Experimental hyperthyroidism and central mediators of stress axis and thyroid axis activity in common carp (Cyprinus carpio L.)

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Abstract

The effect of experimental hyperthyroidism, realized by T4 injection, on central mediators of the hypothalamo–pituitary–interrenal axis (HPI-axis) in common carp (Cyprinus carpio L.) was studied. Our results show that hyperthyroidism evokes a marked 3.2-fold reduction in basal plasma cortisol levels. Corticotropin-releasing hormone-binding protein (CRH-BP) mRNA levels in the hypothalamus, measured by real-time quantitative PCR, were significantly elevated by 40%, but CRH, urotensin-I, prepro-TRH, prohormone convertase-1 (PC1), and POMC mRNA levels were unchanged. In the pituitary pars distalis, PC1, CRH receptor-1, and POMC mRNA levels were unaffected, as was ACTH content. Plasma α-MSH concentrations were significantly elevated by 30% in hyperthyroid fish, and this was reflected in PC1 and POMC mRNA levels in pituitary pars intermedia that were increased 1.5- and 2.4-fold respectively. The α-MSH content of the pars intermedia was unchanged. Hyperthyroidism has profound effects on the basal levels of a central mediator, i.e., CRH-BP, of HPI-axis function in unstressed carp in vivo, and we conclude that HPI- and hypothalamo–pituitary–thyroid-axis functions are strongly interrelated. We suggest that the changes in plasma cortisol, thyroid hormone, and α-MSH levels reflect their concerted actions on energy metabolism.

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Introduction

The synthesis and the secretion of thyroxine (T4) by the thyroid gland are stimulated by thyroid-stimulating hormone (TSH) from the pituitary gland. In mammals, TSH secretion is under positive hypothalamic control by thyrotropin-releasing hormone (TRH), and thyroid hormones exert a classical negative feedback on the expression of TSH (Shupnik 2000) and TRH (Koller et al. 1987, Kakucska et al. 1992, Guissouma et al. 1998). In teleosts also, inhibitory regulation by thyroid hormones of pituitary TSH expression has been confirmed (Larsen et al. 1997, Pradet-Balade et al. 1997, Yoshiura et al. 1999, Chatterjee et al. 2001), but, to the best of our knowledge, the only non-mammalian study showing an inhibitory effect of T3 and T4 on the expression of TRH was conducted on chicken (Lezoualc’h et al. 1992).

Studies on bighead carp (Aristichthys nobilis), arctic charr (Salvelinus alpinus), and Japanese eel (Anguilla japonica) indicate a role for TRH in fishes similar to that in mammals (Eales & Himick 1988, Chatterjee et al. 2001, Han et al. 2004). However, in the lungfish (Protopterus ethiopicus), Nile tilapia (Oreochromis niloticus), and common carp (Cyprinus carpio) TRH does not stimulate TSH secretion (Gorbman & Hyder 1973, Melamed et al. 1995, Kagabu et al. 1998). In several non-mammalian vertebrates, including teleosts, factors other than TRH were found to be thyrotropin-releasing factors. Indeed, in coho salmon (Oncorhynchus kisutch), corticotropin-releasing hormone (CRH) exerts thyrotropic effects on cultured-pituitary cells (Larsen et al. 1998). A direct stimulatory action of CRH was also demonstrated for amphibian, reptilian, and avian pituitary thyrotropes (reviewed by De Groef et al. (2006)).

The principal action of hypothalamic CRH in teleosts is the regulation of the stress response (Huisings et al. 2004, Flik et al. 2006). In teleostean fishes, that lack a median eminence, neurons from the nucleus preopticus (NPO) send axons directly to the pituitary gland where they release CRH upon registration of a stressor. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from corticotrope cells in the pituitary pars distalis (Metz et al. 2004) by the activation of the CRH receptor-1 (CRH-R1; Huisings et al. 2004). ACTH is derived from pro-opiomelanocortin (POMC) through the action of prohormone convertase-1 (PC1; Benjamet et al. 1991, Castro & Morrison 1997), and stimulates the secretion of cortisol from the interrenal cells in the head kidney (Flik et al. 2006).
The effect of CRH on pituitary corticotropes is modulated by CRH-binding protein (CRH-BP), a hypothalamic factor that binds CRH with a higher affinity than does CRH-R1 (Potter et al. 1991, Cortright et al. 1995). The primary action of CRH-BP is generally thought to be inhibitory on the CRH-induced ACTH release from pituitary corticotropes (Potter et al. 1991, Cortright et al. 1995, Westphal & Seasholtz 2006). In common carp, CRH-BP immunoreactivity is localized in perikarya in the recessus opticus, a hypothalamic region distinct from the NPO, and colocalizes in CRH-immunopositive nerve fibers projecting from the NPO to pituitary corticotropes (Huising et al. 2004). Beside CRH, urotensin-I (UI), and CRH-R2, two other members of the teleostean CRH system (Flik et al. 2006), have also been implicated in the stress response of teleosts (Fryer et al. 1983, Bernier & Craig 2005). UI has a potent thyrotropic effect on coho salmon pituitary cells, similar to CRH (Larsen et al. 1998). However, the exact role of UI in the stress response and thyroid metabolism of teleosts has scarcely been addressed. Other members of the teleostean CRH-family are urocortin-2 and -3 orthologs (Hsu & Hsueh 2001, Lewis et al. 2001, Boorse et al. 2005), but, as yet, no data are available on their function in fish.

The dual function of CRH as a corticotropin- and a thyrotropin-releasing hormone suggests a functional relationship between the hypothalamo–pituitary–interrenal axis (HPI-axis) and the hypothalamo–pituitary–thyroid axis (HPT-axis). This is further corroborated by the finding, in fishes, that experimentally elevated plasma cortisol or thyroid hormone levels correlate negatively with plasma T4 and cortisol respectively (Redding et al. 1984, Mustafa & MacKinnon 1999). These findings, and in particular the absence of a thyrotropic effect of TRH in carp and the thyrotropic effect of CRH and UI in coho salmon, led us to postulate that thyroid hormones may have important regulatory effects on mediators of stress axis activity in the central nervous system of common carp.

Materials and methods

Animals and animal procedures

Common carp (Cyprinus carpio L.), hereafter called carp, of the all-male E4×R3R8 isogenic strain (Bongers et al. 1997) and with a body weight of 41 ± 12 g (mean ± s.d.) were obtained from the Department of Fish Culture and Fisheries of Wageningen University (The Netherlands). Fish were kept in stock in 150 l tanks, with circulating filtered Nijmegen city tap water at 22 °C, at a photoperiod ratio of 16 h light:8 h darkness. Fish were fed commercial fish food (Trouvit; Trouw, Putten, The Netherlands) at a ration of 1.5% of the estimated body weight per day.

Fish were injected intraperitoneally (i.p.) on day 1 of the treatment regimen with 10 μg T4 (from Sigma Chemical Co.) per gram body weight in saline (0.9% NaCl), followed by i.p. injections of 1 μg T4 per gram body weight every third day for a period of 2 weeks. Control fish were injected with vehicle only. Fish were sampled on the first day after administration of the final injection. To this end, fish were deeply anesthetized in 0.1% 2-phenoxyethanol; mixed arterial and venous blood was collected by puncture from the caudal vessels using heparinized 23 gauge needles. Animals were then killed by spinal transection, and pituitary gland, hypothalamus and preoptic region (hereafter referred to as hypothalamus), and head kidneys were dissected. Plasma was obtained from whole blood by centrifugation (15 min, 15 000 g, 4°C), and stored at −20 °C until further analysis. Pituitary glands used for pituitary hormone content measurements, and those used for measurements of tissue POMC, PC1, CRH-R1, and CRH-R2 mRNA levels were bisected into pars distalis and pars intermedia. Pituitary glands used for measurements of TSH β-subunit mRNA were left intact. Tissues were immediately frozen in dry ice and stored at −80 °C until further processing.

In vitro superfusion and static incubation of carp head kidney

Head kidneys from two untreated carp were removed directly after anesthesia and placed individually in a superfusion chamber. Tissues were superfused with a Hepes–Tris-buffered medium (pH 7.4) saturated with carbogen (95% O2–5% CO2), at a rate of 30 μl/min as described in detail by Metz et al. (2005). When a stable baseline of cortisol secretion was obtained, T4 (at 1 μM), 3,5,3′-triiodothyronine (T3; 1 μM), or human ACTH(1–39) (hACTH; 0.1 μM), were administered for a period of 30 min. Ten- or twenty-minute fractions were collected, stored on ice, and analyzed for cortisol content as described later. Thyroid hormones and hACTH were obtained from Sigma Chemical Co.

Head kidneys from five untreated carp were obtained. The head kidneys obtained from one animal were pooled, diced into approximately 1 mm3 sized fragments, and suspended in the same buffer that was used for superfusion experiments. The suspension was distributed in a 12-well plate in 2 ml volumes. Ten nanometers of T4 were added; control incubations received the saline vehicle only. After 16 h, the incubation medium was sampled for cortisol, after which 30 nM hACTH were added and left to incubate for 1 h, subsequently, the medium was sampled again.
RNA extraction and cDNA synthesis

To extract total RNA, selected tissues were removed from seven animals from the control and T4-treated group respectively, and homogenized in 500 μl TRIzol reagent (Invitrogen) by ultrasonification. Following a DNase treatment (Invitrogen), 1 μg RNA was reverse transcribed to cDNA in a 20 μl reaction mixture containing 300 ng random primers, 0.5 mM dNTPs, 10 mM dithiothreitol, 10 U RNase inhibitor, and 200 U Superscript II Reverse Transcriptase (Invitrogen) for 50 min at 37 °C and stored at −20 °C.

Cloning and sequencing of carp PC1 cDNA

Primer sequences used are shown in Table 1; oligonucleotides were purchased from Biolegio (Malden, The Netherlands). PC1 sequences were retrieved from the puffer fish (Takifugu rubripes) and zebrafish (Danio rerio) genomes via a BLAST search at the Ensembl site (http://www.ensembl.org) using mouse PC1 cDNA as a query sequence. Mouse PC1 cDNA was aligned with the retrieved puffer fish and zebrafish sequences, and degenerate primers PC1-fw and PC1-rv were designed based on conserved sequences. To obtain a carp PC1 nucleotide sequence, a PCR was carried out on carp pituitary pars distalis cDNA using primers PC1-fw and PC1-rv. PCR products were separated by electrophoresis on a 1% agarose–ethidium bromide gel, ligated into a pCR4-TOPO vector, and introduced into competent TOP10 Escherichia coli cells (TOPO TA Cloning Kit; Invitrogen). Successfully transfected cells were selected on LB–kanamycin agar and checked for appropriately sized inserts using primers T3 and T7. Successfully transfected plasmids were extracted (Miniprep; Bio-Rad), sequenced using primers T3 and T7, and analyzed on an ABI Prism 310 automated sequencer (PE Applied Biosystems, Foster City, CA, USA). The 5'- and 3'-ends were obtained by rapid amplification of cDNA ends (RACE)-PCR using the GeneRacer kit (Invitrogen). One microgram of RNA from carp pituitary pars distalis was processed according to the GeneRacer protocol (Invitrogen) to obtain RACE-ready cDNA. PCR was performed using gene-specific primers (PC1-3’RACE and PC1-5’RACE) and the GeneRacer ligated primers. Template DNA was mixed with 600 nM GeneRacer primer and 200 nM gene-specific primers in

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Y: C or T/U; S: C or G; K: G or T/U.
a volume of 50 µl containing 1·75 mM MgCl₂, 350 µM dNTPs, and 3·75 U Taq polymerase–Tgo polymerase mix (Roche Applied Science). The 5′- and 3′-nested PCRs (primers: PC1-3/RACEn and PC1-5/RACEn) were then performed on the PCR products to further amplify the PC1 cDNA fragments. Nested PCR products were analyzed on agarose gel, cloned into a TOPO vector, and sequenced as described previously. The authenticity of the consensus sequence from four to six clones was confirmed from alignments with PC1 cDNAs from angler fish (Roth et al. 1993) and mouse, and the putative PC1 cDNAs from the puffer fish and zebrafish genomes. The carp PC1 nucleotide sequence thus obtained has been submitted to the DDBJ–EMBL–GenBank databases under accession number AM236095.

Hormone measurements

Pituitary pars distalis and pars intermedia tissues, obtained from five to seven animals, were homogenized on ice in 200 µl 10 mM HCl in a Potter–Elvehjem device. Homogenates were diluted in demineralized water 200-fold before analysis. Blood plasma, obtained from 19 control animals and 17–21 T₄-treated animals, was diluted fivefold in RIA buffer prior to cortisol measurements, plasma analyzed for α-melanocyte-stimulating hormone (MSH) was not diluted. ACTH measurements, plasma analyzed for the α-subunit of human ACTH 1–24 (Biogenesis, Poole, UK) was used. The Journal of Molecular Endocrinology CRH-R2, and TSH was diluted fivefold in RIA buffer prior to cortisol concentration. ACTH- and CRH-BP was measured. In general, cDNA was diluted 50-fold, but cDNA from pituitary pars distalis and pars intermedia was diluted 25 000-fold to measure POMC gene expression. Five microliters of cDNA were used in a 25 µl reaction mixture consisting of 12·5 µl Taq Green Master Mix (PE Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands) and 3 µl of each primer (600 nM final concentration) were added. The primer sets used for PCR are shown in Table 1. RQ-PCR was performed on a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems). The reaction mixture was incubated for 10 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C and 1 min annealing and extension at 60 °C. Melting curves confirmed the identity and specificity of the PCR products. Dissociation plots were analyzed and cycle threshold (Cₜ) values were determined. The expression of genes of interest was calculated relative to β-actin and 40S ribosomal protein S11 expression. Both control genes yielded similar results and therefore all results in this study are expressed relative to β-actin mRNA expression.

Statistical analysis

All data are presented as mean values ± s.e.m. The number of different preparations (n) is given in parentheses. Differences between groups were assessed with Student’s unpaired t-test. Statistical significance was accepted at P < 0·05 (two-tailed), probabilities are indicated by asterisks (*P < 0·05; **P < 0·01; ***P < 0·001).

Results

Animal model validation

Plasma-free T₄ concentrations were elevated 5·3-fold (P < 0·001) in T₄-treated fish (Fig. 1A) and pituitary TSH β-subunit mRNA levels were 3·9-fold (P < 0·001) lower than in control animals (Fig. 1B), which confirmed a successful induction of hyperthyroidism. The validation of our experimental hyperthyroid animal model is further corroborated by the hyperactive behavior of the T₄-injected group, in particular during feeding, compared with the control group (personal observations).

Plasma hormone levels

In control animals, basal plasma cortisol levels were 54 ± 6 ng/ml (equivalent to 149 ± 18 nM; n = 19), which is similar to the values measured in unstressed carp reported elsewhere (van den Burg et al. 2003).
In hyperthyroid carp, basal plasma cortisol levels were reduced 3.2-fold \((P<0.001;\) Fig. 2A). Plasma \(\alpha\)-MSH levels in control carp were 0.30 ± 0.11 nM \((n=19)\), which values are, again, within the range measured in unstressed carp \(\) (van den Burg et al. 2003, 2005a, Metz et al. 2005). In hyperthyroid carp, basal \(\alpha\)-MSH levels had increased by 30\% \((P<0.05;\) Fig. 2B).

**Pituitary pars distalis and hypothalamus**

In the pituitary pars distalis, no significant changes in POMC, PC1, and CRH-R1 mRNA expression levels relative to \(\beta\)-actin in the pituitary glands of saline-injected \((n=7)\) and \(T_4\)-injected \((n=17)\) animals were detected. Concomitantly, the ACTH content of the pituitary pars distalis was similar in saline-injected and \(T_4\)-injected carp \((189\pm16\) and \(176\pm23\) pg/gland respectively; \(n=7;\) \(P=0.65)\).

In the hypothalamus, mRNA levels of POMC, CRH, UI, prepro-TRH, and PC1 were not affected by the \(T_4\) treatment, but CRH-BP mRNA levels were elevated significantly by 40\% in hyperthyroid animals \((P<0.05;\) Fig. 3).

**Pituitary pars intermedia**

In the pituitary pars intermedia of hyperthyroid carp POMC and PC1 mRNA expression levels were elevated 1.5-fold \((P<0.05)\) and 2.4-fold \((P<0.01)\) respectively; whereas the expression of CRH-R1 mRNA was not affected by \(T_4\) treatment. CRH-R2 mRNA was not detectable \((\) Fig. 4). The \(\alpha\)-MSH content of the pars intermedia of hyperthyroid animals was 30 ± 5 pmol/gland \((n=5)\), and had not changed significantly compared with controls, whose value was 32 ± 4 pmol/gland \((n=7;\) \(P=0.77)\).

**In vitro head kidney incubations**

In two pilot experiments, carp head kidneys superfused \(\) in vitro displayed a basal cortisol secretion rate of 58 ± 9 pg/min per head kidney, which is similar to values reported earlier \(\) (Metz et al. 2005). Following a 20-min exposure to 1 \(\mu\)M \(T_4\) or \(T_3\), the basal cortisol release was virtually unchanged; the viability of the head kidney

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**Figure 1** (A) Plasma-free T4 concentrations (pM) in saline-injected \((n=19)\) and \(T_4\)-injected \((n=21)\) animals. (B) Expression levels of TSH \(\beta\)-subunit mRNA relative to \(\beta\)-actin in the pituitary glands of saline-injected \((n=7)\) and \(T_4\)-injected \((n=7)\) animals. Student’s t-test was used for statistical evaluation.

**Figure 2** (A) Plasma cortisol and (B) \(\alpha\)-MSH concentrations in saline-injected \((n=19)\) and \(T_4\)-injected \((n=21 and 17\) respectively) animals. Student’s t-test was used for statistical evaluation.
tissue was confirmed by stimulation with 0.1 μM hACTH, to which the tissue responded with a pronounced rise in the cortisol secretion rate (results not shown). Based on these observations, we decided to perform long-term incubations of head kidneys with thyroid hormones. After a 16-h static incubation, the cortisol concentration in the control medium was 0.26 ± 0.03 nM (n=5). When head kidney tissue was incubated in the presence of 10 nM T4 for the same period of time, the cortisol concentration in the incubate was similar: 0.27 ± 0.07 nM (n=5; P=0.86). Upon subsequent incubation with 0.1 μM hACTH for 1 h, the cortisol concentration in the control medium had increased to 4.4 ± 1.3 nM (n=5), and that in the medium containing 10 nM T4 to 3.1 ± 1.8 nM (n=5; P=0.59), confirming the viability of the head kidney tissue after a prolonged static incubation.

Discussion

Basal plasma cortisol levels in the control group were similar to the values measured in unstressed carp in other studies (van den Burg et al. 2003, Metz et al. 2004). Moreover, the basal cortisol levels we measured, one day after the final i.p. injection was administered, are considerably lower than the concentrations that were measured in carp subjected to an acute or chronic stressor, which displayed plasma cortisol levels that were increased 5- to 26-fold (Weyts et al. 1997, van den Burg et al. 2003, Huising et al. 2004, Metz et al. 2004) and 5- to 10-fold (Huising et al. 2004, Metz et al. 2005) respectively, compared with control levels. It was also observed that after the removal of an acute stressor, the elevated plasma cortisol levels in carp decrease rapidly, i.e., within 90–120 min, to basal control levels (Weyts et al. 1997, Huising et al. 2004), and that no changes occur in the hypothalamic expression of CRH and CRH-BP (Huising et al. 2004). We judge that our injection protocol per se did not constitute a chronic stressor, and did not result in acclimation of the experimental animals. The cortisol concentrations that were measured in the control group reflect basal, unstressed levels.

We here demonstrate in a validated experimental animal model that experimentally induced hyperthyroidism results in a marked hypocortisolemia. The decrease in basal plasma cortisol correlates with an increase in hypothalamic CRH-BP mRNA expression. Since the release of cortisol from viable carp head kidney tissue does not respond to short- and long-term exposure to T4 in vitro, we exclude the interrenal cells as a target for direct thyroid hormone action. Taken together, these results support a role for thyroid hormone as a regulator of the teleostean stress axis, by the modulation of CRH-BP expression and, hence, the bioavailability of ligands from the CRH family. In carp, CRH-BP is expressed in a cell population that is clearly distinct from the CRH-immunoreactive nucleus preopticus (Huising et al. 2004), and these neurons could well be the target for thyroid hormone action.

Our experimental design only allowed the determination of end-point measurements. Earlier changes, if present, in the CRH and thyroid systems of our animals would therefore not be detected. Indeed, van den Burg et al. (2005b) exposed carp to an acute change in ambient temperature and demonstrated, using functional magnetic resonance imaging, that there is a fast response (i.e., already evident after 30 s) and a clear chronology in the activation and deactivation of brain areas that are involved in the stress response. On a time scale of hours to days, chronically stressed carp differentially express members of the CRH system, and have blunted, but still elevated plasma cortisol levels (Huising et al. 2004, Metz et al. 2005).
which reflect acclimation of the fish to the stressor. Our results can then be interpreted as the acclimation of the CRH and thyroid systems to a chronic hyperthyroidism. We are presently designing time-course studies to investigate the dynamics of the interactions between the CRH and the thyroid systems.

The activation of the hypothalamo–pituitary–interrenal (HPI) axis in teleosts results in the release of cortisol, the prime stress hormone. The main function of cortisol in the stress response of teleostean fish is the redistribution of energy away from, e.g., growth and reproduction, and toward physiological functions required for coping with the stressor and to restore homeostasis (Wendelaar Bonga 1997, Schreck 2000). Cortisol stimulates gluconeogenesis and lipolysis (Sheridan 1988, van der Boon et al. 1991), which result in increased plasma glucose and free fatty acid levels. These catabolic actions of cortisol reflect its glucocorticoid potency that is commensurate to the central role of this hormone in the stress response and the regulation of energy metabolism.

As is cortisol, thyroid hormone is fundamentally implicated in the regulation of energy metabolism in vertebrates. Thyroid hormone stimulates basal metabolic rate and oxygen consumption in several teleostean tissues (Peter & Oommen 1989, Lynshiang & Gupta 2000), and has profound effects on lipid, carbohydrate, and protein metabolism in teleosts (Plisetskaya et al. 1983, Sheridan 1986, Ballantyne et al. 1992, Scott-Thomas et al. 1992, Shameena et al. 2000). In addition, during prolonged fasting, plasma T₃ levels decrease in Nile tilapia (Oreochromis niloticus; Van der Geyten et al. 1998), which could well represent a mechanism for the adaptation, by downregulating energy expenditure, to caloric and nutritional deprivation.

Since both the HPI- and HPT-axis are fundamentally involved in the regulation of energy expenditure, it can be anticipated that, of necessity, mutual interactions between these two exist. Indeed, European eel (Anguilla anguilla) and coho salmon (Oncorhynchus kisutch) treated with cortisol showed suppressed plasma T₃ levels (Redding et al. 1984, 1986). It is interesting to note that Larsen et al. (1998) demonstrated thryotropic effects of CRH-family peptides in cultured pituitary cells from the latter species. Two other salmonid species, Atlantic salmon (Salmo salar) and arctic charr (Salvelinus alpinus), treated with cortisol or given iodide supplementation, showed predictably increased plasma cortisol and thyroid hormone levels respectively, and that correlated negatively with plasma thyroid hormone and cortisol levels respectively (Mustafa & MacKinnon 1999). We here suggest that these negative correlations between plasma cortisol and thyroid hormone are caused by the interaction of the HPI- and HPT-axis in the brain of the fish. This would allow the integration of afferent signals and the coordinated regulation of, ultimately, the interrenal cells and the thyroid gland.

To our knowledge, this is the first study to simultaneously investigate the in vivo responsiveness of both the CRH system and TRH for treatment with thyroid hormone in a teleost. The upregulation of hypothalamic CRH-BP gene expression in hyperthyroid carp indicates that a member of the CRH family is the principal TSH-releasing factor in this species. This is further corroborated by the non-responsive of prepro-TRH expression and PC1 that is involved in the processing of pro-TRH to mature TRH in the hypothalamus (Schaner et al. 1997). Indeed, in carp (Kagabu et al. 1998) and a number of other teleosts (Gorbman & Hyder 1973, Melamed et al. 1995), TRH does not stimulate TSH secretion. Instead, CRH is the thyrotropic factor (Larsen et al. 1998). CRH has a widespread distribution in the teleostean brain; hypophysiotropic CRH-positive neurons have been identified in the NPO, and the nucleus lateralis tuberis (NLT) and nucleus recessus lateralis (NRL) in the hypothalamus (Matz & Hofeldt 1999, Pepels et al. 2002, Huisjing et al. 2004). Matz & Hofeldt (1999) showed that, in Chinook salmon (Oncorhynchus tshawytscha), CRH-immunoreactive fibers, originating from the preoptic region and NLT, terminate with bouton-like swellings in the proximity of TSH-ir cells in the pituitary pars distalis. These anatomical data, together with our results, provide evidence for the existence of a CRH–TSH–T₄ axis in teleosts. However, since UI is very potent in stimulating the secretion of TSH from cultured teleostean pituitary cells (Larsen et al. 1998), we cannot exclude UI as a thyrotropic principle. Yulis et al. (1986) showed extensive UI-ir staining in hypothalamic perikarya as well as in fibers that innervate the pituitary proximal pars distalis of the white sucker Catostomus commersoni. Indeed, in carp (our unpublished results) and other teleosts (Ueda et al. 1983), the proximal pars distalis harbors TSH-ir cells. Since CRH and UI have a high affinity for CRH-BP (Baigent & Lowry 2000, Valverde et al. 2001), an increased expression of CRH-BP is likely to affect the bioavailability of UI as well as that of CRH. Experimental data clearly point to UI as a thyrotropic factor, and, until matters are conclusively resolved, the teleostean HPT-axis should be represented as a CRH/UI–TSH–T₄ axis.

TRH has also a wide distribution in the teleostean brain, and hypophysiotropic neurons expressing TRH were found in distinct parvocellular and magnocellular regions in the NPO of salmonids (Ando et al. 1998, Díaz et al. 2001). In carp brain, TRH-ir neurons are located in the NRL, and TRH-ir fibers are present in the NLT and NPO (Hamano et al. 1990), which nuclei, in the same animal species, also express CRH (Huisjing et al. 2004). By their anatomical locations, it thus appears...
that hypothalamic CRH-ir and TRH-ir neurons can communicate bidirectionally.

We found that experimental hyperthyroidism resulted in an increase in plasma α-MSH, which was corroborated by increased pituitary pars intermedia POMC and PC1 mRNA expression. Although α-MSH is considered to be corticotropic in Mozambique tilapia in vitro carp interrenal cells (Metz et al. 2005). It was suggested that elevated plasma α-MSH levels sustain HPI-axis activity through a short-loop positive feedback on CRH neurons in the nucleus preopticus (Metz et al. 2005). The opposite changes in plasma cortisol and α-MSH levels in hyperthyroid carp reported here are compatible with a role of α-MSH in the fine-tuning of HPI-axis activity, but our results do not provide conclusive evidence.

We here postulate that the effects observed in this study are probably related via the physiological actions of cortisol and thyroid hormone in the regulation of energy metabolism. α-MSH can fulfill a similar role, although evidence is scarce. In goldfish (Carassius auratus), POMC mRNA expression was observed in brain regions known to control food intake, and intracerebroventricular injection of the α-MSH agonist [Nle⁴, D-Phe⁷]-α-MSH (NDP-α-MSH) inhibited food intake (Cerdá-Reverter et al. 2003). We interpret our experimental results to reflect the concerted actions of cortisol, thyroid hormone, and α-MSH on energy metabolism in carp.

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