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Research Article

HYPOTHALAMIC NEUROPEPTIDE Y (NPY) mRNA CONTENT DECREASES AFTER CENTRAL ADMINISTRATION OF INSULIN IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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ABSTRACT
Insulin is known to exert its anorectic effect in mammals by reducing expression of the orexigenic signal neuropeptide Y (NPY) in the arcuate nucleus. However, the neural pathways by which insulin regulates food intake in fish are not well known. Bearing in mind that the actions of insulin and NPY on food intake in fish are similar to those observed in mammals, we studied the possible influence of insulin on NPY gene expression in the hypothalamus of rainbow trout. The NPY mRNA content of the hypothalamus-preoptic area (H-POA) was determined by qPCR, 12 h and 24 h after intracerebroventricular (i.c.v.) administration of a single dose of insulin (90 µg kg^{-1}). Anorectic activity was observed 24 h after insulin administration, along with a slight, but significant, reduction in NPY mRNA levels, suggesting that NPY is not a relevant target for insulin-induced inhibition of food intake in rainbow trout.

Keywords*: insulin, NPY mRNA, Hypothalamus, Food Intake, Rainbow Trout*

INTRODUCTION
In vertebrates, regulation of food intake and energy homeostasis are controlled by a complex neural circuitry involving multiple central and peripheral satiety signals that are mainly integrated at the level of the hypothalamus. One of these signals is insulin (Mayer and Belsham, 2009), which enters the central nervous system (CNS), via saturable receptor-mediated uptake across the blood-brain barrier, at a concentration proportional to circulating insulin levels in mammals (Baura *et al*., 1993) and in teleosts (Soengas and Aldegunde, 2004). Thus, central insulin levels increase when circulating hormone levels increase. Insulin receptors are highly expressed in both mammalian and fish brains (Leibush *et al*., 1996). Some studies in mammals have shown that insulin acts as an anorectic signal within the CNS (Air *et al*., 2002; Schwartz *et al*., 1999; Stanley *et al*., 2005; Woods *et al*., 1998). However, the possible action of insulin on feeding behaviour has scarcely been studied in fish. No significant short-term effect on food intake was observed in the channel catfish after central insulin administration (Silverstein and Plisetskaya, 2000). By contrast, an inhibitory effect on food intake was observed in rainbow trout 26 h (central) and 52 h (peripheral) after insulin administration (Soengas and Aldegunde, 2004).

Feeding behaviour in mammals is known to be strongly stimulated by NPY (Schwartz *et al*., 2000; Valassi *et al*., 2008), via activation of Y1 and Y5 receptors (Beck, 2006; Neary *et al*., 2004). Equally, NPY is the principal orexigenic signal in cypriniformes (Narnaware *et al*., 2000; Yokobori *et al*., 2012; Zhou *et al*., 2013), salmoniformes (Aldegunde and Mancebo, 2006) and perciformes (Kiris *et al*., 2007). In rainbow trout, NPY stimulates feeding acting via activation of Y1- and Y2-like receptors (Aldegunde and Mancebo, 2006). In agreement with the orexigenic action of NPY, the hypothalamic levels of this peptide are increased by food deprivation in several fish species (Narnaware and Peter, 2001; Silverstein *et al*., 1999). Such effect of food deprivation on brain NPY mRNA is reversed by refeeding (Narnaware *et al*., 2000).

In mammals, NPY neurons of the arcuate nucleus have been suggested as candidate targets for the anorectic action of insulin (Mayer and Belsham, 2009; Begg and Woods, 2012). Several observations support this hypothesis: 1) insulin administration inhibits hypothalamic NPY gene expression (Wang and Leibowitz, 1997; Sato *et al*., 2005); 2) a decrease in insulin levels leads to an increase in NPY gene expression (Sipols *et al*., 1995); and 3) insulin exerts direct actions on the neurons and acts by repressing NPY/AgRP genes expression through a MAPK MEK/ERK-dependent pathway (Mayer and Belsham, 2009). However, possible neuronal targets for insulin in the hypothalamus of fish have not yet been identified. Given that the actions of insulin and NPY on food intake in rainbow trout are similar to those observed in mammals, the aim of the present study was therefore to investigate if there is a direct link between central insulin administration and the neuropeptide Y in rainbow trout. To accomplish that, the effect of insulin on mRNA NPY abundance in the H-POA was studied. This will enable clarification of some aspects of the pathways mediating the effects of insulin on food intake in rainbow trout.

MATERIAL AND METHODS

Animals and i.c.v. administration

Experiments were carried out with immature (80±10 g body weight) rainbow trout, *Oncorhynchus mykiss*, obtained from a commercial trout farm (Javier de la Calle, Estrada, Pontevedra, Spain) and transported to the experimental aquarium in the Faculty of Biology (University of Santiago de Compostela). Fish were acclimated in 100 l conical tanks for 1 week (20 animals per tank) in constantly running, aerated well water (temperature 11.5 ± 0.5 ºC, pH 6.7 ± 0.3) under a 12h:12h light:dark photoperiod (lights on at 8 am). Fish were fed once a day at 10 am with commercial dry pellets (Dibaq, Proaqua Nutricion S.A., Palencia, Spain; ratio equivalent to 1% of body mass per day). All experiments were carried out in the morning to avoid possible effects of circadian variations. Following acclimation, fish were randomly assigned to experimental tanks (see below for details). Fish were anaesthetized with 2-phenoxyethanol (0.5 ml l^{-1}) before being handled, injected or sampled. The experiments were designed and carried out in accordance with national guidelines (decree 53/2013, BOE 34) for animal experimentation.

Human insulin was purchased from Sigma Chemical Co. (St Louis, USA) and dissolved in 35% 10x PBS, 62.5% 0.005 N HCl, and 2.5% 1 M NaOH, pH 7.4 (vehicle, VEH). Insulin was administered by i.c.v. injection, as previously described (Mancebo *et al*., 2013; Pérez-Maceira *et al*., 2014, 2016). Briefly, a 10 µl Hamilton microsyringe fitted with a 26G needle was used, as follows: each fish was placed with the dorsal side upwards and the tissue covering the skull was removed by scraping, to facilitate rapid visualization of the injection point on the midline, at the level of the anterior point of union of the optic tectum lobes. A small hole was made at this point (with a 25G needle) and the Hamilton microsyringe (fitted with a 26G needle) was immediately placed in the hole leading to the third ventricle, at the level of the preoptic nuclei, and was held in place while the 2 µl dose was slowly infused. The needle was then held in position for 5-10 s to enable clearance. Fish vehicle groups were i.c.v. injected with the VEH alone.

Effect of insulin on food intake

24 h-fasted fish were injected i.c.v. at 10 am with either VEH (15 fish: 5 fish per tank; n = 3) or insulin (90 µg kg^{-1}) (15 fish: 5 fish per tank; n = 3) (Soengas and Aldegunde, 2004). 12 h and 24 h after compounds administration, fish were fed and food intake (FI) was determined as previously described (Mancebo *et al*., 2013; Pérez-Maceira *et al*., 2014). Briefly, food was supplied in batches of four to five pellets every 3-4 s over a 10 min assay period. The uneaten food remaining at the bottom of the tank after the assay period was removed, dried and weighed, for accurate calculation of the total amount of food consumed. The basal FI was calculated for all fish (vehicle and insulin-treated) over four consecutive days, prior to any experimental manipulation. If any significant variation was observed over the four days, the experiment was delayed until a constant 4-day period was obtained. For data analysis, the resulting values (g of food per kg body mass per 10 min assay) were expressed as a percentage of the corresponding basal level (i.e. the mean intake in g kg^{-1} per daily 10 min assay, for the 4-day basal period line).

Effect of insulin on abundance of NPY mRNA in the hypothalamic-preoptic area

Two experiments were carried out to determine the possible involvement of H-POA NPY in the anorectic effect of insulin. In the first experiment, 24 h-fasted fish were injected i.c.v. with either VEH (vehicle group, n = 4) or a 90 µg kg^{-1} dose of insulin (treated group, n = 4). Twelve hours after injection, fish were sacrificed to obtain the H-POA. The same procedure was used for the second experiment, except that fish were sacrificed 24 h after i.c.v. injection (vehicle group, n = 4, and treated group, n = 4). Both experiments were replicated three times (thereby, at the end of each experiment: vehicle group n = 12, and treated group n = 12).

For tissue sampling, fish were removed from the experimental tanks, anaesthetized and rapidly sacrificed by decapitation. The brains were quickly removed and the H-POA was immediately dissected out, frozen in liquid nitrogen and stored at -80 °C until assay.

Total RNA was isolated from H-POA by using the Norgen Animal Tissue RNA Purification Kit (Norgen Biotek Co. Thorold, Ontario, Canada) according to the manufacturer's protocol. DNase treatment was included in the RNA Purification Kit. The RNA concentration obtained and DNA and organic contamination were evaluated by analysis of samples in a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.). Subsequent electrophoresis on a 1% agarose gel and spectrophotometry analysis revealed no DNA contamination and a high stability of total RNA.

A quantitative *real-time* PCR (qPCR) assay was developed to measure the mRNA levels of the studied gene in the H-POAs. In the first step, 1 µg of total RNA was reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The reaction mix provided by the manufacturer contains oligo (dT) and random hexamer primers. Reverse transcription (RT) reaction conditions were as follows: 25 ºC for 5 min, an extension of 30 min at 50 ºC and a denaturation step of 85 ºC for 5 min. Final incubation was carried out after loading 1 µl of *E. coli* RNAse H, at 37 °C for 20 min. All RT reactions were performed in a Biometra T1 Thermocycler (Göttingen, Germany). After synthesis, cDNA was stored at -80 ºC until posterior analysis. In the second step, target gene expression levels were determined by qPCR, which was implemented using SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The specific rainbow trout primer sequences used for target gene *npy* and reference genes *β-actin* and *elongation factor 1α* (*ef-1α*) are shown in Table 1 and were obtained from Thermo Fisher Scientific Inc. Genes were amplified in singleplex qPCR runs by using a 96-well plate loaded with 5 µl of cDNA and 1 µl of each forward and reverse primer (500 nM) in a final volume of 20 µl.

Table 1. Nucleotide sequences of *Oncorhynchus mykiss* specific primers used to evaluate mRNA NPY abundance by qPCR analysis.

	Primer sequence	ACCN
npy	F: 5'-CCCTGACACACTGGACACAC-3' R: 5'-CATTGGGGTTGTTAGGATGG-3'	AF203902.1
β-actin	F: 5'-ACCCTCAGCTCGTTGTAGA-3' R: 5'-ACGGATCCGGTATGTGCAA-3'	AJ438158
ef-1α	F: 5'-GGGCAAGGGCTCTTTCAAGT-3' R: 5'-CGCAATCAGCCTGAGAGGT-3'	AF498320

ACCN, GenBank Accession Number; F, forward primer; R, reverse primer; npy: neuropeptide Y; ef-1α: elongation factor 1α.

Each PCR run included a standard curve for the corresponding gene including three replicates of five serial dilution points. An end-point PCR was carried out using the Dream Taq Green PCR Master Mix (Thermo Fisher Scientific Inc.), and the primers included in Table 1 were used to obtain the templates for standard curves. The PCR products were then visualized on a 2.5% agarose gel and finally isolated and purified using NucleoSpin Extract II (Macherey-Nagel GmbH & Co, KG) following the manufacturer's instructions. The DNA concentration of the purified products was measured using the NanoDrop® ND-1000. The qPCR cycling conditions consisted of a ramp of 95 ºC for 30 sec, 40 cycles of a two-step amplification program (95 ºC for 5 sec and 57 ºC for 30 sec), and a final extension of 95 ºC for 20 sec and 72 ºC for 20 sec. A melting curve was systematically monitored at the end of each run to confirm the specificity of the amplification reaction. All runs were performed using an iCycler Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). We followed the MIQE Guidelines (Bustin *et al*., 2009) in order to establish meaningful outcomes of the technique and to assess correctly whether the *npy* gene expression was regulated by insulin.

Statistical analysis

Data are expressed as means ± S.E.M. Treatments were compared using a Student's *t*-test at a significance level of $P < 0.05$. Analyses were carried out using SigmaPlot 12.0. For the experiments involving NPY gene expression, the Kolmogorov-Smirnov normality test and Levene's test for homogeneity of variances were used to check for normality of distribution and homocedasticity of the *npy*, *β-actin* and *ef-1α* gene expression data. A student's t test and nested ANOVA were used to evaluate the validity of *β-actin* and *ef-1α* as good reference gene for purposes of normalization and to assert statistical differences in normalized NPY Ct values between vehicle and treatment groups.

RESULTS AND DISCUSSION

The results of food intake experiments (12 h and 24 h after i.c.v. administration of insulin) are shown in Figure 1. The FI decreased significantly ($P < 0.05$) 24 h after i.c.v. administration of insulin (90 μg kg^{-1}). However, no effects on FI were observed 12 h after the administration of insulin.

The effects of the administration of insulin (90 μg kg^{-1}) on the abundance of NPY mRNA in the H-POA are shown in Figure 2. The i.c.v. administration of insulin produced a small but significant ($P < 0.05$) decrease in the NPY mRNA abundance 24 h after i.c.v. injection. Administration of insulin did not have any significant effect on NPY mRNA abundance after 12 h.

The *β-actin* and *ef-1α* genes are the most commonly used as references for studying gene expression in rainbow trout (Mancebo *et al*., 2013; Palti *et al*., 2010). However, in the present study, insulin administration significantly modified *β-actin* expression relative to vehicle group, but did not affect *ef-1α* expression. Each sample was therefore normalized to the expression level of the reference gene *ef-1α*.

We first checked the effect of insulin administration on food intake in rainbow trout and observed an inhibitory effect, although only 24 h after its administration. These results are consistent with our previous findings demonstrating that treatment with insulin inhibits FI in rainbow trout 26 h or 52 h after of administration, but not after a shorter period of time (Soengas and Aldegunde, 2004). The insulin-induced inhibition of FI seems to be species-specific, as no effect was observed after central administration of insulin in channel catfish (Silverstein and Plisetskaya, 2000). These discrepancies may be due to the feeding nature of these two species (carnivorous, the rainbow trout, and omnivorous, the channel catfish). The effects on FI observed in rainbow trout are similar to those described in mammals in relation to the central action of insulin on FI, in which the effects of the hormone can last from hours to days (Schwartz *et al*., 1999). Once the anorectic action of insulin was confirmed, thus validating the experimental design, our objective was to determine whether the anorectic action of insulin can alter the H-POA NPY neuron activity.

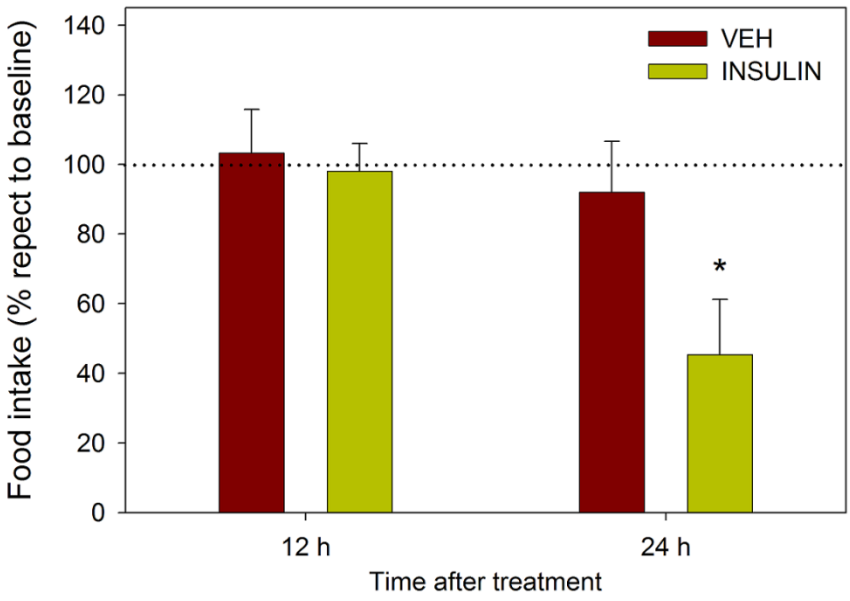

Figure 1. Effects of i.c.v. administration of VEH (vehicle) or insulin (90 µg kg^{-1}) on food intake after 12 h and 24 h in rainbow trout. Food intake is shown as mean ± SEM of food intake (g per kg body mass per 10-min assay) expressed as a percentage of the basal level (calculated as mean food intake over the four days prior to the experiment, g kg^{-1} per 10-min assay). The values are shown as means ± SEM of three replicate experiments with 5 fish per group (n = 3). Asterisk * indicates a significant difference relative to the corresponding vehicle group (*P* < 0.05).

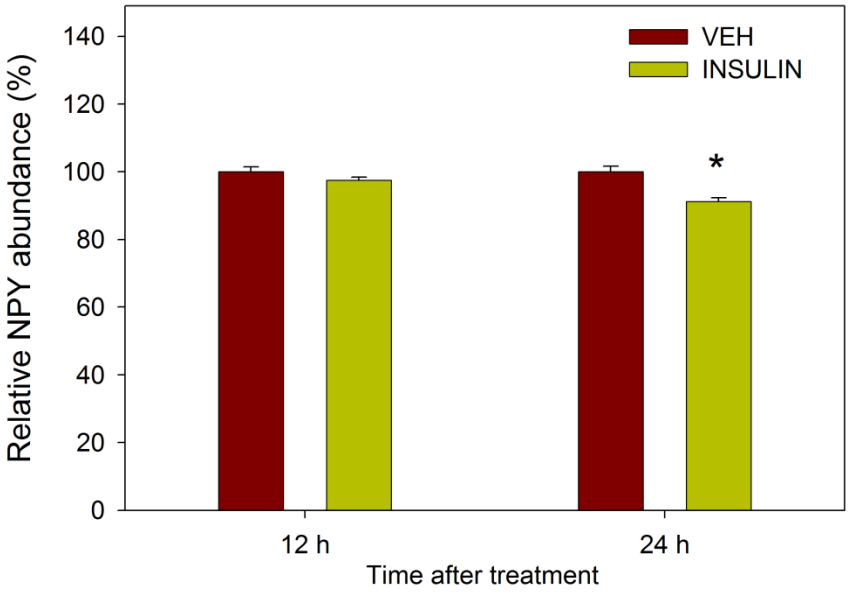

Figure 2. Relative abundance of mRNA NPY in the hypothalamus-preoptic area of rainbow trout 12 h and 24 h after i.c.v. administration of VEH (vehicle) or insulin (90 µg kg^{-1}). Each value is the mean ± S.E.M of 12 fish per group. Data represent the change relative to the vehicle group (VEH), which was considered as 100% expression in each experiment. An asterisk * indicates a significant difference relative to the corresponding vehicle group (*P* < 0.05).

We demonstrate here for the first time in fish that insulin decreased the abundance of NPY mRNA in the H-POA after 24 h, in parallel with its inhibitory action on food intake. We observed a correlation between insulin administration, inhibition of food intake and the decrease in NPY abundance in rainbow trout, similarly to observations in mammals (Begg and Woods, 2012; Mayer and Belsham, 2009; Sato *et al*., 2005; Wang and Leibowitz, 1997). Nevertheless, while the observed insulin-induced FI intake inhibition in this study was around 50%, the reduction in NPY mRNA levels was significantly lower (around 9%). This suggests that NPY is not the main target for insulin-induced food intake inhibition in rainbow trout. In mice, selective ablation of insulin receptors in POMC or AgRP neurons does not affect body weight or food intake (Könner *et al*., 2007). As indicated for mice, different neurons other than NPY neurons could mediate the ability of insulin to suppress FI in rainbow trout.

In mammals, NPY stimulates food intake with a preferential effect on carbohydrate intake (Beck, 2006). However, rainbow trout is a carnivorous fish whose natural diet is poor in carbohydrates. This fish is known for its inefficiency in using high levels of dietary carbohydrates, and ingestion or administration of glucose leads to prolonged hyperglycemia (Baños *et al*., 1998; Blasco *et al*., 1996), as occurs in mammals with non-insulin-dependent diabetes (Moon, 2001). We therefore postulate that the minor role of NPY in the anorectic effect of insulin may be related to adaptation to a diet low in carbohydrates, which could be linked to fewer, or less sensitive, insulin receptors in the NPY/AgRP neurons in the H-POA.

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