhibited by glucose 6-phosphate and therefore no product inhibition can occur, this probably indicates that at least part of the glucose being phosphorylated by GK resulted from lactate synthesis. DCA treatment decreased GK activity in all tissues, which could be the result of the effect of DCA on glycogen metabolism (stimulating glycogen storage) in a way similar to that observed in mammalian liver (Kato-Weinstein et al., 1998). Comparing this response with that previously observed when the same tissues were incubated with glucose alone (Polakof et al., 2007a) this suggests that the presence of lactate is altering the action of glucose on GK activity possibly because it is replacing at least part of the glucose to be used as fuel.

Altogether, it seems that lactate is not able to induce similar metabolic changes to those elicited by glucose in the glucosensing regions assessed *in vitro*, and therefore does not support the hypothesis of lactate mimicking glucose effects in fish glucosensing regions (Polakof et al., 2007a), in contrast to that occurring in mammals (Yang et al., 1999; Schuit et al., 2001). However, several lines of evidence obtained *in vivo* such as the similar increase in GK activity after either glucose or lactate ICV treatments may suggest an involvement of lactate in the activity of glucosensing tissues, in a way similar to that proposed in mammals (Song and Routh, 2005).

In summary, data obtained in the present study provide several pieces of indirect evidence allowing us to suggest the presence in trout brain of an astrocyte–neuron lactate shuttle similar to that proposed in mammals (Routh, 2002; Marty et al., 2007), reinforcing previous data obtained in rainbow trout brain of the use of lactate as fuel (Soengas et al., 1998). However, since other findings were contradictory, additional studies should be carried out to obtain direct evidence for an ANLS, such as pharmacological disruption of metabolic coupling between neurons and astrocytes followed by histological analysis of neural activation. Furthermore, lactate metabolism is also apparently involved in glucose homeostasis through changes in plasma glucose levels and glucose production in liver. Finally, evidence obtained in the present study *in vitro* do not support our previous hypothesis regarding a mimicking effect of lactate (compared with glucose) in fish glucosensing regions (Polakof et al., 2007a). However, evidence obtained *in vivo* are compatible with such a role, making necessary more studies to clearly assess the role of lactate in glucosensing tissues.

LIST OF ABBREVIATIONS

2-DG	2-deoxy-D-glucose
4-CIN	α-cyano-4-hydroxy cinnamate
ANLS	astrocyte-neuron lactate shuttle
BB	Brockmann bodies
DCA	dichloroacetate
DHAP	di-hydroxiacetone phosphate
DIDS	4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate
G6Pase	glucose 6-phosphatase (EC. 3.1.3.9.)
GK	hexokinase IV or glucokinase (EC. 2.7.1.2.)
GSase	glycogen synthase (EC. 2.4.1.11.)
HK	hexokinase I (EC. 2.7.1.11.)
ICV	intracerebroventricular
IP	intraperitoneal
LDH-O	lactate dehydrogenase oxidase (EC. 1.1.1.27.)
LDH-R	lactate dehydrogenase reductase (EC. 1.1.1.27.)
MCT	monocarboxylate transporter
PK	pyruvate kinase (EC. 2.7.1.40.)

This study was supported by research grants from Ministerio de Educación y Ciencia and European Fund for Regional Development (AGL2004-08137-c04-03/ACU and AGL2007-65744-C03-01/ACU), and Xunta de Galicia (PGIDT05PXIC31202PN). S.P. was the recipient of a predoctoral fellowship from the Xunta de Galicia (Program Maria Barbeito).

REFERENCES

Bliss, T. M. and Sapolsky, R. M. (2001). Interactions among glucose, lactate and adenosine regulate energy substrate utilization in hippocampal cultures. *Brain Res.* **899**, 134-141.

Borg, M. A., Tamborlane, W. V., Shulman, G. I. and Sherwin, R. S. (2003). Local lactate prefusion of the ventromedial hypothalamus suppresses hypoglycemic counterregulation. *Diabetes* **52**, 663-666.

Brown, A. M., Wender, R. and Ransom, B. R. (2001). Metabolic substrates other than glucose support axon function in central white matter. *J. Neurosci. Res.* **66**, 839-843.

Cassady, C. J., Philis, J. W. and O'Regan, M. H. (2001). Further studies on the effects of topical lactate on amino acid efflux from the ischemic rat cortex. *Brain Res.* **901**, 30-37.

Dienel, G. A. and Hertz, L. (2001). Glucose and lactate metabolism during brain activation. *J. Neurosci. Res.* **66**, 824-838.

Himmi, T., Perrin, J., Dallaporta, M. and Orsini, J.-C. (2001). Effects of lactate on glucose-sensing neurons in the solitary tract nucleus. *Physiol. Behav.* **74**, 391-397.

Ishihara, H., Wang, H., Drewes, L. R. and Wollheim, C. B. (1999). Overexpression of monocarboxylate transporter and lactate dehydrogenase alters insulin secretory responses to pyruvate and lactate in beta cells. *J. Clin. Invest.* **104**, 1621-1629.

Itoh, Y., Esaki, T., Shimoji, K., Cook, M., Law, M. J., Kaufman, E. and Sokoloff, L. (2003). Dichloroacetate effects on glucose and lactate oxidation by neurons and astroglia *in vitro* and on glucose utilization by brain *in vivo*. *Proc. Natl. Acad. Sci. USA* **100**, 4879-4884.

Kang, L., Routh, V. H., Kuzhikandathil, E. V., Gaspers, L. D. and Levin, B. E. (2004). Physiological and molecular characteristics of rat hypothalamic ventromedial nucleus glucosensing neurons. *Diabetes* **53**, 549-559.

Kato-Weinstein, J., Lingohr, M. K., Orner, G. A., Thrall, B. D. and Bull, R. J. (1998). Effects of dichloroacetate on glycogen metabolism in B6C3F1 mice. *Toxicology* **130**, 141-154.

Keppler, D. and Decker, K. (1974). Glycogen. Determination with amyloglucosidase. In *Methods of Enzymatic Analysis* (ed. H. U. Bergmeyer), pp. 1127-1131. New York: Academic Press.

Kusakabe, M., Nakamura, I., Evans, J., Swanson, P. and Young, G. (2006). Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. *J. Endocrinol.* **189**, 541-554.

Lam, T. K. T., Gutierrez-Juarez, R., Pocai, A. and Rossetti, L. (2005). Regulation of blood glucose by hypothalamic pyruvate metabolism. *Science* **309**, 943-947.

Marty, N., Dallaporta, M. and Thorens, B. (2007). Brain glucose sensing, counterregulation, and energy homeostasis. *Physiology* **22**, 241-251.

Matschinsky, F. M., Magnuson, M. A., Zelent, D., Jetton, T. L., Doliba, N., Han, Y., Taub, R. and Grimsby, J. (2006). The network of glucokinase-expressing cells in glucose homeostasis and the potential of glucokinase activators for diabetes therapy. *Diabetes* **55**, 1-12.

Milligan, C. L. and Girard, S. S. (1993). Lactate metabolism in rainbow trout. *J. Exp. Biol.* **180**, 175-193.

Moore, S. (1968). Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the nynhidrin reaction. *J. Biol. Chem.* **1242**, 6281-6283.

Panserat, S., Médale, F., Blin, C., Brèque, J., Vachot, C., Plagnes-Juan, E., Gomes, E., Krishnamoorthy, R. and Kaushik, S. (2000). Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilhead seabream, and common carp. *Am. J. Physiol.* **278**, R1164-R1170.

Pellerin, L. (2003). Lactate as a pivotal element in neuron-glia metabolic cooperation. *Neurochem. Int.* **43**, 331-338.

Pellerin, L. and Magistretti, P. J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc. Natl. Acad. Sci. USA* **91**, 10625-10629.

Philis, J. W., Ren, J. and O'Regan, M. H. (2001). Studies on the effects of lactate transport inhibition, pyruvate, glucose and glutamine on amino acid, lactate and glucose release from the ischemic rat cerebral cortex. *J. Neurochem.* **76**, 247-257.

Polakof, S,., Míguez, J. M. and Soengas, J. L. (2007a). *In vitro* evidences for glucosensing capacity and mechanisms in hypothalamus, hindbrain and Brockmann bodies of rainbow trout. *Am. J. Physiol.* **293**, R1410-R1420.

Polakof, S., Míguez, J. M., Moon, T. W. and Soengas, J. L. (2007b). Evidence for the presence of a glucosensor in hypothalamus, hindbrain, and Brockmann bodies of rainbow trout. *Am. J. Physiol.* **292**, R1657-R1666.

Poole, R. C. and Halestrap, A. P. (1993). Transport of lactate and other monocarboxylates across mammalian plasma membranes. *Am. J. Physiol.* **264**, C761-C782.

Routh, V. H. (2002). Glucose-sensing neurons: are they physiologically relevant? *Physiol. Behav.* **76**, 403-413.

Sangiao-Alvarellos, S., Bouça, P., Míguez, J. M. and Soengas, J. L. (2003). Intracerebroventricular injections of noradrenaline affect brain energy metabolism of rainbow trout. *Physiol. Biochem. Zool.* **76**, 663-671.

Sangiao-Alvarellos, S., Lapido, M., Míguez, J. M. and Soengas, J. L. (2004). Effects of central administration of arginine vasotocin on monoaminergic neurotransmitters and energy metabolism of rainbow trout brain. *J. Fish Biol.* **64**, 1313-1329.

Schuit, F. C., Huypens, P., Heimberg, H. and Pipeleers, D. G. (2001). Glucose sensing in pancreatic β-cells. A model for the study of other glucose-regulated cells in gut, pancreas, and hypothalamus. *Diabetes* **50**, 1-11.

Smith, P. K. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.

Soengas, J. L. and Moon, T. W. (1995). Uptake and metabolism of glucose, alanine and lactate by red blood cells of the American eel *Anguilla rostrata*. *J. Exp. Biol.* **198**, 877-888.

Soengas, J. L., Strong, E. F. and Andrés, M. D. (1998). Glucose, lactate, and β-hydroxybutyrate utilization by rainbow trout brain: changes during food deprivation. *Physiol. Zool.* **71**, 285-293.

Soengas, J. L., Polakof, S., Chen, X., Sangiao-Alvarellos, S. and Moon, T. W. (2006). Glucokinase and hexokinase expression and activities in rainbow trout tissues: changes with food deprivation and refeeding. *Am. J. Physiol.* **291**, R810-R821.

Song, Z. and Routh, V. H. (2005). Differential effects of glucose and lactate on glucosensing neurons in the ventromedial hypothalamic nucleus. *Diabetes* **54**, 15-22.

Song, Z. and Routh, V. H. (2006). Recurrent hypoglycemia reduces the glucose sensitivity of glucose-inhibited neurons in the ventromedial hypothalamus nucleus. *Am. J. Physiol.* **291**, R1283-R1287.

Vavaiya, K. V. and Briski, K. P. (2007). Caudal hindbrain lactate infusion alters glucokinase, SUR1, and neuronal substrate fuel transporter gene expression in the dorsal vagal complex, lateral hypothalamic area, and ventromedial nucleus hypothalamus of hypoglycemic male rats. *Brain Res.* **1176**, 62-70.

Wender, R., Brown, A. M., Fern, R., Swanson, R. A., Farrell, K. and Ransom, B. R. (2000). Astrocytic glycogen influences axon function and survival during glucose deprivation in central white matter. *J. Neurosci.* **200**, 6804-6810.

Wong, C., Rodríguez-Páez, L., Nogueda, B., Pérez, A. and Baeza, I. (1997). Selective inhibition of the sperm-specific lactate dehydrogenase isozyme-C4 by *N*-isopropyl oxamate. *Biochim. Biophys. Acta* **1343**, 16-22.

Yang, X.-J., Kow, L.-M., Funabashi, T. and Mobbs, C. V. (1999). Hypothalamic glucose sensor, similarities to and differences from pancreatic β-cell mechanisms. *Diabetes* **48**, 1763-1772.

Youson, J. H., Al-Mahrouki, A. A., Anemiya, Y., Graham, L. C., Montpetit, C. J. and Irwin, D. M. (2006). The fish endocrine pancreas: research update and future directions in ontogenic and phylogenetic development. *Gen. Comp. Endocrinol.* **148**, 105-115.