The Journal of Experimental Biology 213, 3858-3865

doi:10.1242/jeb.048439

Effect of different glycaemic conditions on gene expression of neuropeptides involved in control of food intake in rainbow trout; interaction with stress

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Accepted 10 August 2010

SUMMARY

To assess mechanisms relating to food intake and glucosensing in fish, and their interaction with stress, we evaluated changes in the expression of orexigenic (NPY) and anorexigenic (POMC, CART and CRF) peptides in central glucosensing areas (hypothalamus and hindbrain) of rainbow trout subjected to normoglycaemic (control), hypoglycaemic (4 mg insulin kg^{-1}) or hyperglycaemic (500 mg glucose kg^{-1}) conditions for 6 h under normal stocking density (NSD; 10 kg fish mass m^{-3}) or under stress conditions induced by high stocking density (HSD; 70 kg fish mass m^{-3}). Hyperglycaemic NSD conditions resulted in decreased mRNA levels of NPY and increased levels of CART and POMC in the hypothalamus as well as increased mRNA levels of CART and CRF in the hindbrain compared with hypo- and normoglycaemic conditions. HSD conditions in normoglycaemic fish induced marked changes in the expression of all peptides assessed: mRNA levels of NPY and CRF increased and mRNA levels of POMC and CART decreased in the hypothalamus, whereas the expression of all four peptides (NPY, POMC, CART and CRF) decreased in the hindbrain. Furthermore, HSD conditions altered the response to changes in glycaemia of NPY and POMC expression in the hypothalamus and CART expression in the hypothalamus and the hindbrain. The results are discussed in the context of food intake regulation by glucosensor systems and their interaction with stress in fish.

Key words: trout, glucosensing, stress, hypothalamus, hindbrain, CART, POMC, CRF, NPY.

INTRODUCTION

In mammals, several brain regions, including the hypothalamus, medulla oblongata and mesencephalon, integrate endocrine (leptin, insulin, ghrelin, etc.) and metabolic (glucose, fatty acids, etc.) information to elaborate a coordinated response using neural effector pathways that produce key factors that either stimulate (orexigenic) or inhibit (anorexigenic) food intake (Marty et al., 2007). These areas contain specialized neurons that utilize glucose as a signalling molecule rather than as an energy substrate (Levin et al., 2004). Thus, glucose-excited (GE) neurons increase, whereas glucose-inhibited (GI) neurons decrease their firing rate as glucose levels rise (Levin et al., 2004; Marty et al., 2007). In both GE and GI neurons, the role of glucokinase (GK) is essential for glucosensing capacity (Marty et al., 2007). In previous studies, we demonstrated the existence of glucosensor systems based on GK in the hypothalamus, the hindbrain and Brockmann bodies of a carnivorous teleost species, the rainbow trout *Oncorhynchus mykiss* (Polakof et al., 2007a; Polakof et al., 2007b). These systems 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., 2008a; Polakof et al., 2008b).

It is well known that neurons in glucosensing areas produce peptides involved in the control of food intake in mammals (Schwartz et al., 2000). Thus, for instance, neurons from the arcuate nucleus in the hypothalamus that produce neuropeptide Y (NPY) and/or Agouti-related protein (AgRP) appear to be GI, whereas neurons producing pro-opiomelanocortin (POMC) and/or cocaine- and amphetamine-related transcript (CART) appear to be GE (Dunn-Meynell et al., 2002), resulting in increased expression of POMC and CART and decreased expression of NPY and AgRP when glucose levels rise (Mobbs et al., 2005). The mRNA of those genes has been detected in brain of different fish species in areas analogous to those characterized in mammals (Cerdá-Reverter and Canosa, 2009). We also obtained evidence in rainbow trout for the presence of GK in specific brain areas related to food intake regulation and energy homeostasis, some of these areas homologous to those known to contain glucosensing neurons in mammals, such as the ventromedial nuclei, arcuate nuclei, paraventricular nuclei and lateral hypothalamus in the hypothalamus (Polakof et al., 2009). Therefore, the areas in which GK (the basis of the glucosensor mechanism) and peptides involved in the control of food intake are present are generally coincident, allowing us to suggest a functional relationship between them. However, no studies have been carried out to date in fish regarding this issue, although indirect evidence, such as the finding that NPY expression increased in response to increased circulating glucose levels in the preoptic area of goldfish *Carassius auratus auratus* (Narnaware and Peter, 2002) and in tilapia *Oreochromis mossambicus* brain (Riley et al., 2009), supports the relationship.

Despite an appreciation for the negative impact of stress in food intake in fish (Wendelaar Bonga, 1997), our knowledge of the neuroendocrine mechanisms responsible for the appetite-suppressing effects of stress is superficial (Bernier and Peter, 2001; Bernier, 2006). In a previous study on rainbow trout, we observed that the glucosensing mechanisms did not work properly under the stress

conditions induced by high stocking density (HSD), providing no information related to changes in plasma glucose levels. This resulted in the inability of the fish to compensate for an increase in circulating glucose levels with changes in food intake, as has been observed in fish under non-stressed conditions (Conde-Sieira et al., 2010). The changes in the activity of glucosensor systems, as well as in the food intake response, might be related to an altered expression of orexigenic or anorexigenic factors in the same areas owing to the interaction between different glycaemic conditions and stress. Several studies have reported changes in the expression of peptides in several brain areas when fish were exposed to stress conditions such as decreased corticotrophin-releasing factor (CRF) and increased NPY expression in the preoptic area of goldfish (Bernier et al., 2004), increased CRF expression in the forebrain (Bernier and Craig, 2005) and preoptic area (Bernier et al., 2008) of rainbow trout and decreased POMC expression in the brain of sole *Solea solea* (Palermo et al., 2008). However, there are no studies available in the literature regarding changes in the expression of the same peptides under different glycaemic conditions when fish are simultaneously exposed to a stressful situation.

Therefore, the objectives of the present study were to: (1) evaluate changes in the expression of orexigenic (NPY) and anorexigenic (POMC, CART and CRF) peptides involved in the control of food intake in fish (Volkoff et al., 2009) in central glucosensing areas (hypothalamus and hindbrain) in rainbow trout subjected to different glycaemic conditions to relate expression of these peptides to the activity of glucosensor systems; and (2) evaluate the interaction between changes in glucose levels and stress in the expression of the same peptides to obtain evidence for their role in the deregulation of glucosensor systems and changes in food intake occurring in rainbow trout under stress conditions (Conde-Sieira et al., 2010).

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-litre tanks under laboratory conditions and a 12 h:12 h light:dark photoperiod in dechlorinated tapwater at 15°C. Fish mass was 131±1 g. Fish were fed once daily (10.00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg, Segovia, Spain; proximate food analysis was 48% crude protein, 6% carbohydrates, 25% crude fat and 11.5% ash; 20.2 MJ kg^{-1} feed). The experiments described complied with the guidelines of the European Union Council (86/609/EU) and the Spanish Government (RD 1201/2005) for animal care and use.

Experimental design

Following acclimation, fish were randomly assigned to 100-litre experimental tanks. Fish were fasted for 24 h before injection to ensure that basal hormone levels were achieved. On the day of the experiment, at 10.00 h, fish were lightly anaesthetized with MS-222 (50 mg l^{-1}) buffered to pH 7.4 with sodium bicarbonate, weighed and intraperitoneally injected with 5 ml kg^{-1} body mass of Cortland saline, alone (normoglycaemic treatment) or containing insulin (hypoglycaemic treatment, 4 mg bovine insulin kg^{-1} body mass; Sigma Chemical, St Louis, MO, USA) or D-glucose (hyperglycaemic treatment, 500 mg kg^{-1} body mass). Immediately after injection, fish were returned to their tanks, where they remained without any access to food for 6 h. Half of the tanks (two replicates per glycaemic treatment) were kept stocked at 10 kg fish mass m^{-3} [normal stocking density (NSD)]. In the remaining tanks (two replicates per glycaemic treatment), a quantity of water was removed until reaching a stressful HSD (70 kg fish mass m^{-3}) was reached. Therefore, the six experimental groups (two replicates per treatment) used were: (1) normoglycaemic fish under NSD, (2) hypoglycaemic fish under NSD, (3) hyperglycaemic fish under NSD, (4) normoglycaemic fish under HSD, (5) hypoglycaemic fish under HSD and (6) hyperglycaemic fish under HSD. Fish in all tanks were sampled after 6 h, with N=6 per treatment and density.

Sampling

Fish were removed from holding tanks, anaesthetized as above and weighed. Blood was collected by caudal puncture with ammonium-heparinized syringes. Fish were sacrificed rapidly by decapitation, and plasma samples were obtained after blood centrifugation and divided into two aliquots. One aliquot was immediately frozen in liquid nitrogen for the assessment of plasma cortisol levels, whereas the other aliquot, for the assessment of plasma glucose, was deproteinized immediately (using 6% perchloric acid) and neutralized (using 1 mmol l^{-1} potassium bicarbonate) before freezing in liquid nitrogen and storage at –80°C until further analysis. The brain was removed, placed on a chilled Petri dish, and the hypothalamus and hindbrain were obtained as described previously (Polakof et al., 2007b), frozen in liquid nitrogen and stored at –80°C until assayed.

Assessment of glucose and cortisol levels

Plasma glucose levels were determined enzymatically using commercial kits (Spinreact, Barcelona, Spain) adapted to a microplate format. Plasma cortisol levels were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA).

Gene expression analysis by real-time quantitative reverse transcription 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). One µg total RNA was reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen) and random hexaprimers (Invitrogen). Gene expression levels were determined by real-time quantitative reverse transcription PCR (qPCR) using the iCycler iQ™ (Eppendorf, Hamburg, Germany). Analyses were performed on 1 µl of the diluted cDNA [further dilution 1/25 for elongation factor 1-alpha (EF1α) and 1/4 for POMC] using the ABsolute™ qPCR SYBR® Green Mix (Abgene, Cambridge, UK), in a total PCR reaction volume of 15 µl, containing 50 to 500 nmol l^{-1} of each primer. GK (Panserat et al., 2000), POMC1 (Leder and Silverstein, 2006), EF1α (Bernier et al., 2008) and CRF (Bernier et al., 2008) qPCRs were performed using primers previously described. Primers for NPY were designed according to the available sequence in public databases (GenBank accession number NM_001124266). The forward NPY primer was 5′-CTCGTCTGGACCTTTATATGC-3′ and the reverse primer was 5′-GTTCATCATATCTGGACTGTG-3′. For CART, a fragment of rainbow trout CART was cloned using primers that were designed according to the alignment of known fish CART sequences. Briefly, brain cDNA was primed with omCART-Fw (5′-ACCATGGAGAGCTCCAG-3′) and omCART-Rv (5′-GCGCACTGCTCTCCAA-3′) and amplified by standard PCR. After agarose purification, the fragment of 275 bp was subcloned into pGEM-T easy vector (Promega) and sequenced on both strands. The nucleic sequence was 97% identical to the CART sequence reported for lake trout *Salvelinus namaycush* (accession

number DQ836925). These pair of primers was then used for CART qPCR. All new primers were designed using Generunner free software (http://www.generunner.net/).

Relative quantification of the target gene transcript was done using EF1α gene expression as a reference (Bernier et al., 2008), which was stably expressed in this experiment. Thermal cycling was initiated using hot-start iTaq^{TM} DNA polymerase activation with incubation at 95°C for 15 min. Forty steps of PCR were performed, each of which consisted of heating at 95°C for 15 s for denaturing, at specific annealing tempeatures (58°C for NPY and 60°C for the remaining peptides) for 30 s and extension at 72°C for 30 s. Following the final PCR cycle, melting curves were systematically monitored (0.5 deg s^{-1} temperature gradient from 55 to 95°C) to ensure that only one fragment was amplified. Each sample was analysed three times. All the replicates of each sample were located in the same plate for each gene to allow comparisons. We included in all the plates the standard curve (by triplicate) and blanks for DNA, PCR and retrotranscription (by duplicate). Only efficiency values between 85 and 100% were accepted, and the R^2-value for all the genes assessed was always >0.985. Relative quantification of the target gene transcript with the EF1α reference gene transcript was made following the Pfaffl method (Pfaffl, 2001) with the Relative Expression Software Tool (REST©; Qiagen, Hilden, Germany). This mathematical algorithm computes an expression ratio (R) based on qPCR efficiency and the crossing point deviation of the unknown sample *versus* a control group (ΔCT): $R=[E_T{}^{\Delta CT T(\bar{S}C-\bar{S}U)}]/[E_{EF1\alpha}{}^{\Delta CT EF1\alpha(\bar{S}C-\bar{S}U)}]$, where E is PCR efficiency determined using a standard curve of cDNA serial dilutions (cDNA dilutions from 1/32 up to 1/512), T is the target gene, $\bar{S}_C$ is the mean of the control sample and $\bar{S}_U$ is the mean of the unknown sample.

Statistics

Comparisons among groups were performed by two-way ANOVA with glycaemic levels (normo-, hypo- or hyper-) and stocking density (NSD and HSD) as main factors. When a significant effect was noticed within a factor, *post hoc* comparisons were carried out within that factor using a Student–Newman–Keuls (SNK) test, and differences were considered statistically significant at $P<0.05$.

RESULTS

Plasma glucose levels decreased after hypoglycaemic treatment and increased after hyperglycaemic treatment in fish under NSD and HSD, whereas levels were higher in fish under HSD than in fish under NSD in hypoglycaemic and hyperglycaemic fish (Fig. 1A). Plasma cortisol levels were higher in fish under HSD than in fish under NSD in any glycaemic condition whereas, in fish under HSD, levels in hypo- and hyperglycaemic conditions were higher than those under normoglycaemic conditions (Fig. 1B).

GK mRNA levels in the hypothalamus increased with glycaemia in fish under NSD whereas, in fish under HSD, levels were higher in the normoglycaemic condition and decreased in the hypo- and hyperglycaemic conditions. Fish under HSD displayed higher levels than in NSD under normoglycaemic conditions but lower levels under hyperglycaemic conditions (Fig. 2A). In the hindbrain, GK mRNA levels in fish under NSD showed no significant changes with glycaemia whereas, under HSD, levels were higher in the hypo- and hyperglycaemic conditions than in normoglycaemic conditions (Fig. 2B). Levels were always higher in fish under HSD than in fish under NSD.

NPY mRNA levels in the hypothalamus of fish under NSD decreased in the hyperglycaemic condition compared with the hypo- and normoglycaemic conditions whereas, in fish under HSD, no changes were observed with glycaemia. Levels in fish under HSD were always higher than those of fish in NSD (Fig. 3A). In the hindbrain, no changes with glycaemia were observed in NPY mRNA levels for fish under NSD and HSD, although levels in fish under HSD were always lower than under NSD (Fig. 3B).

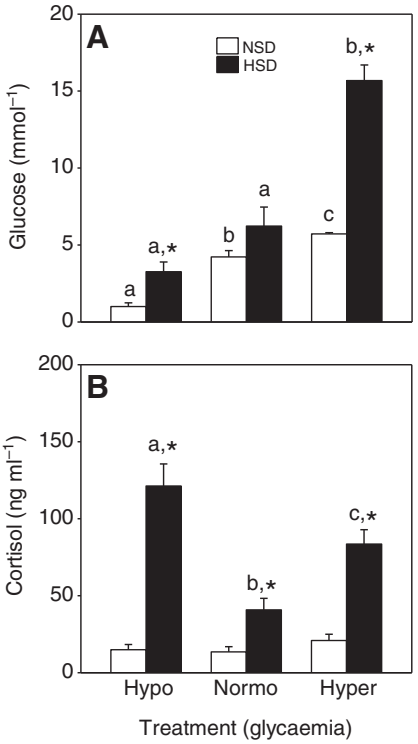

Fig. 1. (A) Glucose (B) and cortisol levels in plasma of rainbow trout under different glycaemic conditions, elicited by intraperitoneal injection of Cortland saline alone (normoglycaemic; 5 ml kg^{-1} body mass) or containing insulin (hypoglycaemic; 4 mg bovine insulin kg^{-1} body mass) or D-glucose (hyperglycaemic; 500 mg D-glucose kg^{-1} body mass), kept at normal stocking density (NSD; 10 kg m^{-3}) or high stocking density (HSD; 70 kg m^{-3}) for 6 h. Data represent means + s.e.m. of six measurements. *, significantly different ($P<0.05$) from NSD at the same glycaemic condition. Different letters indicate significant differences ($P<0.05$) from different glycaemic conditions at the same density.

CART mRNA levels increased in hyperglycaemic *versus* normo- and hypoglycaemic fish in the hypothalamus (Fig. 4A) and hindbrain (Fig. 4B) of fish under NSD whereas no changes with glycaemia were observed for fish under HSD. Levels in fish under HSD were always lower than those in fish under NSD.

POMC mRNA levels in the hypothalamus increased in the hyperglycaemic condition *versus* normo- and hypoglycaemic conditions in fish under NSD whereas no changes were observed in fish under HSD (Fig. 5A). In the hindbrain, no changes in POMC mRNA levels were observed with the increase in glycaemia in fish under NSD and HSD (Fig. 5B). Both hypothalamic and hindbrain POMC mRNA levels in fish under HSD were lower than in fish under NSD (Fig. 5A,B).

CRF mRNA levels in the hypothalamus displayed no changes with the increase in glycaemia in fish under NSD and HSD; levels in fish under HSD were higher than those under NSD (Fig. 6A). In the hindbrain, CRF mRNA levels increased in hyperglycaemic fish *versus* hypo- and normoglycaemic fish under NSD and HSD conditions. Levels were lower in fish under HSD than under NSD (Fig. 6B).

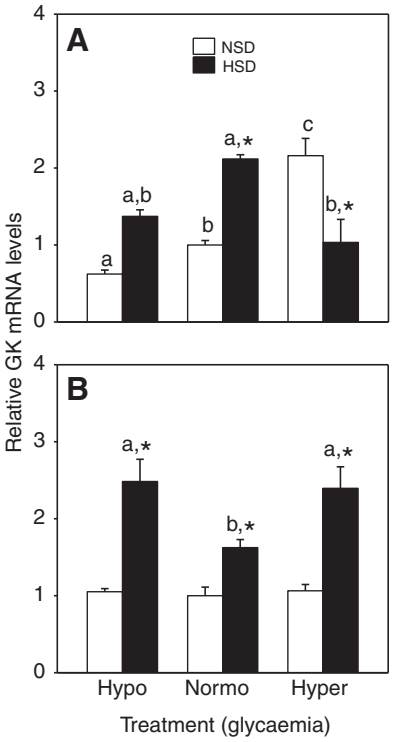

Fig. 2. GK mRNA levels in the (A) hypothalamus and (B) hindbrain of rainbow trout under different glycaemic conditions, elicited by intraperitoneal injection of of Cortland saline alone (normoglycaemic; 5 ml kg^{-1} body mass) or containing insulin (hypoglycaemic; 4 mg bovine insulin kg^{-1} body mass) or D-glucose (hyperglycaemic; 500 mg D-glucose kg^{-1} body mass), kept at normal stocking density (NSD; 10 kg m^{-3}) or high stocking density (HSD; 70 kg m^{-3}) for 6 h. Differences in mRNA levels between treatments are presented as an *x*-fold-induction with respect to the normoglycaemic NSD group. Expression results were normalised by EF1α mRNA levels (EF1α mRNA levels – no variation). Data represent means + s.e.m. of six measurements. *, significantly different (P<0.05) from NSD at the same glycaemic condition. Different letters indicate significant differences (P<0.05) from different glycaemic conditions at the same density.

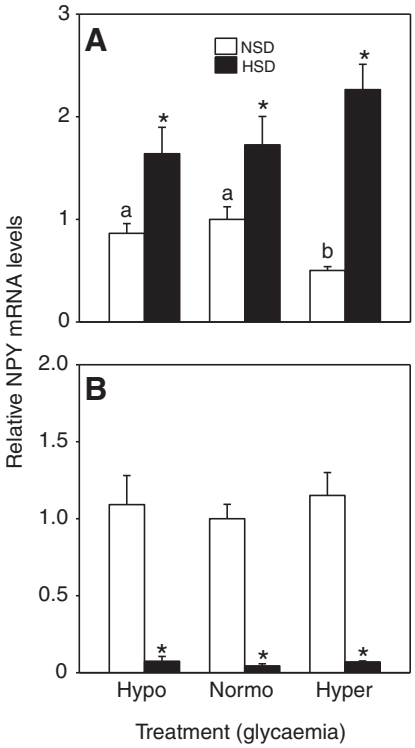

Fig. 3. NPY mRNA levels in the (A) hypothalamus and (B) hindbrain of rainbow trout. Glycaemic conditions and other details as in Fig. 2.

DISCUSSION

Effects of different glycaemic conditions in fish under NSD

The different treatments used in fish kept under NSD were effective because decreased plasma glucose levels after hypoglycaemic treatment and increased plasma glucose levels after hyperglycaemic treatment were observed, thus validating the experimental design. However, because hypoglycaemic conditions were obtained by insulin treatment, some caution must be taken when interpreting results in hypoglycaemic groups because at least some of the effects could be attributed to the action of insulin alone. In addition, no significant changes were noticed in plasma cortisol levels among glycaemic treatments in fish under NSD, suggesting that fish were not stressed by the experimental glycaemic conditions. Both of these results are similar to those previously observed in rainbow trout that were subjected to similar changes in glucose levels, but for a longer duration (Conde-Sieira et al., 2010).

Increased GK mRNA levels were observed in the hypothalamus in parallel with the increased levels of plasma glucose. This response is similar to that found in previous studies, in which changes in the expression of GK were assessed 6 h after induction of changes in glucose (Polakof et al., 2007b; Polakof et al., 2008c). This result suggests once more that the glucosensor system based on GK activity is activated when glucose levels rise and is inhibited when glucose levels fall. However, no changes were observed in GK mRNA levels in the hindbrain, which is in contrast to the increase already reported in the same species after similar changes in glucose for a similar time period (Polakof et al., 2007b). Glucosensor areas in mammals are known to produce neuropeptides involved in the control of food intake in response to changes in the levels of glucose (Schwartz et al., 2000; Marty et al., 2007). The mammalian model suggests that glucose (a signal of plentiful energy) excites neurons that produce anorexigenic factors such as POMC or CART and inhibits neurons that produce orexigenic factors such as NPY and AgRP (Burdakov et al., 2005). In fish, there are no previous references for the possible involvement of these or any other peptides in the changes in food intake associated with the activation/inhibition of glucosensor systems.

Our results show a decrease in NPY mRNA levels in hyperglycaemic fish compared with hypo- and normoglycaemic fish in the hypothalamus of rainbow trout under NSD, whereas no changes were observed in the hindbrain. In the only study carried out in fish regarding changes in NPY expression in response to changes in glucose, an increase rather than a decrease was observed in the preoptic area (but no changes were observed in the hypothalamus) of goldfish (Narnaware and Peter, 2002) and in the whole brain of tilapia (Riley et al., 2009) when glucose levels rose. However, a decreased expression of NPY is known to occur in the mammalian hypothalamus when glucose levels rise either *in vivo* (Lynch et al., 2000; Mobbs et al., 2005) or *in vitro* (Lee et al., 2005) or in the starved-to-fed transition, when circulating glucose levels are also known to increase (Stolarczyk et al., 2010). Considering that, in several mammalian hypothalamic nuclei (such as the arcuate nucleus), NPY neurons appear to be GI (Dunn-Meynell et al., 2002), we suggest that an increase in glucose levels

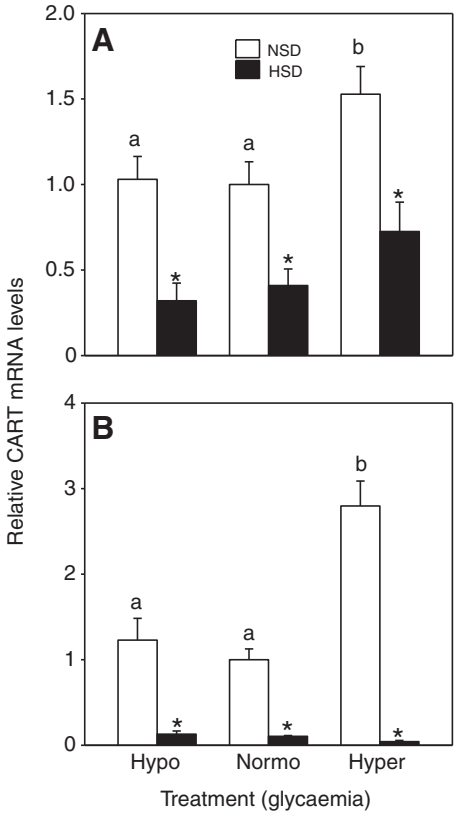

Fig. 4. CART mRNA levels in the (A) hypothalamus and (B) hindbrain of rainbow trout. Glycaemic conditions and other details as in Fig. 2.

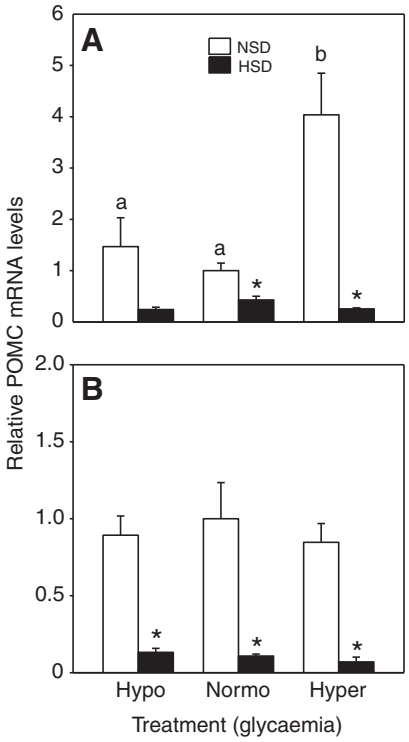

Fig. 5. POMC mRNA levels in the (A) hypothalamus and (B) hindbrain of rainbow trout. Glycaemic conditions and other details as in Fig. 2.

in rainbow trout hypothalamus induces decreased synthesis of NPY as a result of the activation of the glucosensor mechanism (enhanced GK expression) in a way similar to that observed in mammals but in contrast to that observed in other fish species. Considering the orexigenic role of NPY in fish (Volkoff et al., 2009), a decreased expression should correlate with a decreased food intake, which has been shown to occur in rainbow trout subjected to hyperglycaemic conditions (Polakof et al., 2008a; Polakof et al., 2008b). The absence of an effect of different glucose concentrations on mRNA production in the hindbrain indicates that NPY expression in that area is not as closely related to glucosensing capacity as in the hypothalamus.

CART mRNA levels increased in the hypothalamus and hindbrain in hyperglycaemic versus normo- and hypoglycaemic fish. No similar studies have been carried out in fish to date. In mammals, the increase in glucose levels during the starved-to-fed transition is not accompanied by changes in CART expression in the hypothalamus (Stolarczyk et al., 2010), although the decrease in glucose levels during the fed-to-fasted transition is accompanied by a decrease in hypothalamic CART mRNA levels (Fekete et al., 2006). This suggests a specificity of the response in rainbow trout compared with that known in mammals, in which changes were observed not only in the hypothalamus but also in the hindbrain and occurred only under hyperglycaemic conditions. CART is a well-known anorexigenic factor in fish (Volkoff et al., 2009) and is widely expressed in fish brain (Cerdá-Reverter and Canosa, 2009). Considering the decrease in food intake that occurs in rainbow trout when glucose levels rise (Polakof et al., 2008a; Polakof et al., 2008b), an increased expression of CART could also be involved in this response and therefore could be associated with the increased activity of the glucosensor systems in those areas.

POMC mRNA levels increased in rainbow trout hypothalamus (but not in the hindbrain) under hyperglycaemic conditions compared with fish under hypo- and normoglycaemic conditions. Again, there have been no similar studies carried out in fish to date. In mammals, an increase in circulating glucose levels is known to produce increased POMC expression in the hypothalamus (Lynch et al., 2000; Mobbs et al., 2005; Fekete et al., 2006; Cai et al., 2007; Stolarczyk et al., 2010) in a manner similar to that described in the present study. In fish, POMC is produced in the hypothalamus in Atlantic salmon *Salmo salar* and goldfish brains (Cerdá-Reverter and Canosa, 2009), and preliminary observations (J.M.C.-R., unpublished data) have also pointed to the expression of POMC in the hindbrain. The response of POMC expression in the hypothalamus to changes in glycaemia in rainbow trout was in agreement with the anorexigenic role of that peptide in fish (Volkoff et al., 2009), as well as with the decrease in food intake observed under hyperglycaemic conditions in the same species (Polakof et al., 2008a; Polakof et al., 2008b) suggesting that changes in food intake occurring in parallel with changes in the activation/inhibition of the glucosensor system are mediated, at least in part, by changes in the expression of POMC.

CRF expression in the hypothalamus did not change in response to glucose in fish in NSD whereas an increase in parallel with changes in glucose was noticed in the hindbrain. The absence of changes in the hypothalamus is similar to the response observed in mammals, where no changes in hypothalamic CRF expression were observed in animals under the hyperglycaemic conditions occurring after the fasted-to-fed transition (Stolarczyk et al., 2010). Owing to the anorexigenic nature of CRF in fish, one would expect an increase rather than a decrease in expression with an increase in glucose

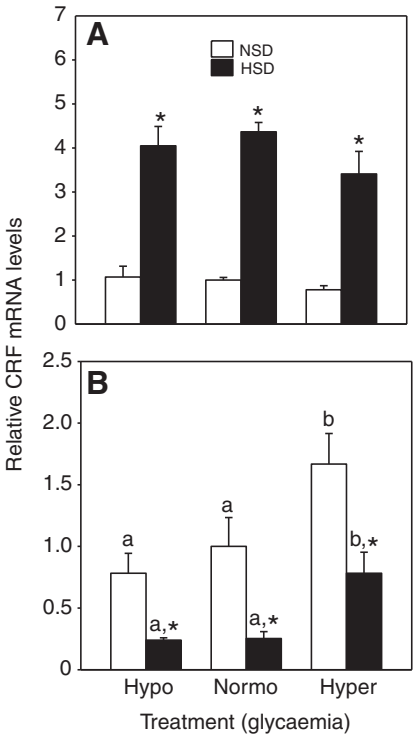

Fig. 6. CRF mRNA levels in the (A) hypothalamus and (B) hindbrain of rainbow trout. Glycaemic conditions and other details as in Fig. 2.

levels, a situation in which the activation of glucosensor systems is accompanied by anorexia (Polakof et al., 2008a; Polakof et al., 2008b). Considering that such an increase occurred in the hindbrain, this allows us to suggest a role for hindbrain CRF in the changes in food intake associated with the activity of glucosensor systems in rainbow trout.

Effects of HSD in normoglycaemic fish

Stress is known to produce increased levels of cortisol and glucose in plasma in fish (Wendelaar Bonga, 1997). The HSD to which rainbow trout were exposed increased levels of both parameters in plasma in normoglycaemic fish, reinforcing the validity of the experimental design used. GK mRNA levels in normoglycaemic fish were higher under HSD than under NSD conditions, both in the hypothalamus and hindbrain. This is in contrast with the decrease observed after 2 days (hindbrain) and 5 days (hypothalamus) in rainbow trout that were subjected to similar changes in glucose (Conde-Sieira et al., 2010), suggesting that the effect of stress in GK expression is clearly dependent on time. The activation of GK under stress conditions suggests an activation of the glucosensor systems in both areas, a situation known to induce a decrease in food intake in the same species (Polakof et al., 2008a; Polakof et al., 2008b); this is coincident with the known decrease in food intake under stress conditions in fish (Wendelaar Bonga, 1997). It allows us to suggest that the decrease in food intake during stress in fish is at least partly associated with changes induced in the expression of orexigenic/anorexigenic factors by the activation of the glucosensor system. In the only available study in fish literature regarding this issue, Doyon et al. provided evidence for a correlation between NPY and CRF gene expression in stress-subordinate rainbow trout (Doyon et al., 2003).

Stress induced by HSD in normoglycaemic fish resulted in increased NPY mRNA levels in the hypothalamus but a decrease in the hindbrain. In another study carried out in rainbow trout, glucocorticoid treatment (a condition analogous to that observed after stress) induced decreased expression of NPY in the preoptic area of rainbow trout (Doyon et al., 2006) whereas, in goldfish, cortisol treatment induced opposite effects in the preoptic area with no changes observed within the hypothalamus (Bernier et al., 2004). In previous studies, we observed that the glucosensing areas in rainbow trout brain are the hypothalamus and hindbrain, with no activity reported in other brain regions (Polakof et al., 2007b). Therefore, we suggest that changes in peptide expression occurring in the preoptic area, although related to the control of food intake, are probably not related to the part of the control of food intake that is influenced by the glucosensor systems.

POMC mRNA levels decreased in the hypothalamus and hindbrain of fish under HSD when compared with those under NSD. In a recent study carried out in sole, HSD resulted in a decrease in brain POMC expression (Palermo et al., 2008), in agreement with the results of the present study. However, the decrease in POMC expression with stress is not coincident with the expected increase of expression for an anorexigenic factor in a situation known to reduce food intake, such as stress. Expression of POMC gene is probably regulated by CRF and is under negative feedback control by glucocorticoids through the glucocorticoid-responsive element, suggesting a complex relationship between both factors.

CART mRNA levels decreased in the hypothalamus and hindbrain of fish under HSD compared with those under NSD. Because CART is an anorexigenic factor, a decrease in mRNA levels could result in increased food intake, which does not occurr under similar stress conditions (Conde-Sieira et al., 2010). Therefore, changes in CART expression cannot be easily correlated to those of food intake, suggesting the existence of a more complex regulation, in a manner similar to that suggested for POMC.

CRF mRNA levels displayed marked differences when comparing the response of hypothalamus and hindbrain to HSD. Thus, while levels sharply increased in the hypothalamus (more than fourfold), they decreased in the hindbrain. This might be related to the fact that CRF synthesis and release in fish is mostly associated with several hypophysiotropic areas, including caudal telencephalon, preoptic area and tuberal hypothalamus (Cerdá-Reverter and Canosa, 2009), allowing us to suggest that changes observed in the hypothalamus are more relevant in terms of the changes in food intake occurring during stress. Decreased CRF mRNA levels were also observed in the preoptic area of cortisol-treated goldfish (Bernier et al., 2004) whereas, in rainbow trout, different stressors induced increased CRF mRNA levels in the forebrain (Bernier and Craig, 2005; Bernier et al., 2008).

The sharp increase in CRF expression in the hypothalamus of rainbow trout under HSD matches very well with the known decrease of food intake in stressed fish. In fact, the increase observed was so high that it could produce an anorexigenic effect even considering the decreases observed in POMC and CART under the same conditions. Because changes occurred in glucosensing areas (especially the hypothalamus), we suggest that they are related to the control of food intake mediated by glucosensor systems.

Interaction of different glycaemic conditions with HSD

Changes in plasma cortisol levels depending on glycaemic conditions were observed only in fish under HSD. The high levels of cortisol under those glycaemic conditions could be responsible, at least in part, for changes in the expression of neuropeptides

because enhanced glucocorticoid levels are known to alter neuropeptide expression in fish (Bernier et al., 2004; Doyon et al., 2006).

GK mRNA levels in the hypothalamus – which, in fish under NSD, followed levels of glucose – changed in fish under HSD, showing decreased levels under normo- and hyperglycaemic conditions. This result is similar to the changes in GK activity previously observed in fish with similar glycaemic conditions after 2 and 5 days under HSD (Conde-Sieira et al., 2010). This result indicates that the responses of glucosensor mechanisms to changes in glucose are altered (attenuated or diminished) under stress conditions. Because GK activity can be modified by insulin treatment, we cannot disregard that at least some of the differences observed in hypoglycaemic fish could be attributed to the action of insulin alone.

The observed decrease in NPY expression in the hypothalamus of hyperglycaemic fish compared with normoglycaemic fish under NSD conditions disappeared under HSD conditions whereas, in the hindbrain, the trend in both groups of fish was the same. It seems that stress interacts with the glycaemic control of neuropeptide synthesis and release, resulting in the disappearance of the normal response to glycaemia in NPY expression in the hypothalamus and thus resulting in a deregulation of the control of food intake caused by the activation of glucosensor systems (Conde-Sieira et al., 2010). Based on the present results, we suggest that the lack of regulation is caused, at least in part, by the altered NPY expression. POMC expression displayed a result similar to that of NPY in the hypothalamus, i.e. the changes observed with glycaemia in fish under NSD (in this case an increase) disappeared in fish under HSD, suggesting that stress alters the normal synthesis and release of POMC involved in the activation of the glucosensor systems. CART mRNA levels in the hypothalamus of fish under HSD displayed the same trend in response to glycaemia as did fish under NSD whereas, in the hindbrain, the increased expression in parallel with the increase in glucose in NSD fish changed to a decrease in fish under HSD. As in the case mentioned above for POMC and NPY, it seems that stress alters the normal response of CART synthesis in glucosensor areas, such as the hypothalamus and hindbrain, in response to changes in glucose; therefore, changes in this peptide might be involved in the deregulation of food intake by glucose levels under stress conditions. In contrast to the peptides mentioned above, changes in CRF mRNA levels with glycaemia in NSD were not modified by HSD in any of the brain regions assessed.

CONCLUSIONS AND PERSPECTIVES

In summary, we have demonstrated, for the first time in fish, that the expression of several neuropeptides involved in the regulation of food intake in glucosensing central areas (hypothalamus and hindbrain) of rainbow trout is regulated by changes in glycaemia in a way that is compatible with the effects of changes in glucose on food intake already reported for the same species (Polakof et al., 2008a; Polakof et al., 2008b). The most important changes observed were the decreased levels of NPY mRNA and increased levels of CART and POMC mRNA in the hypothalamus of hyperglycaemic fish whereas increased levels of CART and CRF mRNA were observed in the hindbrain. It is also interesting to emphasise that changes in peptide expression were mainly observed in fish under hyperglycaemic conditions. These results suggest that these peptides are produced in those areas in a manner similar to that addressed in the mammalian glucosensing neurons in response to changes in glucose levels (Marty et al., 2007). We also demonstrated that stress induced by HSD conditions induced marked changes in the expression of all peptides assessed in the glucosensing areas of fish under normoglycaemic conditions. These changes include increased levels of NPY and CRF mRNA as well as decreased levels of POMC and CART mRNA in the hypothalamus. In the hindbrain, the expression of the four neuropeptides assessed decreased in stressed fish. This suggests that the decrease in food intake observed in fish under stress conditions (Wendelaar Bonga, 1997) is related, at least in part, to the activation of glucosensor systems through changes in the expression of these peptides. In a previous study of rainbow trout under HSD (Conde-Sieira et al., 2010), we found that the glucosensing mechanisms did not work properly, providing no information related to changes in plasma glucose levels. This resulted in an inability of the fish to compensate for circulating glucose levels with changes in food intake, as has been observed in fish under non-stressed conditions (Conde-Sieira et al., 2010). These changes in the regulation of food intake under stress conditions might be associated with changes in the expression of neuropetides involved in the control of food intake. Accordingly, we demonstrated that HSD conditions alter the response of neuropeptide expression to changes in glycaemia observed under NSD conditions for NPY and POMC expression in the hypothalamus and CART expression in the hypothalamus and hindbrain. The fact that the expression of these peptides in fish under HSD was independent of glycaemic conditions suggests that the deregulation of food intake by glucose levels observed in fish under HSD (Conde-Sieira et al., 2010) is partially associated with the changes herein described in neuropeptide expression. Future studies are necessary to assess the specific mechanisms underlying this response.

LIST OF ABBREVIATIONS

AgRP	Agouti-related protein
CART	cocaine- and amphetamine-related transcript
CRF	corticotrophin-releasing factor
EF1α	elongation factor 1-alpha
GE	glucose-excited neurons
GI	glucose-inhibited neurons
GK	glucokinase
HSD	high stocking density
NPY	neuropeptide Y
NSD	normal stocking density
POMC	pro-opiomelanocortin

ACKNOWLEDGEMENTS

This study was supported by research grants from Ministerio de Ciencia e Innovación (AGL2007-65744-C03-01/ACU and AGL2007-65744-C03-03/ACU), Xunta de Galicia (Consolidación e estructuración de unidades de investigacion competitivas, INCITE09ENA310002ES) and Universidade de Vigo (Contrato-Programa con grupos de investigación consolidados). M.C.-S., M.J.A. and A.J.A. were recipients of an FPI predoctoral fellowship (Spanish Science and Innovation Ministry), a “Juan de la Cierva” research contract (Spanish Science and Innovation Ministry) and an AECI predoctoral fellowship (Spanish Foreign Affairs and Cooperation Ministry), respectively.

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