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RESEARCH ARTICLE

CRF treatment induces a readjustment in glucosensing capacity in the hypothalamus and hindbrain of rainbow trout

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SUMMARY

Stress conditions induced in rainbow trout a readjustment in the glucosensing response of the hypothalamus and hindbrain such that those sensors did not respond properly to changes in glucose levels, as demonstrated in previous studies. To evaluate the hypothesis that corticotropin-releasing factor (CRF) could be involved in that response, we have incubated the hypothalamus and hindbrain of rainbow trout at different glucose concentrations in the presence of different concentrations of CRF. Under those conditions, we evaluated whether parameters related to glucosensing [the levels of glucose, glycogen and glucose 6-phosphate, the activities of glucokinase (GK), glycogen synthase (GSase) and pyruvate kinase (PK), and mRNA abundance of transcripts for GK, Glut2, Kir.6-like and sulfonylurea receptor (SUR)-like] are modified in the presence of CRF in a way comparable to that observed under stress conditions. We obtained evidence allowing us to suggest that CRF could be involved in the interaction between stress and glucosensing as CRF treatment of the hypothalamus and hindbrain *in vitro* induced a readjustment in glucosensing parameters similar to that previously observed under stress conditions *in vivo*. We had also previously demonstrated that stress elicits alterations in food intake in parallel with the readjustment of glucosensing systems. Here, we provide evidence that the mRNA abundance of several of the neuropeptides involved in the regulation of food intake, such as neuropeptide Y (NPY) or cocaine- and amphetamine-regulated transcript (CART), is affected by CRF treatment, in such a way that their expression does not respond to changes in glucose levels in the same way as controls, allowing us to suggest that the food intake response that is integrated by changes in those peptides and known to be reduced by stress could be also mediated by CRF action in glucosensing areas.

Key words: trout, glucosensing, CRF, hypothalamus, hindbrain.

INTRODUCTION

In mammals, hypothalamic neurons producing corticotropin-releasing factor (CRF) play a crucial role in the control of the hypothalamic–pituitary–adrenal axis and in the coordination of the autonomic and behavioral responses to stress (Cheng et al., 2007). Moreover, members of the CRF family of peptides are known to produce a global negative energy profile by reducing food intake and increasing energy expenditure, and the effects on energy expenditure are thought to depend on the activation of thermogenesis mediated by corticotropin-releasing factor receptor type 1 (CRF-R-1, also known as CRHR1) (Doyon et al., 2007). Moreover, CRF has been suggested to change the sensitivity of hypothalamic glucosensing neurons to glucose (McCrimmon et al., 2006) and to regulate the glucosensing machinery (Evans et al., 2004).

In fish, CRF plays a key role in regulating and integrating the neuroendocrine, autonomic, immune, and behavioural responses to stressors (Bernier, 2006) and is a key factor involved in the control of food intake (Volkoff et al., 2009), although the CRF receptor subtypes mediating the anorectic effects of CRF in fish have yet to be identified (Bernier, 2006). There are no studies available regarding the possible effect of CRF on the glucosensing capacity in fish.

In previous *in vivo* studies in rainbow trout, we demonstrated the existence of glucosensor systems in hypothalamus, hindbrain and Brockmann bodies based on glucokinase (GCK, hereafter referred to as GK) that are similar in their functioning to those in pancreatic β-cells – requiring glucose uptake by the low-affinity glucose transporter type 2 (SLC2A2, hereafter referred to as GLUT2), glucose phosphorylation by GK and the subsequent metabolism of glucose by means of glycolysis to increase the intracellular ATP:ADP ratio. This leads to the closure of K_{ATP}-channels, membrane depolarization and the entry of Ca^{2+}, which triggers increased neuronal activity and neurotransmitter secretion in brain regions and insulin release in pancreatic β-cells (reviewed by Polakof et al., 2011). Those glucosensing systems in the hypothalamus and hindbrain are activated when glucose levels increase at the same time that food intake decreases; conversely, when glucose levels decrease, the glucosensors are inactivated and food intake increases (Polakof et al., 2007a; Polakof et al., 2008a; Polakof et al., 2008b). Moreover, those glucosensing mechanisms in hypothalamus, hindbrain and Brockmann bodies are readjusted under stress conditions, such as those associated with high stocking density, resulting in an inability of the system to respond to changes in levels of circulating glucose (Conde-Sieira et al., 2010a). Furthermore, the same stress conditions elicit changes in the mRNA abundance of several peptides related to the control of food intake, including CRF (Conde-Sieira et al., 2010b), that could be related to the well-known anorectic effect of stress in fish (Wendelaar Bonga, 1997; Bernier,

2006). Accordingly, we hypothesized that CRF could be a candidate for mediating the changes in the sensitivity or machinery of glucosensing mechanisms in fish and their possible effects in food intake.

Therefore, to evaluate such a hypothesis, we incubated the hypothalamus and hindbrain of rainbow trout at different glucose concentrations (indicative of hypo-, normo- and hyper-glycemic conditions in rainbow trout) in the presence of different concentrations of CRF to assess whether the responses to changes in glucose levels of factors related to glucosensing are modified in the presence of CRF in a way comparable to that observed under stress conditions. Under those conditions, we have assessed several factors involved in the glucosensing response, such as the levels of metabolites (glycogen, glucose and glucose 6-phosphate), glucose transport capacity (Glut2 mRNA abundance), glucose phosphorylation capacity (GK activity and mRNA abundance), glycolytic potential [pyruvate kinase (PK) activity], glycogen metabolism [glycogen synthase (GSase) activity] and potential of the ATP-dependent potassium channel (K^+_{ATP}) [mRNA abundance for the potassium inward rectifier Kir.6-like and sulfonylurea receptor (SUR)-like].

MATERIALS AND METHODS

Fish

Immature rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (Soutorredondo, Spain). Fish were maintained at a normal stocking density (10 kg m^{-3}) for 1 month in 100 l tanks under laboratory conditions and a 12 h:12 h light:dark photoperiod in dechlorinated tap water at 15°C. Fish mass was 57±5 g. Fish were fed once daily (10:00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Segovia, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat and 11.5% ash; 20.2 MJ kg^{-1} of feed). The experiments described comply with the guidelines of the European Union Council (2010/63/EU) and of the Spanish Government (RD 1201/2005) for the use of animals in research.

Experimental protocol

On the morning of an experiment, fish that were fasted for 24 h (to ensure that basal levels of metabolic hormones were achieved) were dip-netted, anaesthetized with MS-222 (50 mg l^{-1}) buffered to pH 7.4 with sodium bicarbonate, weighed and euthanized by decapitation. The hypothalamus and hindbrain were dissected as described previously (Polakof et al., 2007b). Tissues were rinsed by immersion in 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 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 a 0.5% CO_2 and 99.5% O_2 mixture. In order to have sufficient mass, tissues were pooled from different fish, resulting in pools of three to four tissue samples (hypothalamus or hindbrain). On each pool, tissue was finely sliced and mixed and then placed in 48-well culture plates (25 mg of tissue in 250 µl of modified Hanks' medium per well).

For each experiment, freshly obtained tissues were incubated as described previously (Polakof et al., 2007b) in 48-well culture plates at 15°C for 1 h with 250 µl of modified Hanks' medium per well containing 25 mg of tissue that was gassed with a 0.5% CO_2 and 99.5% O_2 mixture. In control wells, medium contained three different concentrations of D-glucose: 2, 4 and 8 mmol l^{-1} (indicative of hypo-, normo- and hyperglycaemic conditions in rainbow trout). Treated wells contained the same glucose concentrations and different concentrations (1, 10 or 100 nmol l^{-1}) of CRF (from sheep, Sigma Chemical Co., St Louis, MO, USA). CRF of mammalian origin (sheep, human, rat) has been used in other *in vivo* studies performed in fish (De Pedro et al., 1993; De Pedro et al., 1998; Bernier and Peter, 2001). The concentrations of CRF were selected according to previous *in vitro* studies performed in fish (Seale et al., 2002). After incubation for 1 h, tissues were quickly removed, freeze-clamped in liquid nitrogen and stored at –80°C until assayed.

For each experiment, three different sets of tissue pools (N=12; four treatments at three glucose concentrations) were used. The first set was used to assess enzyme activities (GK, GSase and PK). The second set was assayed for the measurement of tissue metabolites (glucose, glycogen and glucose 6-phosphate levels). Finally, the third set was used for quantification of the mRNA abundance of factors related to glucosensing capacity (GK, Glut2, Kir.6-like and SUR-like) and peptides related to the control of food intake [neuropeptide Y (NPY), pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART)]. The same procedure was performed in four independent replicates per set (N=4 experiments).

Assessment of metabolite levels and enzyme activities

The pieces of tissue used for the assessment of metabolite levels were homogenized immediately by ultrasonic disruption with 7.5 volumes of ice-cooled 6% perchloric acid and neutralized (using 1 mol l^{-1} potassium bicarbonate). The homogenate was centrifuged, and the resulting supernatant was immediately assayed. Tissue 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, Spain). Glucose 6-phosphate levels were assessed as described previously (Polakof et al., 2008a; Polakof et al., 2008b).

Tissue pieces used to assess enzyme activities were homogenized by ultrasonic disruption with nine 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 (P-2714; Sigma Chemical Co.). The homogenate was centrifuged and the supernatant used immediately for enzyme assays. Enzyme activities were determined using the 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 homogenates (10–15 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 (3–15 min). Enzyme activities were normalized to a value per milligram of protein. Protein was assayed in triplicate in homogenates using microplates according to the Bradford method, with bovine serum albumin (Sigma Chemical Co.) as a standard. Enzymatic analyses were all performed at maximum rates, with the reaction mixtures set up in preliminary tests to render optimal activities. The activities of GK, GSase and PK were estimated as described previously (Polakof et al., 2007a; Polakof et al., 2007b; Polakof et al., 2008a; Polakof et al., 2008b).

Gene expression analysis by real-time quantitative RT-PCR

Total RNA was extracted from rainbow trout hypothalamus and hindbrain using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RQ1-DNAse (Promega, Madison, WI, USA). Total

RNA (2 μg) was reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen) and random hexaprimers (Invitrogen). Gene expression levels were determined by real-time quantitative RT-PCR (q-PCR) using the iCycler iQ (Bio-Rad, Hercules, CA, USA). Analyses were performed on 1 μl of the diluted cDNA (with a further dilution by 1:4 for POMC) using the Maxima SYBR Green qPCR Mastermix (Fermentas, Vilnius, Lithuania), in a total PCR reaction volume of 15 μl, containing 50–500 nmol l^{-1} of each primer. The abundance of mRNAs for factors related to glucosensing capacity (GK, Glut2, Kir.6-like and SUR-like) and for peptides related to the control of food intake (NPY, POMC and CART) was assessed by qPCR as described previously for rainbow trout (Polakof et al., 2008a; Polakof et al., 2008b; Polakof et al., 2008c; Conde-Sieira et al., 2010b).

Relative quantification of the target gene transcripts was performed using β-actin gene expression as a reference, which was stably expressed in this experiment. Thermal cycling was initiated with incubation at 95°C for 15 min using hot-start iTaq DNA polymerase activation; 40 steps of PCR were performed, each one consisting of heating at 95°C for 15 s for denaturing, and specific annealing for 30 s and extension at 72°C for 30 s. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C s^{-1} from 55 to 95°C) to ensure that only one fragment was amplified. Each sample was analysed in triplicate. All the replicates of each sample were located in the same plate for each gene in order to allow comparisons. We included in all the plates the standard curve (in triplicate), and blanks for DNA, PCR and retrotranscription (in duplicate). Only efficiency values between 85 and 100% were accepted (the R^2 value for all the genes assessed was always higher than 0.985). Relative quantification of the target gene transcript with the β-actin reference gene transcript was made by following the method of Pfaffl (Pfaffl, 2001).

Statistics

Comparisons among groups were performed using two-way ANOVA tests, with glucose concentration (2, 4 and 8 mmol l^{-1}) and treatment (CRF concentrations) as the main factors. When necessary, 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

The content of metabolites in the hypothalamus is shown in Fig. 1. The levels of glucose (Fig. 1A), glycogen (Fig. 1B) and glucose 6-phosphate (Fig. 1C) increased in controls in parallel with the increase of glucose concentration in the medium, whereas, in CRF-treated groups, this trend was maintained and also enhanced for glucose levels (Fig. 1A). The increase in hypothalamic glycogen content was further enhanced by CRF supplementation, especially in those samples exposed to 4 mmol l^{-1} glucose (Fig. 1B). In addition, the increase of hypothalamic glucose 6-phosphate levels was enhanced by CRF, with independence of the CRF dose in samples incubated with 2 mmol l^{-1} glucose. By contrast, only 10 or 100 nmol l^{-1} CRF further enhanced the increase when hypothalamus was incubated with concentrations of 4 mmol l^{-1} or 8 mmol l^{-1} glucose (Fig. 1C).

Enzyme activities in the hypothalamus are shown in Fig. 2. GK (Fig. 2A) and GSase (Fig. 2B) activities increased in controls in parallel to the glucose concentration in the medium, whereas no change was noticed for PK activity (Fig. 2C). While 1 and 10 nmol l^{-1}

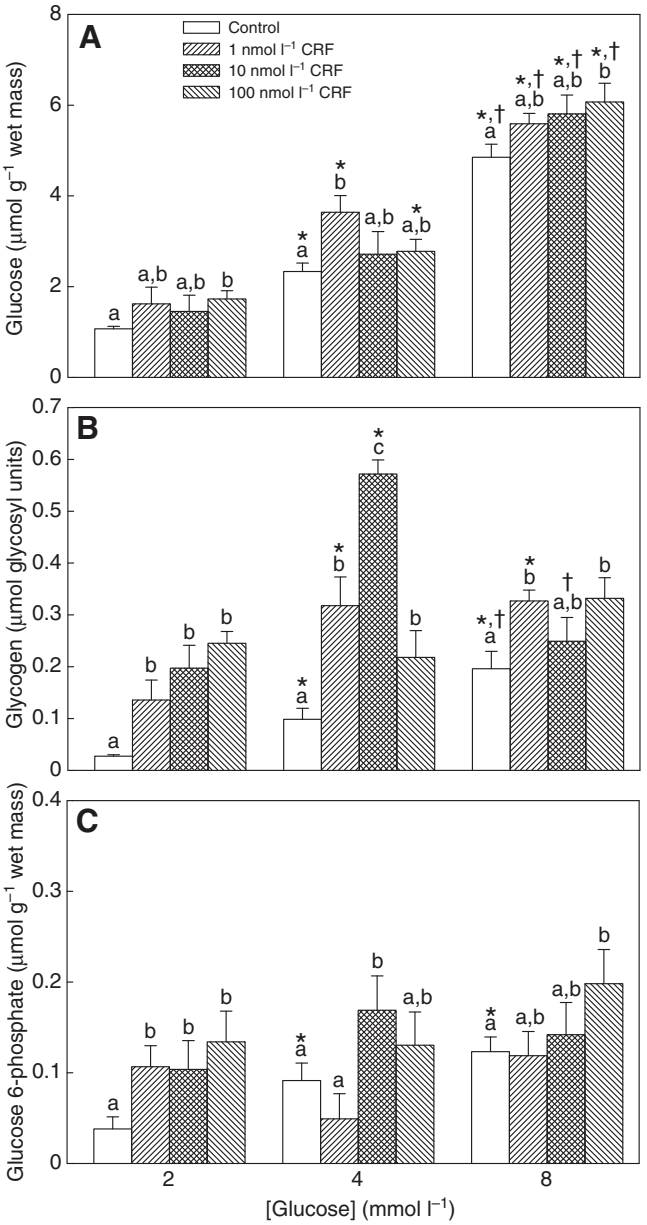

Fig. 1. Glucose (A), glycogen (B) and glucose 6-phosphate (C) levels in hypothalamus of rainbow trout incubated *in vitro* for 1 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} D-glucose alone (control) or containing 1, 10 or 100 nmol l^{-1} corticotropin-releasing factor (CRF). Each value is the mean + s.e.m. of four independent experiments performed with pools of hypothalami from three or four different fish. Different letters indicate significant differences ($P<0.05$) among treatments within each glucose concentration. *Significantly different from groups incubated with 2 mmol l^{-1} glucose at the same treatment ($P<0.05$); †significantly different from groups incubated with 4 mmol l^{-1} glucose at the same treatment ($P<0.05$).

CRF treatment enhanced the increase in GK activity at 4 and 8 mmol l^{-1} glucose concentrations, such an effect was not observed at the highest CRF concentration (100 nmol l^{-1}) assayed (Fig. 2A). When hypothalamus was cultured with low (2 mmol l^{-1}) and medium (4 mmol l^{-1}) concentrations of glucose, the increase in GSase activity was elevated by CRF addition in a non-dose-dependent way; by contrast, CRF addition had no effect when samples were cultured

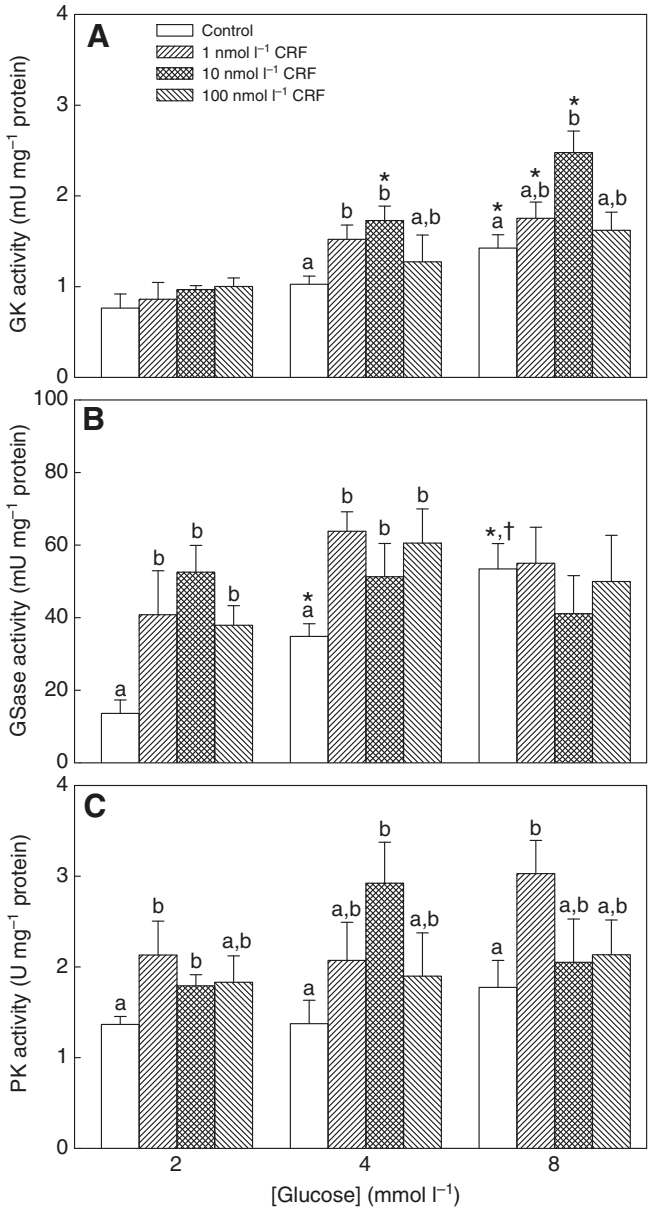

Fig. 2. Activities of glucokinase (GK, A), glycogen synthase (GSase, B) and pyruvate kinase (PK, C) in hypothalamus of rainbow trout incubated *in vitro* for 1 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} D-glucose alone (control) or containing 1, 10 or 100 nmol l^{-1} CRF. Each value is the mean + s.e.m. of four independent experiments performed with pools of hypothalami from three or four different fish. For details of the letters and symbols, see the legend to Fig. 1.

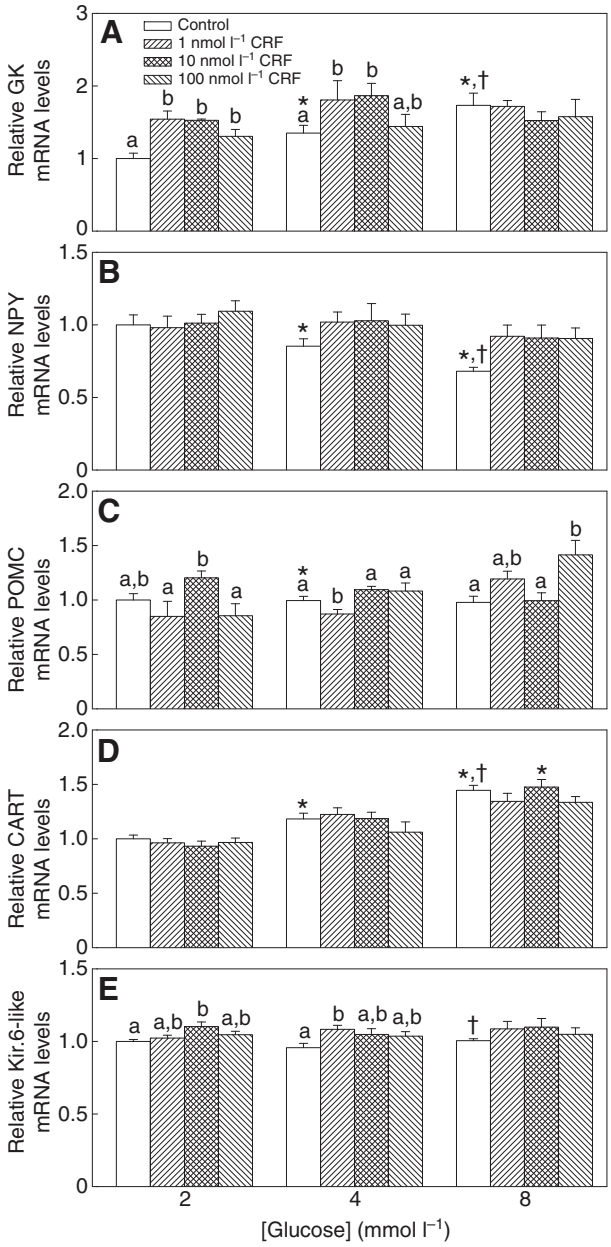

Fig. 3. The levels of mRNA encoding GK (A), neuropeptide Y (NPY, B), pro-opiomelanocortin (POMC, C), cocaine- and amphetamine-regulated transcript (CART, D) and Kir.6-like (E) in the hypothalamus of rainbow trout incubated *in vitro* for 1 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} D-glucose alone (control) or containing 1, 10 or 100 nmol l^{-1} CRF. Differences in mRNA levels between treatments are presented as an x-fold-induction with respect to a 2 mmol l^{-1} glucose control. Expression results were normalized by β-actin mRNA levels (no variation). Each value is the mean + s.e.m. of four independent experiments performed with pools of hypothalami from three or four different fish. For details of the letters and symbols, see the legend to Fig. 1.

with a high (8 mmol l^{-1}) concentration of glucose (Fig. 2B). PK activity was not affected by a medium glucose concentration in controls (Fig. 2C); however, the CRF effect was dependent on a medium glucose concentration as the lowest CRF dose (1 nmol l^{-1}) increased PK activity in those samples incubated with low and high glucose concentrations, and 10 nmol l^{-1} CRF showed the same effect at 2 and 4 mmol l^{-1} glucose concentrations, whereas the highest CRF dose (100 nmol l^{-1}) had no effect at any of the CRF doses assayed.

The hypothalamic mRNA abundance of factors related to glucosensing and for peptides related to the control of food intake is shown in Fig. 3. No changes were observed for the mRNA abundance of Glut2 and SUR-like (data not shown). The abundance of mRNA encoding GK (Fig. 3A) and CART (Fig. 3D) increased in a glucose-concentration-dependent way in controls. In general, CRF addition further enhanced GK mRNA abundance in hypothalamus cultured with 2 and 4 mmol l^{-1} glucose, but it had no effect when samples were cultured in 8 mmol l^{-1} glucose (Fig. 3A). Treatment with 10 nmol l^{-1} CRF increased CART mRNA abundance

only at 8 mmol l^{-1} glucose (Fig. 3D). The expression of NPY decreased with increasing glucose concentrations in controls, and CRF addition abolished this effect in a non-concentration-dependent way (Fig. 3B). The concentration of glucose had no effect on the abundance of mRNA for POMC (Fig. 3C), and the abundance of POMC mRNA decreased after adding 1 nmol l^{-1} CRF to those samples incubated in the presence of 4 mmol l^{-1} glucose but increased after adding the highest CRF dose (100 nmol l^{-1}) at 8 mmol l^{-1} glucose (Fig. 3C). By contrast, CRF treatment increased the abundance of Kir.6-like mRNA at 2 mmol l^{-1} glucose (10 nmol l^{-1} CRF) and 4 mmol l^{-1} glucose (1 nmol l^{-1} CRF) (Fig. 3E).

The content of hindbrain metabolites is shown in Fig. 4. The content of glucose (Fig. 4A), glycogen (Fig. 4B) and glucose 6-phosphate (Fig. 4C) increased in controls in parallel with the increase of glucose concentration in the medium. In contrast to that observed in hypothalamus, only addition of 10 and 100 nmol l^{-1} CRF partially abolished the glucose-concentration-dependent increase of hindbrain glucose content when samples were incubated in the presence of 8 mmol l^{-1} glucose (Fig. 4A). In addition, CRF treatment at particular doses induced minor changes in the levels of metabolites at different concentrations of glucose in the medium for glucose (decrease at 8 mmol l^{-1} glucose), glycogen (dose-dependent increase at 2 mmol l^{-1} and decrease at 4 mmol l^{-1} glucose) and glucose 6-phosphate (increase at 8 mmol l^{-1} glucose after 10 nmol l^{-1} CRF addition), as observed in Fig. 4A–C.

Enzyme activities in hindbrain are shown in Fig. 5. The activity of GK (Fig. 5A) increased in controls in parallel with the increase of glucose concentration in the medium, whereas GSase activity increased at 8 mmol l^{-1} glucose (Fig. 5B). By contrast, no changes were noticed for PK activity (Fig. 5C). CRF treatment further enhanced the increase in GSase activity in a dose-dependent way at each of the glucose concentrations tested (Fig. 5B); however, the effective dose of CRF increased in parallel to glucose concentration, with only 10 nmol l^{-1} CRF potentiating GSase activity at 8 mmol l^{-1} glucose (Fig. 5B). In addition, no significant changes were noticed for PK activity in hindbrain after adding CRF at each glucose concentration (Fig. 5C).

The abundance of mRNA for factors related to glucosensing and for peptides related to control of food intake in hindbrain is shown in Fig. 6. No changes upon increasing the glucose concentration were observed for Glut2, POMC and CART (data not shown), as well as for GK (Fig. 6A), Kir 6-like (Fig. 6C) and SUR-like (Fig. 6D). Administration of CRF did not produce any effect on GK mRNA abundance (Fig. 6A). By contrast, in a similar way to that observed in the hypothalamus, the abundance of mRNA encoding NPY (Fig. 6B) decreased in controls in parallel to the increase of glucose concentration. Moreover, the glucose-concentration-dependent decrease of NPY expression was decreased by CRF administration (Fig. 6B); thus, 100 nmol l^{-1} CRF was effective at 4 mmol l^{-1} glucose, and 10 nmol l^{-1} CRF at 8 mmol l^{-1} glucose; by contrast, no effect was observed for CRF addition on NPY expression when samples were incubated with a low (2 mmol l^{-1}) glucose concentration (Fig. 6B). CRF treatment induced a trend to decrease Kir.6-like and SUR-like mRNA abundance, but no differences of statistical significance were produced (Fig. 6C,D).

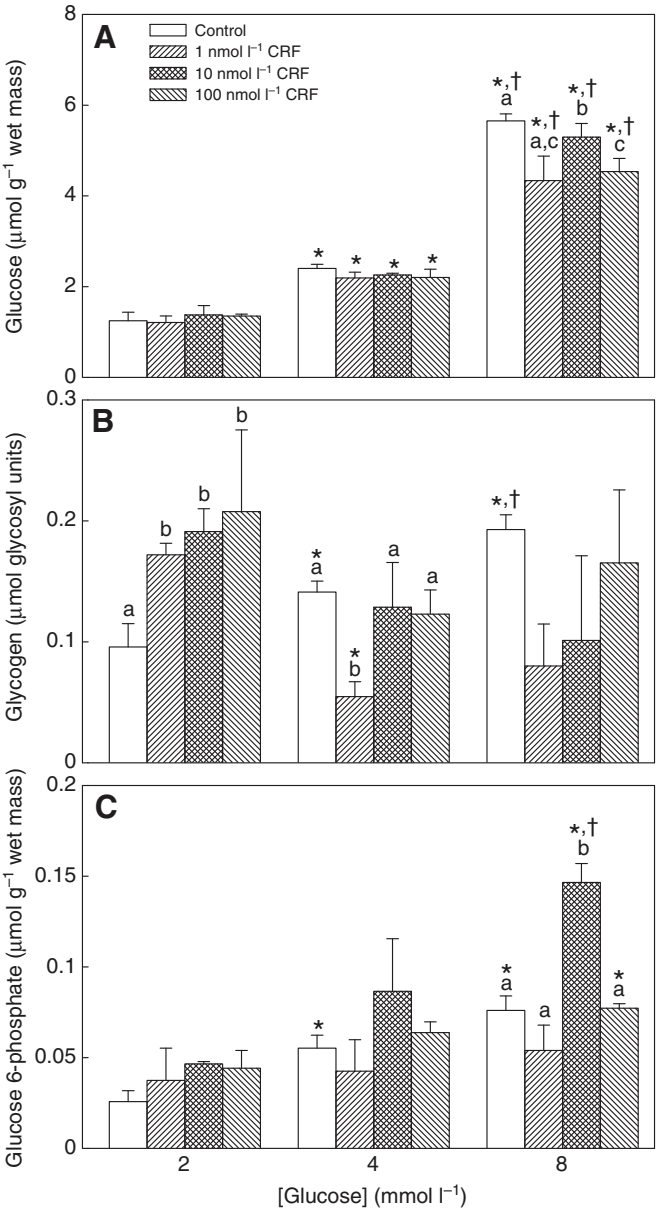

Fig. 4. Glucose (A), glycogen (B) and glucose 6-phopshate (C) levels in hindbrain of rainbow trout incubated *in vitro* for 1 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} D-glucose alone (control) or containing 1, 10 or 100 nmol l^{-1} CRF. Each value is the mean + s.e.m. of four independent experiments performed with pools of hindbrain from three or four different fish. For details of the letters and symbols, see the legend to Fig. 1.

DISCUSSION

The effects of CRF on glucosensing-related parameters

Increased content of metabolites (glucose, glycogen and glucose 6-phosphate) and increased activities of GK (also GK mRNA abundance in hypothalamus) and GSase were observed in hypothalamus and hindbrain of all control groups in parallel to the increase in the concentration of glucose in the medium, in a way similar to that addressed under similar *in vitro* conditions in the same species previously (Polakof et al., 2007b; Aguilar et al., 2011). All these results validate the experimental design used.

In a previous study (Conde-Sieira et al., 2010b), we had described in rainbow trout that the glucosensing response (at central and peripheral levels), characterized by changes in parameters such as glycogen and glucose levels, as well as GK, PK, GSase and GPase activities, was attenuated or even suppressed under the stress conditions elicited by high stocking density. Those changes suggested a readjustment in the activity

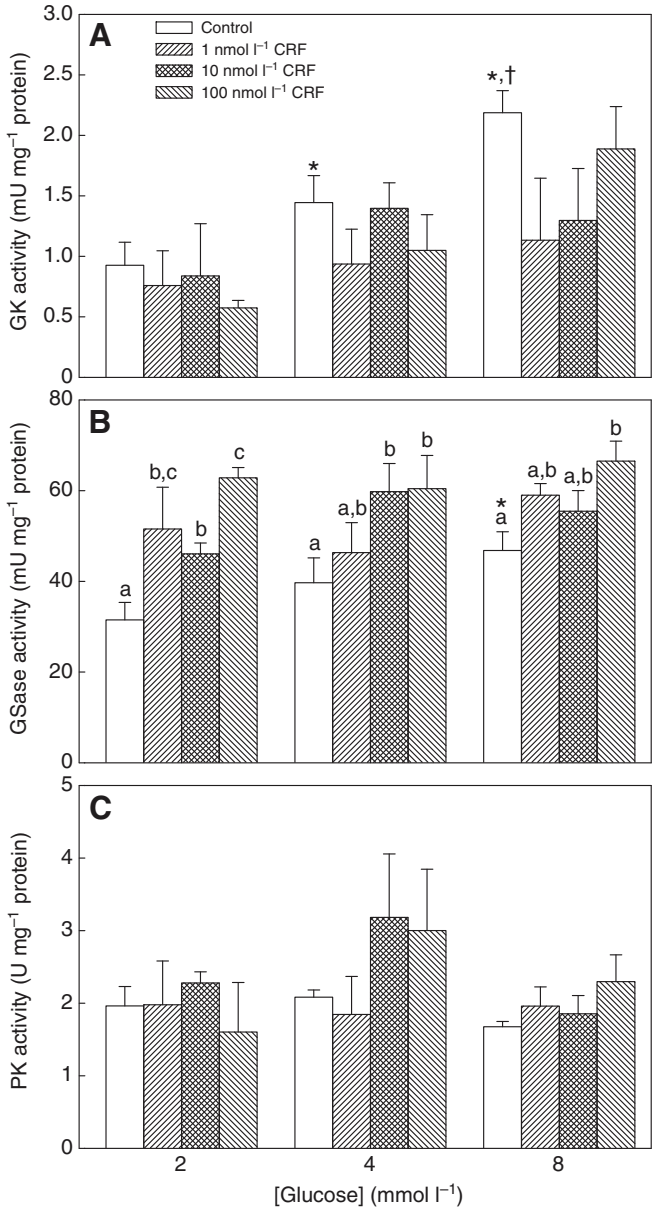

Fig. 5. Activities of GK (A), GSase (B) and PK (C) in hindbrain of rainbow trout incubated *in vitro* for 1 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} D-glucose alone (control) or containing 1, 10 or 100 nmol l^{-1} CRF. Each value is the mean + s.e.m. of four independent experiments performed with pools of hindbrain from three or four different fish. For details of the letters and symbols, see the legend to Fig. 1.

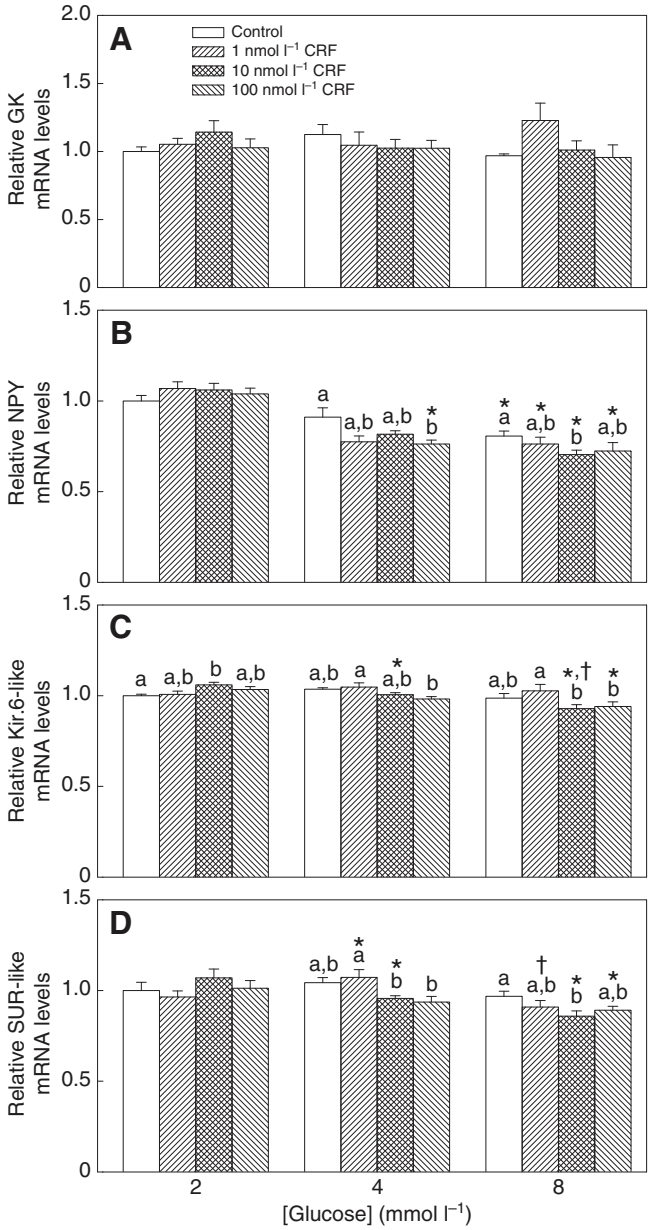

Fig. 6. The levels of mRNA encoding GK (A), NPY (B), Kir.6-like (C) and sulfonylurea receptor (SUR)-like (D) in hindbrain of rainbow trout incubated *in vitro* for 1 h at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l^{-1} D-glucose alone (control) or containing 1, 10 or 100 nmol l^{-1} CRF. Differences in mRNA levels between treatments are presented as an x-fold-induction with respect to a 2 mmol l^{-1} glucose control. Expression results were normalized by β-actin mRNA levels (no variation). Each value is the mean + s.e.m. of four independent experiments performed with pools of hindbrain from three to four different fish. For details of the letters and symbols, see the legend to Fig. 1.

of glucosensing mechanisms under conditions of stress, resulting in the system being unable to provide information related to changes in plasma glucose levels. In the same study, we found that cortisol levels described a very complex interaction between glycemic conditions and the stress induced by stocking density conditions, suggesting that glucosensor mechanisms are probably influenced by the hypothalamus–pituitary–interrenal (HPI) axis. As, in mammals, CRF amplifies the counter-regulatory response to acute hypoglycaemia in hypothalamic glucosensor areas (McCrimmon et al., 2006), whereas urocortin (another member of the CRF family) suppresses the glucosensing response (Cheng et al., 2007), we hypothesized that CRF could be involved in the readjustment of the glucosensing response observed in rainbow trout under stress conditions.

To assess this hypothesis, in the present study, we subjected hypothalamus and hindbrain to increased concentrations of CRF at different glucose concentrations. We basically observed two different responses in CRF-treated groups compared with controls when considering the response to different glucose concentrations. First, CRF-treated tissues tended to display, in response to changes

in the glucose levels of the medium, the same response of controls – that is, indicative that the glucosensing system is still activated by increased glucose levels – and in which CRF was able to increase the response further, at least under hypo- and normo-glycemic conditions. This response was basically restricted to glucose levels in the hypothalamus, which might suggest a main role of that tissue in mediating the response to stress. The second type of response was more common, and was indicative of a lack of response of the glucosensing parameters when tissues were subjected to increased glucose concentrations. Under those conditions, CRF treatment induced values similar to those observed in a control group at concentrations up to 8 mmol l^{-1} glucose (response independent of the glucose concentration), with some exceptions. This response occurred in both tissues for the levels of glycogen and glucose 6-phosphate and for the activities of GK (also mRNA abundance in hypothalamus) and GSase. In general, CRF treatment paralleled or potentiated the response to glucose of the factors assessed, meaning as a consequence that the glucosensing system is therefore not properly conveying information regarding changes in the levels of glucose in the medium. The action of CRF on glucosensing systems seems to operate mainly under hypo- and normo-glycemic conditions, which is interesting considering that the activation of the HPI axis normally results in hyperglycemia (Wendelaar Bonga, 1997).

In most of the cases assessed, CRF treatment elicited an increase in the parameters related to glucosensing – that is, it induced the same response that, in control conditions, would be induced by increased glucose levels in the medium (Polakof et al., 2007b), but further enhancing the measured levels or activities. High glucose levels are anorectic in the same species, as described previously (Polakof et al., 2008a; Polakof et al., 2008b), and coincide with an activation of the glucosensing system, producing a response similar to that we have found in the present study. There are no similar studies performed in fish to compare against, but, in rats, a hyperglycemic effect of central CRF administration has been observed (Brown et al., 1982a; Brown et al., 1982b), which would be similar to the activation of the glucosensing system observed in the present study. The response to CRF in rainbow trout was observed in most of the parameters related to glucosensing capacity, such as increased levels of glycogen and glucose 6-phosphate, increased activities of GK, GSase and PK, and increased abundance of mRNA encoding GK and Kir.6-like in hypothalamus. In hindbrain, the magnitude of effects of CRF was lower than that observed in hypothalamus, such as increased levels of glycogen and GSase activity, and decreased abundance of mRNA for Kir.6-like and SUR-like. Interestingly, the effect of CRF in both tissues was not observed at all glucose concentrations used, which is apparently indicative of an interaction between the effect of the hormone and the amount of glucose present in the incubation medium. As the amount of glucose was intended to be related to the glycemic conditions normally experienced by this species, we can conclude that the regulatory effect of CRF was apparently more important under hypo- and normo-glycemic conditions than under hyperglycemic conditions. Taking into account that, in a previous study, we had demonstrated that plasma cortisol levels in the same species were also dependent on glycemic conditions (Conde-Sieira et al., 2010a), these results allow us to suggest that the functioning of the HPI axis is modulated by glucose not only at the final step of the HPI axis (cortisol) but also at the very beginning within the brain (CRF), basically under hypo- or normo-glycemic conditions. The precise mechanism through which CRF is involved in the readjustment of glucosensing capacity remains to be elucidated. However, in mammals, CRFR1 and CRFR2 receptors are present in hypothalamic glucosensing neurons (Cheng et al., 2007), and it has been suggested that CRF changes the sensitivity of VMH-glucosensing neurons to glucose (McCrimmon et al., 2006) and that it regulates the glucosensing machinery (Evans et al., 2004). Therefore, any of these possibilities could be valid for rainbow trout.

CRF effects on mRNA abundance of neuropeptides related to the control of food intake

The protein CRF has effects on the control of food intake in fish (Volkoff et al., 2009) and is considered a powerful anorexigenic agent, as demonstrated repeatedly in several species, including rainbow trout (Bernier and Craig, 2005). In a previous study in the same species (Conde-Sieira et al., 2010a), we had observed that the readjustment in the activity of glucosensor systems was associated with changes in food intake, resulting in an inability of the fish to compensate with changes in food intake for changes in circulating glucose levels, as observed in fish under non-stress conditions (Polakof et al., 2008a; Polakof et al., 2008b). The mechanisms through which stress alters the food intake response induced by the activation of the glucosensor systems are not known, but probably some of the factors involved in the activation of the HPI axis, such as CRF, are probably involved (Bernier and Craig, 2005). The experimental design used (*in vitro*) in the present study does not allow us to evaluate changes in food intake. However, we measured changes in the abundance of mRNA for different neuropeptides, such as NPY, POMC and CART, that are involved in the regulation of food intake (Volkoff et al., 2009) and for which we have demonstrated that their mRNA abundance was dependent on the glucose concentration in the medium (Conde-Sieira et al., 2010b), allowing us to suggest their relationship with central glucosensing mechanisms in rainbow trout.

The increased glucose concentration in the medium elicited in controls decreased the mRNA abundance of NPY in hypothalamus and hindbrain and increased CART mRNA abundance in hypothalamus, in agreement with our previous *in vivo* (Conde-Sieira et al., 2010b) and *in vitro* results (Aguilar et al., 2011) in the same species, thus reinforcing the validity of our experimental design. In the present study, we found that, in a way similar to that observed for glucosensing factors, CRF treatment induced a change in the response of mRNA abundance to changes in glucose concentration in the medium. Thus, while NPY mRNA abundance in controls decreased with the amount of glucose in the medium, CRF administration was able to suppress such an effect. The response to CRF was not dependent on either glucose concentration or CRF dose in the hypothalamus of CRF-treated fish. Thus, CRF did counteract the glucose effect on NPY mRNA. Considering that CRF treatment is anorectic in this species, the maintenance of NPY mRNA abundance (an orexigenic factor) under CRF treatment is the converse of that expected. By contrast, CART mRNA abundance significantly increased in parallel with the increase in glucose levels in controls but mostly displayed no changes for CRF-treated fish relative to controls. Moreover, several other minor effects of CRF were also noticed in the abundance mRNAs for neuropeptides, such as the increase noticed for POMC in the hypothalamus. The slight amount of changes observed in abundance of mRNAs for peptides due to CRF treatment agrees with results obtained in mammals (Doyon et al., 2007), reinforcing the view that the main action of CRF on peptide expression is altering the pattern of response to changes in glucose levels. In general, it seems that CRF treatment altered the normal response to increased glucose concentration in the medium of mRNA abundance of several neuropeptides in the

hypothalamus and hindbrain, resulting in a similar response that became independent of glucose concentration. Moreover, in the few cases where CRF induced effects, they were indicative of an enhancement of the anorexigenic factors and a depression of orexigenic factors, which is in general agreement with the anorectic role of CRF in different fish species (Volkoff et al., 2009), including rainbow trout (Bernier and Craig, 2005). Only the exception of the maintenance of NPY mRNA abundance in hypothalamus indicates that food intake could not be inhibited, helping fish to maintain high glucose concentrations under conditions of stress, or that the neuropeptide might be stored in order to be released post-stress to then enhance food intake.

In summary, we had demonstrated in a previous study (Conde-Sieira et al., 2010a) that stress conditions induced in rainbow trout a readjustment in the tissue-specific glucosensing response of hypothalamus and hindbrain such that those sensors did not respond properly to changes in glucose levels, especially under hyperglycemic conditions. We had hypothesized that any of the factors belonging to the HPI axis could be involved in that response. Accordingly, in the present study, we provide evidence allowing us to suggest that CRF could be involved in such an interaction between stress and glucosensing as treatment *in vitro* of hypothalamus and hindbrain with CRF induced a readjustment in glucosensing parameters similar to that previously observed under stress conditions *in vivo* (Conde-Sieira et al., 2010a). We had also demonstrated previously (Conde-Sieira et al., 2010a) stress-elicited alterations in food intake of rainbow trout in parallel with the readjustment of glucosensing systems. Here, we have provided evidence that the abundance of mRNA for several of the neuropeptides involved in the regulation of food intake is affected by CRF treatment, in such a way that their expression does not respond to changes in glucose levels in the same way as controls, allowing us to suggest that the food intake response that is integrated by changes in those peptides and known to be reduced by stress could be also be mediated by CRF action in glucosensing areas.

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