Hormones and Behaviour

Neuro
endocrinology

Neuroendocrinology 2001;73:248–260

Received: August 24, 2000
Accepted after revision: January 11, 2001

Appetite-Suppressing Effects of Urotensin I and Corticotropin-Releasing Hormone in Goldfish *(Carassius auratus)*

Nicholas J. Bernier Richard E. Peter

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

Key Words

Urotensin · Corticotropin-releasing hormone · Food intake · Anorexigenic peptides · Adrenal steroids · Stress · Fish · Molecular neuroendocrinology · Telencephalon

Abstract

Fish urotensin I (UI), a member of the corticotropin-releasing hormone (CRH) family of peptides, is a potent inhibitor of food intake in mammals, yet the role of UI in the control of food intake in fish is not known. Therefore, to determine the acute effects of UI on appetite relative to those of CRH, goldfish were given intracerebroventricular (i.c.v.) injections of carp/goldfish UI and rat/human CRH (0.2–200 ng/g) and food intake was assessed for a 2-hour period after the injection. UI and CRH both suppressed food intake in a dose-related manner and UI (ED_{50} = 3.8 ng/g) was significantly more potent than CRH (ED_{50} = 43.1 ng/g). Pretreatment with the CRH receptor antagonist, α-helical $CRH_{(9–41)}$, reversed the reduction in food intake induced by i.c.v. UI and CRH. To assess whether endogenous UI and CRH modulate fish appetite, goldfish were given intraperitoneal implants of the glucocorticoid receptor antagonist, RU-486 (50 and 100 μg/g), or the cortisol synthesis inhibitor, metyrapone (100 and 200 μg/g), and food intake was monitored over the following 72 h. Fish treated with either RU-486 or metyrapone were characterized by a sustained and dose-dependent reduction in food intake. Pretreatment with i.c.v. implants of α-helical $CRH_{(9–41)}$ partially reversed the appetite-suppressing effects of RU-486 and metyrapone. In a parallel experiment, the effects of RU-486 (100 μg/g) and metyrapone (200 μg/g) intraperitoneal implants on brain UI and CRH gene expression were assessed. Relative to sham-implanted controls, fish treated with RU-486 or metyrapone had elevated UI mRNA levels in the hypothalamus and CRH mRNA levels in the telencephalon-preoptic brain region. Together, these results suggest that UI is a potent anorectic peptide in the brain of goldfish and that endogenous CRH-related peptides can play a physiological role in the control of fish appetite.

Introduction

Considerable evidence suggests that the neuropeptide corticotropin-releasing hormone (CRH), in addition to being the major regulator of hypothalamic-pituitary-adrenocortical axis activity, is an endogenous inhibitor of food intake in mammals [1, 2]. Central administration of CRH diminishes food intake [3, 4], and the anorectic effects of

KARGER

Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0028–3835/01/0734–0248$17.50/0

Accessible online at:
www.karger.com/journals/nen

R.E. Peter
Department of Biological Sciences, University of Alberta
Edmonton, Alberta, T6G 2E9 (Canada)
Tel. +1 780 492 4757, Fax +1 780 492 7033
E-Mail dick.peter@ualberta.ca

CRH can be reversed by CRH receptor blockade [5, 6]. Conditions that elevate endogenous hypothalamic CRH levels, such as stress [5, 7] and loss of negative feedback by glucocorticoids [8, 9], also suppress appetite. Further, whereas involuntary overfeeding and satiation lead to an increase in CRH gene expression in the hypothalamic paraventricular nucleus, food restriction has an opposite effect [10, 11]. However, recent evidence has brought into question the role of CRH as a physiologically relevant signal of appetite regulation. In CRH-deficient mice (CRH gene knockout), CRH is not necessary for decreases in food intake induced by either chronic stress [12] or adrenalectomy [13].

The CRH-related peptides fish urotensin I (UI), amphibian sauvagine and mammalian urocortin, are also potent anorexigenic signals in mammals [14–16]. In fact, although the results are equivocal, most studies have found that UI, sauvagine and urocortin are more potent than CRH at inhibiting food consumption [14–19]. Since CRH_2 receptors play an important role in mediating the anorectic effects of CRH-related peptides [2, 6], the differential effects of CRH-related peptides in appetite regulation may be partly explained by the fact that UI, sauvagine and urocortin have a higher affinity for CRH_2 receptors than does CRH [20]. Comparison of the primary structure of CRH-related peptides suggests that UI, sauvagine aud urocortin form a distinct lineage from CRH that arose by gene duplication [21]. At present, the specific involvement of either lineage of the CRH family of peptides in the regulation of food intake and in mediating the impact of stress on appetite is unclear.

Among nonmammalian vertebrates, central injections of ovine CRH (oCRH) have been shown to inhibit food intake in fish [22], amphibian tadpoles [23], pigeons [1] and chickens [24]. As in mammals, the anorectic effects of CRH in goldfish appear to be independent of the activation of the hypothalamic-pituitary-interrenal (HPI) axis and centrally mediated [25]. To our knowledge, however, although originally identified in lower chordates [26, 27], the anorectic properties of either UI or sauvagine have yet to be investigated in their species of origin. Moreover, in nonmammalian vertebrates, whether endogenous CRH-related peptides play a physiological role in the control of food intake or are involved in mediating the effects of stress on appetite remains to be ascertained. Given the longer evolutionary history of UI and CRH peptides in fish, and their distinct neural circuitry [21], studies in teleosts may help to resolve the physiological significance of CRH-related peptides in the control of appetite among vertebrates.

The present study was undertaken to determine the potential role of UI in the control of food intake in goldfish, and to assess whether UI and CRH are endogenous regulators of ingestive behavior in fish. This was achieved in part by assessing the effects of intracerebroventricular (i.c.v.) injections of a wide range of doses of carp/goldfish UI (c/gUI) and rat/human CRH (r/hCRH) on food intake. Given the central role of CRH-related peptides in regulating the HPI axis, we also determined the effects of c/gUI and r/hCRH i.c.v. injections on plasma cortisol. In addition, since blocking the negative feedback action of cortisol appears to stimulate CRH and UI synthesis in fish [28–30], food intake was assessed in goldfish treated with the glucocorticoid receptor antagonist, RU-486, and the cortisol synthesis inhibitor, metyrapone. Finally, experiments using the competitive CRH receptor antagonist, α-helical $CRH_{(9–41)}$, were carried out to determine the potential involvement of central CRH receptors in mediating the effects of exogenous and endogenous CRH-related peptides on food intake.

Materials and Methods

Animals

Goldfish *(Carassius auratus)* of the common or comet variety weighing between 36 and 58 g (47.3 ± 0.2 g, mean ± SEM; n = 510) were obtained from Mount Parnell Fisheries (Mercersburg, Pa., USA) and maintained in either 65-liter glass aquaria (experiments 1–4) or 80-liter fiberglass tanks (experiment 5) at 17 °C under a simulated photoperiod at Edmonton, Alta., Canada. The holding aquaria and tanks received a constant flow of aerated water. Fish were fed ad libitum once daily (10.00 a.m.) with commercially prepared fish food (New Age Pacific 5.0 mm: protein 43%, fat 18%, ash 7%, mineral 2.6% and fiber 2.4%; Moore Clark, Vancouver, B.C., Canada). Fish were acclimated under these standard conditions for a minimum of 3 weeks prior to experimentation, at a density ranging between 7.1 and 10.8 g/l.

Reagents

c/gUI was kindly provided by Dr. J.E. Rivier (Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, Calif., USA). r/hCRH and the CRH receptor antagonist α-helical $CRH_{(9–41)}$ were purchased from Peninsula Laboratories (Belmont, Calif., USA). c/gUI, r/hCRH and α-helical $CRH_{(9–41)}$ stock solutions for i.c.v. injections were dissolved in teleost physiological saline [31], solubilized with 1 *N* NaOH (2%) and subsequently diluted. The saline vehicle for controls was treated in a similar manner.

Experimental Procedures

Assessment of Food Intake. Seventy-two hours prior to experimentation, individual fish of either sex were removed from the holding tanks and placed in separate 65-liter glass aquaria. Thereafter, each goldfish received a daily excess [4% of body weight (BW)] of preweighted food at 10.00 a.m. Uneaten food was collected 2 h later,

desiccated at 100°C for 1 h and weighed. Food intake was calculated as the difference between the initial dry food weight and the adjusted uneaten dry food weight. As a control procedure, the weight reduction of food remaining in water for 2 h and dessicated in parallel with the daily experimental samples was used to adjust the uneaten dry food weights from the experimental aquaria. The uneaten dry food weight was adjusted in order to account for the effects of pellet dissolution during the feeding interval (<4% pellet weight), for potential day-to-day differences in dessication efficiency and for loss in initial food moisture content due to the drying process.

Intracerebroventricular Injections. Brain i.c.v. injections were performed following procedures described by Peter and Gill [32]. Briefly, once fish were deeply anesthetized in a buffered ($NaHCO_3$, 1 g/l) solution of tricaine methanesulfonate (0.5 g/l; MS-222; Syndel, Vancouver, B.C., Canada), a three-sided flap was cut in the frontal bones and folded along its uncut margin to view the dorsal surface of the brain. The fish was then positioned in the holder of a specifically designed stereotaxic apparatus and, viewing under a dissecting microscope, the point zero for the stereotaxic procedure was located. The needle of a 5-µl 26-gauge microsyringe was placed in the preoptic region of the brain third ventricle (coordinates: +1.0 anterior, midline, +1.2 down) according to the stereotaxic atlas of the goldfish brain [32]. Following injection, the needle was withdrawn and the space in the cranial cavity overlying the brain was filled with teleost saline [31]. The bone flap was hinged back into place and secured by surgical thread. Fish were then returned to their tanks and normally recovered from anesthesia within 5 min. Each fish was submitted to only one i.c.v. procedure.

Implants. Solid silastic pellets manufactured as previously described [33] were used to administer RU-486 (Mifepristone; Sigma, St. Louis, Mo., USA) intraperitoneally (i.p.) (1 mm/g BW) through a 2- to 3-mm incision in the body wall. Metyrapone (Metopirone; Aldrich, Milwaukee, Wisc., USA) was mixed with melted cocoa butter (30°C) and injected i.p. (5 µl/g BW) with a 250-µl 18-gauge microsyringe in fish placed on ice to promote solidification of the implants. The RU-486 and metyrapone implantations were carried out under MS-222 anesthesia (0.2 g/l) and the incision in the body wall was closed prior to returning the fish to their original tanks. Pellets for i.c.v. implantation were prepared as previously described [34]. One milligram of α-helical $CRH_{(9–41)}$ was mixed into 5 mg of melted cocoa butter in a small vial. Upon solidification at –20°C, the CRH antagonist-cocoa butter mixture was tamped into a pellet-making apparatus consisting of a length of 26-gauge stainless steel tubing and an inner wire plunger. The wire plunger was then adjusted to a specific length (0.031 mm/g BW) with a dissecting microscope and excess extruded pellet material was removed. Following implantation of the tube into the brain third ventricle (see above), lowering the wire plunger expelled the pellet.

RNA Extraction. Fish were anesthetized in a buffered solution of MS-222 (0.5 g/l) prior to decapitation and excision of two discrete goldfish brain areas: the telencephalon-preoptic and hypothalamic regions [30]. Tissue samples were placed in microcentrifuge tubes and immediately frozen on dry ice. Total RNA was extraced using Trizol Reagent (Life Technologies, Gaithersburg, Md., USA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentrations were determined by ultraviolet spectrophotometry at 260 nm, and samples were stored at –80°C until used.

Slot-Blot Quantification of mRNA. CRH and UI mRNA levels in the telencephalon-preoptic and hypothalamic brain regions of goldfish were quantified by slot-blot analysis [35]. Ten micrograms of total RNA from each brain region (initially diluted with sterile water to 10 µl), to which was added 30 µl of denaturing solution [19.7 µl of formamide, 6.4 µl of formaldehyde (37%) and 3.9 µl of 10 × MOPS], was incubated at 65°C for 15 min. The samples were immediately placed on ice, diluted further with 60 µl of ice-cold 20 × SSC and slotted directly onto Hybond-N membranes (Amersham Life Sciences, Buckinghamshire, England) using a Bio-Dot SF manifold apparatus (Bio-Rad, Richmond, Calif., USA). The RNA was fixed by baking the membranes in a vacuum at 80°C for 2 h and was cross-linked by UV irradiation for 30 s. Goldfish CRH (579 bp) and UI (456 bp) hybridization probes were prepared as described by Bernier et al. [30]. The DNA probes were labeled using a random priming kit (T7 QuickPrime kit, Pharmacia Biotech, Baie d'Urfé, Que., Canada) with [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham) to a specific activity of 1.6×10^8 dpm/µg. Hybridization was performed using the methods of Church and Gilbert [36]. In brief, the membranes were prehybridized in hybridization solution (0.5 *M* Na_2HPO_4, pH 7.2, 7% SDS, 1 m*M* EDTA and 1% BSA) for 3 h at 65°C. The hybridization solution was then changed and the labeled CRH or UI probe was added. After overnight hybridization at 65°C, the membranes were washed four times (2 × 1 min and 2 × 10 min) with washing solution (40 m*M* Na_2HPO_4, pH 7.2, 1 m*M* EDTA and 1% SDS). Signal detection was achieved by exposing the CRH and UI membranes to a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif., USA) screen for 1 and 3 days, respectively, and quantified by ImageQuant software (Molecular Dynamics). To serve as an internal control, the membranes were stripped and reprobed with a [α-^{32}P]dCTP-labeled partial cDNA for goldfish β-actin. The CRH and UI mRNA levels were expressed as a ratio of the hybridization signal for β-actin mRNA and then normalized as a percentage of the time 0 and control value for each individual treatment and brain region analyzed. The linearity of the mRNA signals obtained by slot-blot analysis was assessed by determining the CRH and UI signals from blotting dilutions of total RNA (15, 12.5, 10, 7.5 µg) from the hypothalamic brain region. The number and size of CRH and UI transcripts in the telencephalon-preoptic and hypothalamic goldfish brain regions and the specificity of the CRH and UI hybridization probes were previously ascertained by Northern analysis [30]. Such analysis identified a single gene transcript for both the CRH and UI precursors in the hypothalamus and the telencephalon preoptic brain region of goldfish [30].

Plasma Cortisol Determinations. Plasma cortisol concentrations were measured in duplicate from unextracted samples with a commercial radioimmunoassay kit (ImmuChem Coated Tube Cortisol ^{125}I RIA Kit; ICN Biochemicals, Costa Mesa, Calif., USA). The validity of the RIA for measuring cortisol titers in goldfish plasma was previously determined [30].

Experimental Design

Experiment 1: Effects of UI and CRH i.c.v. Injections on Food Intake and Plasma Cortisol. Individual fish adapted for 72 h to separate glass aquaria were randomly injected i.c.v. with c/gUI, r/hCRH or saline between 9.00 and 10.00 a.m. (n = 10–14). Dosages of c/gUI and r/hCRH were 0.2, 2, 20 and 200 ng/g BW. The fish received food 30 min after the injection and food intake was assessed over a 120-min period. At the end of the food intake trial, fish were terminally anesthetized (1 g/l MS-222), blood was collected by caudal puncture and centrifuged at 10,000 *g* for 5 min, and the separated plasma was stored at –20°C for later analysis of cortisol.

Experiment 2: Effects of α-Helical CRH$_{(9–41)}$ i.c.v. Injections on UI- and CRH-Induced Changes in Food Intake and Plasma Cortisol. Fish were randomly injected i.c.v. with either c/gUI (10 ng/g BW), r/hCRH (10 ng/g BW) or saline alone, or in combination with α-helical CRH$_{(9–41)}$ (100 ng/g BW) between 9.00 and 10.00 a.m. (n = 12–15). The fish received food 30 min after the injection and both food intake and plasma cortisol levels were assessed as in experiment 1. In addition, food intake and plasma cortisol levels were assessed in fish left undisturbed (control).

Experiment 3: Effects of RU-486 and Metyrapone Intraperitoneal Injections on Food Intake. Food intake was assessed on the third day of a 72-hour acclimation period, following which fish received either a blank (silastic or cocoa butter, sham treatments), RU-486 (50 or 100 μg/g BW) or metyrapone (100 or 200 μg/g BW) i.p. implant (n = 10–13). Food intake was then assessed daily over the following 72 h.

Experiment 4: Effects of α-Helical CRH$_{9–41}$ i.c.v. Implants on RU-486- and Metyrapone-Induced Changes in Food Intake. Once acclimated and fed for 72 h in their experimental aquaria, fish were given either a sham or α-helical CRH$_{(9–41)}$ (300 ng/g BW) cocoa butter i.c.v. implant. The i.c.v. injection was followed immediately with either a sham (silastic or cocoa butter), RU-486 (100 μg/g BW) or metyrapone (200 μg/g BW) i.p. implant (n = 8–12). Food intake was then assessed daily over the following 72 h. In a separate trial, food intake was assessed over a 72-hour interval in fish given either a sham or α-helical CRH$_{(9–41)}$ (300 ng/g BW) cocoa butter i.c.v. implant followed immediately by a sham cocoa butter i.p. implant (n = 9).

Experiment 5: Effects of RU-486 and Metyrapone i.p. Injections on Plasma Cortisol and CRH and UI mRNA Levels in the Telencephalon-Preoptic and Hypothalamic Brain Regions. Eight groups of 12 fish each were adapted for a 3-week period to individual 80-liter fiberglass tanks. After this acclimation period, three groups of fish each received either a blank (sham treatment) or RU-486 (100 μg/g BW) i.p. silastic implant between 10.00 and 11.00 a.m. Immediately prior to the implantation of the sham and RU-486 groups, two control groups were terminally anesthetized (1 g/l MS-222) for collection of blood and brain tissues. Two control groups were sampled to assess for potential tank effects between groups. Twenty-four, 48 and 72 h after receiving the implants, the sham- and RU-486-treated groups were sampled. Brains were obtained by decapitation and regionally dissected to determine the expression levels of CRH and UI mRNAs in the hypothalamus and telencephalon-preoptic region. Blood was collected and centrifuged as in experiment 1, and the separated plasma was stored at −20 °C for later analysis of cortisol. In a second trial, eight more groups of 12 fish each were acclimatized as above. Three groups were implanted intraperitoneally with cocoa butter (sham treatment), three with metyrapone (200 μg/g BW) and two were left undisturbed and used as controls. Using the same sampling regime as above, blood and brain tissues were collected from these fish to assess the effects of metyrapone on plasma cortisol and on CRH and UI gene expression.

Statistics

All data are presented as means ± SEM. Differences among treatments were assessed by a one-way analysis of variance (ANOVA) followed by pairwise Student-Newman-Keuls multiple comparison test. The statistical significance of the observed effects of an injection within a treatment was tested using a one-way ANOVA followed by Dunnett's multiple comparison test to compare the preinjection control data point with values at subsequent times.

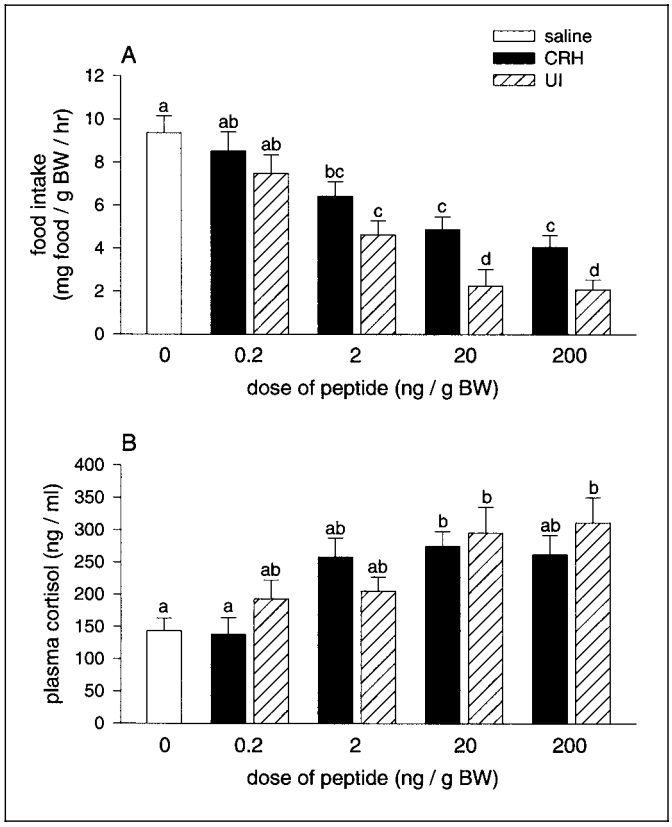

Fig. 1. Effects of a saline, r/hCRH and c/gUI i.c.v. injection on food intake (**A**) and plasma cortisol concentrations (**B**) in goldfish (n = 10–14). Fish received food 30 min after i.c.v. injection and food intake was assessed over a 120-min period. Plasma samples assayed for their cortisol content were taken at the end of the food intake trial, 2.5 hour after i.c.v. injection. Bars that do not share a common letter are significantly different from each other as determined by one-way ANOVA and by pairwise Student-Newman-Keuls test ($p < 0.05$). Values are means ± SEM.

Comparison of the potency [half-maximal effective dose (ED_{50}) values] of c/gUI and r/hCRH in suppressing food intake was assessed by an analysis of covariance to test for parallelism and differences between regression slopes of Hill plots. The significance level for all statistical tests was $p < 0.05$.

Results

Experiment 1: Effects of UI and CRH i.c.v. Injections on Food Intake and Plasma Cortisol

Relative to the effects of i.c.v. saline injection on appetite, both c/gUI and r/hCRH i.c.v. injections suppressed food intake in a dose-dependent manner, with significant effects being observed at dosages of 2 ng/g BW and higher (fig. 1A). Overall, the Hill plots of figure 2 demonstrate

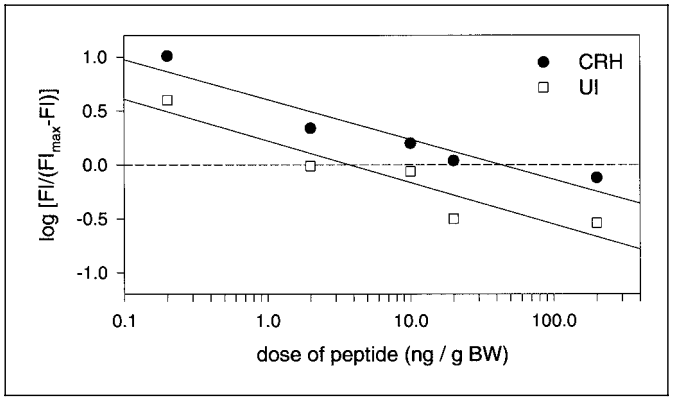

Fig. 2. Hill plots demonstrating the ED_{50} of r/hCRH (ED_{50} = 43.1 ng/g BW) and c/gUI (ED_{50} = 3.8 ng/g BW) i.c.v. injections on food intake (FI) in goldfish. Regression analysis and test for parallelism (analysis of covariance) indicates that UI was significantly ($p < 0.05$) more potent than CRH in suppressing food intake.

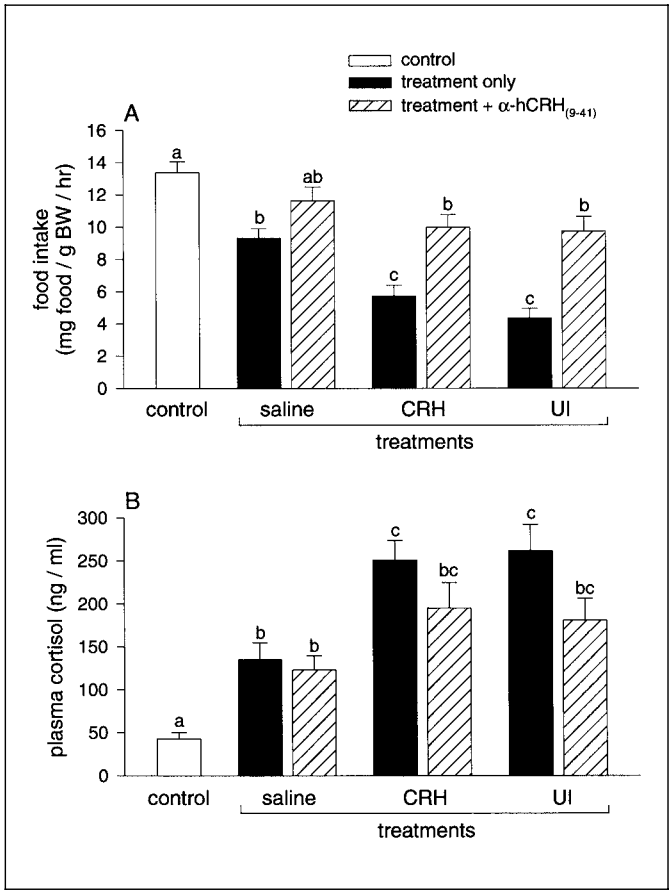

Fig. 3. Effects of a saline, r/hCRH (10 ng/g BW) and c/gUI (10 ng/g BW) i.c.v. injection on food intake (**A**) and plasma cortisol concentrations (**B**) in goldfish (n = 12–15). Fish were untreated (control) or injected i.c.v. with saline, CRH or UI alone, or in combination with the CRH receptor antagonist, α-helical $CRH_{(9-41)}$ (100 ng/g BW). Assessment of food intake and plasma cortisol levels was carried out as in figure 1. Bars that do not share a common letter are significantly different from each other as determined by one-way ANOVA and by pairwise Student-Newman-Keuls test ($p < 0.05$). Values are means ± SEM.

that c/gUI (ED_{50} = 3.8 ng/g BW) is significantly more potent than r/hCRH (ED_{50} = 43.1 ng/g BW) in producing anorectic effects. UI and CRH i.c.v. injections also elicited increases in plasma cortisol relative to i.c.v. saline injections (fig. 1B). However, unlike the differences in potency between UI and CRH on food intake, both peptides were equipotent in stimulating an increase in plasma cortisol and higher doses of either peptide were required to elevate plasma cortisol than to decrease appetite.

Experiment 2: Effects of α-Helical $CRH_{(9-41)}$ i.c.v. Injections on UI- and CRH-Induced Changes in Food Intake and Plasma Cortisol

The i.c.v. injection of the CRH receptor antagonist, α-helical $CRH_{(9-41)}$ (100 ng/g BW), in combination with either UI or CRH (10 ng/g BW), prevented the UI- and CRH-induced suppression of food intake (fig. 3A). Relative to the amount of food eaten by undisturbed control fish, administration of α-helical $CRH_{(9-41)}$ alone reversed the decrease in food intake elicited by i.c.v. injection of saline. The α-helical $CRH_{(9-41)}$ i.c.v. injection partially, but not significantly, reduced the increase in plasma cortisol elicited by either central UI or CRH injection, and did not prevent the rise in plasma cortisol associated with i.c.v. injection of saline alone (fig. 3B).

Experiment 3: Effects of RU-486 and Metyrapone i.p. Injections on Food Intake

While basal food consumption was not affected by i.p. injection of a sham silastic implant, goldfish treated with implants of the glucocorticoid receptor antagonist, RU-486, were characterized by a sustained (72 h) and dose-dependent reduction in food intake (fig. 4A). Similarly, implants of the cortisol synthesis inhibitor, metyrapone, elicited a marked and sustained (48–72 h) decrease in appetite in comparison to sham cocoa butter-implanted goldfish (fig. 4B). In general, the appetite-suppressing effects of RU-486 and metyrapone i.p. injections were most acute 24 h after implantation and lessened over time (fig. 4A, B).

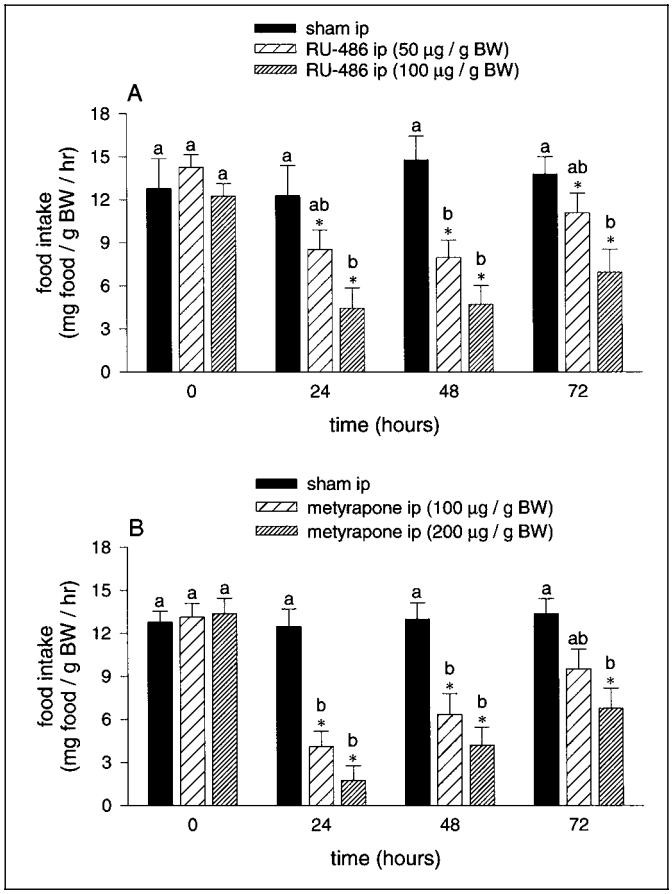

Fig. 4. Time course of changes in food intake in goldfish given an i.p. sham silastic implant (n = 10) or an implant of the glucocorticoid receptor antagonist, RU-486 (n = 12 for the 50 and 100 µg/g BW doses) (**A**) or an i.p. sham cocoa butter implant (n = 11) or an implant of the cortisol synthesis inhibitor, metyrapone (n = 13 and 12 for the 100 and 200 µg/g BW doses, respectively) (**B**). Food intake was assessed daily over a 120-min period. Implants were given immediately after assessment of food intake at time 0. Treatments that do not share a common letter for a given time are significantly different from each other as determined by one-way ANOVA and by pairwise Student-Newman-Keuls test. * $p < 0.05$ compared to the 0-min preinjection control value for a given treatment as determined by one-way ANOVA and by Dunnett's multiple comparison test. Values are means ± SEM.

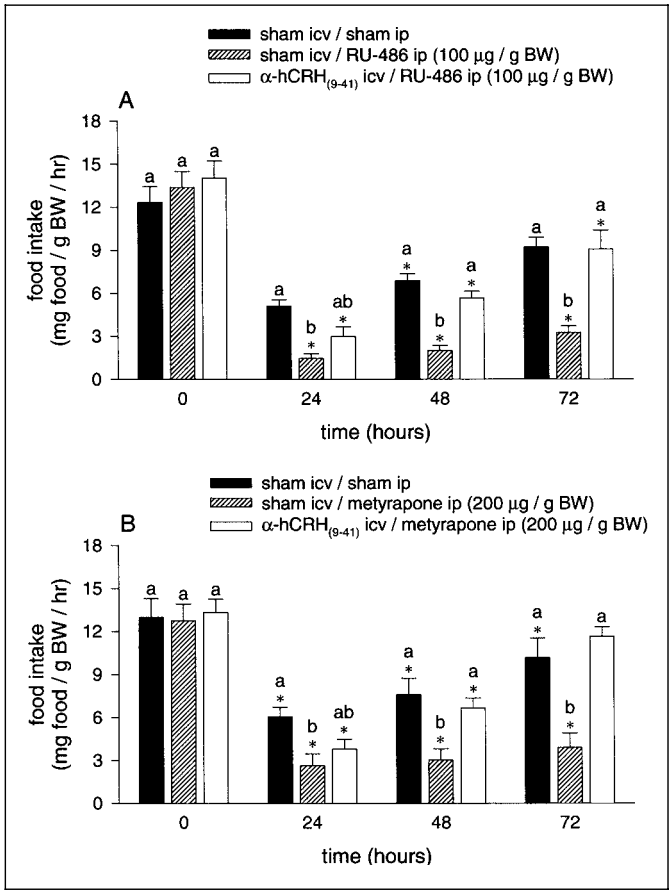

Fig. 5. Time course of changes in food intake in goldfish given different treatments. **A** Fish were sequentially given a combination of either i.c.v. and i.p. sham implants (n = 10), an i.c.v. sham implant and an RU-486 (100 µg/g BW) implant (n = 9), or an i.c.v. implant of the CRH receptor antagonist, α-helical $CRH_{(9–41)}$ (300 ng/g BW), and an i.p. RU-486 (100 µg/g BW) implant (n = 8). **B** Fish were given either i.c.v. and i.p. sham implant (n = 12), an i.c.v. sham implant and an i.p. metyrapone (200 µg/g BW) implant (n = 11), or an i.c.v. implant of α-helical $CRH_{(9–41)}$ (300 ng/g BW) and an i.p. metyrapone (200 µg/g BW) implant (n = 8). Assessment of food intake and injection of implants were carried out as described for figure 4. Treatments that do not share a common letter for a given time are significantly different from each other as determined by one-way ANOVA and by pairwise Student-Newman-Keuls test. * $p < 0.05$ compared to the 0-min preinjection control value for a given treatment as determined by one-way ANOVA and by Dunnett's multiple comparison test. Values are means ± SEM.

Experiment 4: Effects of α-Helical $CRH_{(9–41)}$ i.c.v. Implants on RU-486- and Metyrapone-Induced Changes in Food Intake

Whereas sham i.p. implants had no effect on basal food intake (fig. 4A, B), central injection of a sham cocoa butter implant prior to a sham i.p. implant elicited a marked reduction in basal food consumption (fig. 5A, B, 6). In general, among the fish treated with an RU-486 (100 µg/g BW) or a metyrapone (200 µg/g BW) implant in experiments 3 and 4, those first given a sham i.c.v. cocoa butter implant had lower food intake (fig. 4, 5). Pretreatment with central implants of α-helical $CRH_{(9–41)}$ (300 ng/g

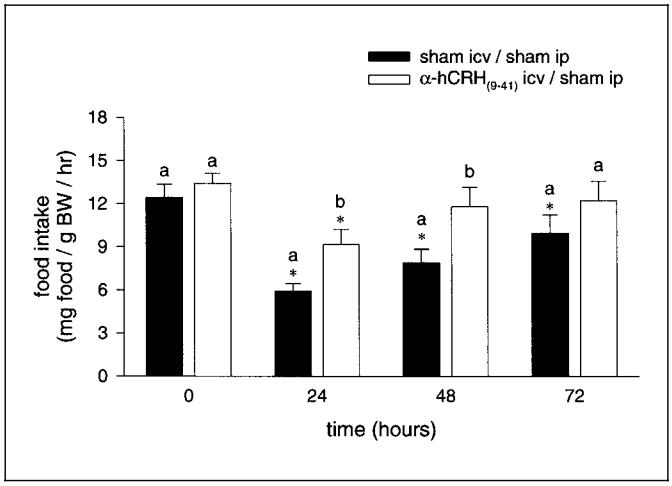

Fig. 6. Time course of changes in food intake in goldfish sequentially given an i.c.v. and i.p. sham implant (n = 9) or an i.c.v. implant of the CRH receptor antagonist, α-helical $CRH_{(9–41)}$ (300 ng/g BW), and an i.p. sham implant (n = 9). Assessment of food intake and injection of implants were carried out as described for figure 4. Treatments that do not share a common letter for a given time are significantly different from each other as determined by Student's t test. * $p < 0.05$ compared to the 0-min preinjection control value for a given treatment as determined by one-way ANOVA and by Dunnett's multiple comparison test. Values are means ± SEM.

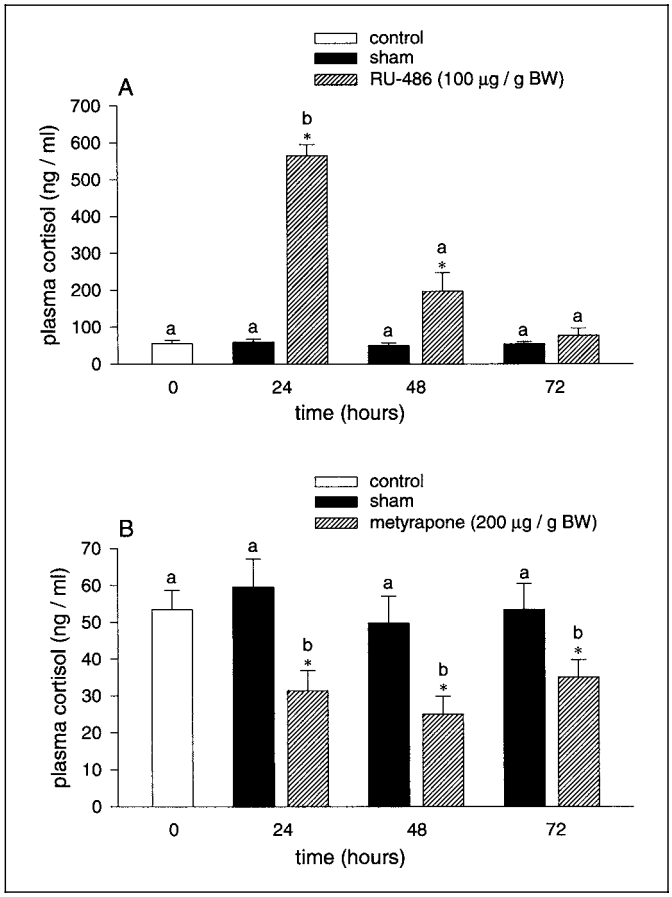

Fig. 7. Time course of changes in plasma cortisol in goldfish given different treatments. **A** Fish were given either a sham silastic implant (n = 12) or an implant of the glucocorticoid receptor antagonist, RU-486 (100 µg/g BW; n = 12). **B** Fish were given either a sham cocoa butter implant (n = 12) or an implant of the cortisol synthesis inhibitor, metyrapone (200 µg/g BW; n = 12). Treatment that do not share a common letter for a given time are significantly different from each other as determined by Student's t test. * $p < 0.05$ compared to the 0-min preinjection control value (n = 24) as determined by one-way ANOVA and by Dunnett's multiple comparison test. Values are means ± SEM.

BW) partially reversed the appetite-suppressing effects of i.p. RU-486 (fig. 5A) and metyrapone (fig. 5B). While fish treated with the combined sham i.c.v. RU-486 or metyrapone i.p. implants experienced a chronic reduction in food intake, fish first given an i.c.v. implant of α-helical $CRH_{(9–41)}$ prior to the RU-486 or metyrapone i.p. injection were characterized by a more transient reduction in food intake that paralleled the changes observed in the i.c.v./i.p. sham-treated fish (fig. 5A, B). Finally, central α-helical $CRH_{(9–41)}$ pretreatment caused a significant reversal, which was complete by 48 h, of the hypophagia induced by the combined sham i.c.v./i.p. treatment (fig. 6).

Experiment 5: Effects of RU-486 and Metyrapone i.p. Injections on Plasma Cortisol and CRH and UI mRNA Levels in the Telencephalon-Preoptic and Hypothalamic Brain Regions

No significant differences were observed between the two control groups in terms of the preinjection plasma cortisol values and the levels of CRH and UI mRNA. Therefore, the values from the two control groups were combined for statistical analysis.

Relative to the time 0 control value, injection of either silastic (fig. 7A) or cocoa butter (fig. 7B) implants had no effect on resting plasma cortisol concentrations. Fish treated with the RU-486 implants were characterized by an acute elevation in plasma cortisol that peaked 24 h after the implant procedure and returned to basal levels 72 h later (fig. 7A). In contrast, metyrapone implants chronically suppressed resting plasma cortisol levels over the 72-hour duration of the experiment (fig. 7B).

CRH and UI mRNA levels in the telencephalon-preoptic and hypothalamic brain regions of goldfish were

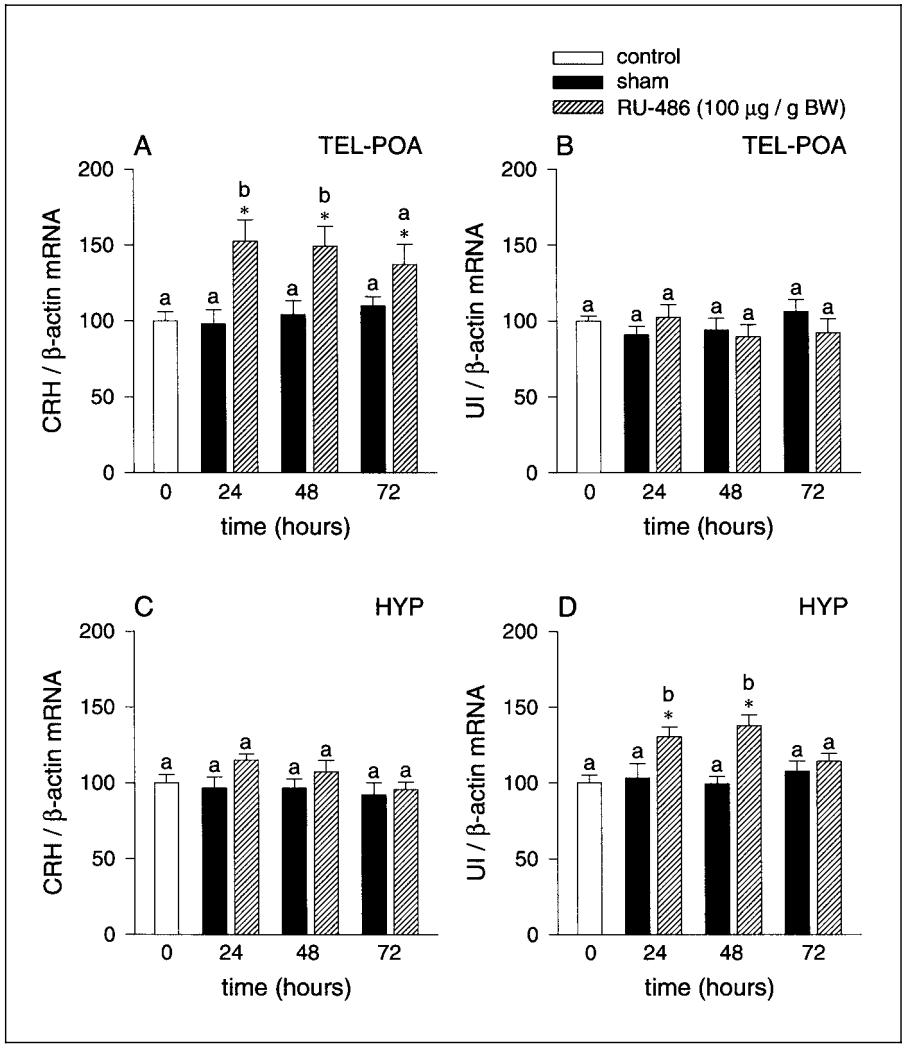

Fig. 8. Time course of changes in CRH (**A**, **C**) and UI (**B**, **D**) mRNA levels in the telencephalon-preoptic (TEL-POA; **A**, **B**) and hypothalamic (HYP; **C**, **D**) brain regions of goldfish given either a sham silastic implant (n = 12) or an implant of the glucocorticoid receptor antagonist, RU-486 (100 µg/g BW; n = 12). Treatments that do not share a common letter for a given time are significantly different from each other as determined by Student's t test. * $p < 0.05$ compared to the 0-min preinjection control value (n = 24) as determined by one-way ANOVA and by Dunnett's multiple comparison test. Values are means ± SEM.

not altered by either silastic (fig. 8) or cocoa butter (fig. 9) sham i.p. implants. Meanwhile, both RU-486 (fig. 8) and metyrapone (fig. 9) implants elicited significant increases in CRH and UI gene expression in the telencephalon-preoptic and hypothalamic brain regions, respectively. Overall, relative to their respective sham treatments, the RU-486- and metyrapone-elicited increases in telencephalon-preoptic CRH gene expression were more pronounced and sustained over a longer period of time than the increases in hypothalamic UI gene expression. In contrast, RU-486 (fig. 8) and metyrapone (fig. 9) implants had no effect on CRH and UI gene expression in the hypothalamic and telencephalon-preoptic brain regions, respectively.

Discussion

Results from the present study provide the first evidence that UI is a potent anorectic peptide in fish. Administered in the brain's third ventricle, homologous UI produces appetite-suppressing effects that are significantly more potent than those of r/hCRH. Moreover, an endogenous role for CRH-related peptides in the control of appetite is suggested by the effects of the CRH antagonist α-helical $CRH_{(9-41)}$ in reversing the reduction in food intake induced by pharmacological treatments that elevate central CRH and UI gene expression and by stress.

In agreement with studies carried out on rats [14–16, 18], central injections of UI in goldfish suppressed food intake in a dose-related manner. The low doses of UI (2 ng/g BW) required to reduce feeding in goldfish and the

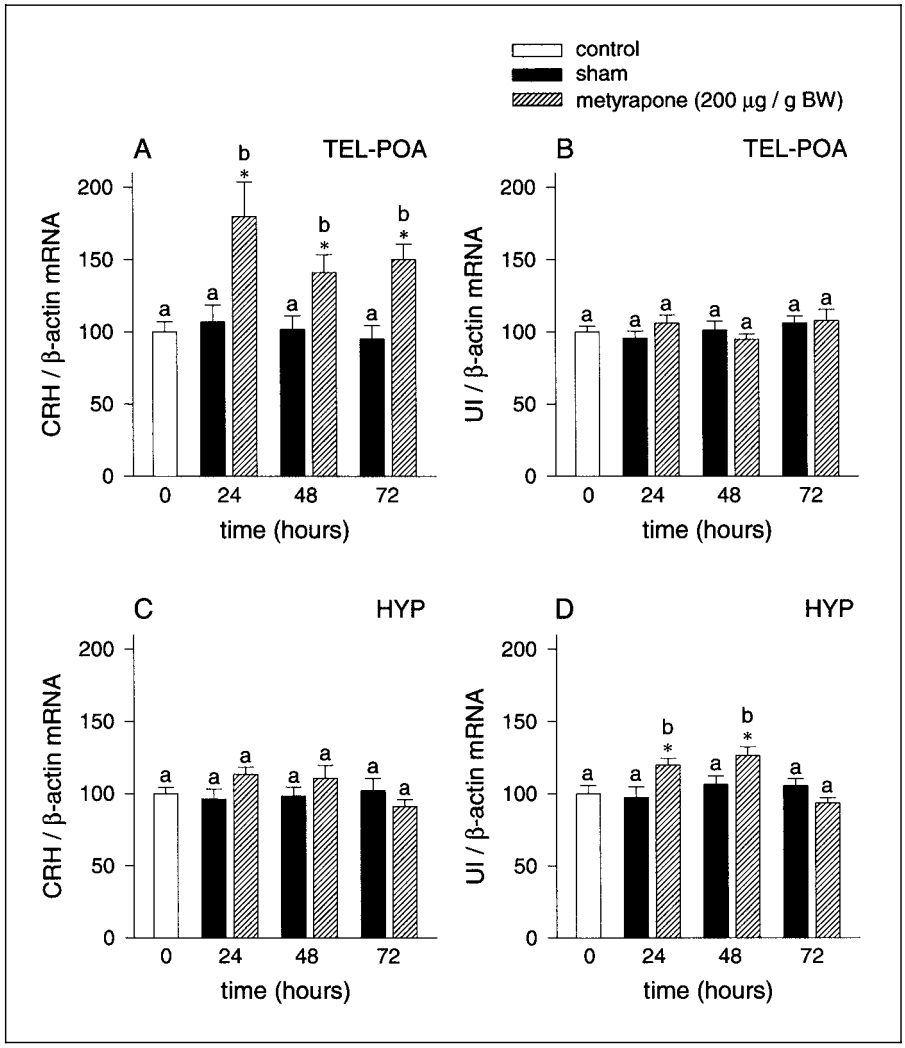

Fig. 9. Time course of changes in CRH **A**, **C**) and UI (**B**, **D**) mRNA levels in the telencephalon-preoptic (TEL-POA; **A**, **B**) and hypothalamic (HYP; **C**, **D**) brain regions of goldfish given either a sham cocoa butter implant (n = 12) or an implant of the cortisol synthesis inhibitor, metyrapone (200 µg/g BW; n = 12). Treatments that do not share a common letter for a given time are significantly different from each other as determined by Student's t test. * $p < 0.05$ compared to the 0-min preinjection control value (n = 24) as determined by one-way ANOVA and by Dunnett's multiple comparison test. Values are means ± SEM.

ability of α-helical $CRH_{(9–41)}$ to antagonize these effects, suggest that the appetite-suppressing actions of UI i.c.v. injections are specifically mediated through central mechanisms involving CRH receptors. Therefore, in addition to the proposed role of hypothalamic UI in the regulation of adrenocorticotropic hormone (ACTH) and thyrotropin secretion from the pituitary [37, 38], our results suggest that central UI may also be involved in the hypothalamic regulation of food intake in fish.

The anorectic effects of CRH i.c.v. injections observed in this study and their reversal by α-helical $CRH_{(9–41)}$ coinjection concur with the earlier reports of De Pedro et al. [22, 25] and together suggest that CRH may be involved in the regulation of food intake in goldfish. Overall, the dose-dependent decrease in goldfish food intake recorded in this study with doses of r/hCRH ranging from 2 to 200 ng/g BW (approximately 0.1–10 µg) is similar to the effects that r/hCRH i.c.v. injections have on food intake in rats [1]. In contrast, De Pedro et al. [22] found that only intracranial doses of oCRH ≥1 µg (approximately 150 ng/g BW) and <3.3 µg inhibited the food consumption of goldfish. While the discrepancies between this study and that of De Pedro et al. [22] are likely the result of methodological differences in the administration of the drugs, they may also result from differences in the affinity of the CRH peptides administered for the CRH receptors of goldfish. For example, r/hCRH binds with a significantly higher affinity than oCRH to both CRH_1 and CRH_2 receptor subtypes in *Xenopus laevis* [39]. In addition, goldfish CRH shares a much greater degree of sequence identity with r/hCRH (92%) than with oCRH (78%) [30].

Interestingly, although peripheral administration of CRH does not affect food intake in either goldfish or rats [1, 22, 25], peripheral injection of UI decreases food intake in the latter [15]. Similarly, although peripheral injection of CRH can decrease food intake in mice, this effect is much weaker than that induced by urocortin [40]. In mice, the potent anorectic effect of i.p. administered urocortin is associated with a sharp reduction in the rate of gastric emptying [40]. Given the distinct UI-synthesizing caudal neurosecretory system of fish and the diverse array of regulatory functions attributed to urophyseal UI [41–43], a potential role for peripheral UI in the regulation of food intake in fish merits future consideration.

Overall, i.c.v. c/gUI was significantly more potent than i.c.v. r/hCRH in inhibiting food intake in goldfish. This result corroborates previous observations that UI has a higher potency than CRH in suppressing appetite in rats [15–17], but also contrasts with another study in rats that reported similar hypophagic effects for all CRH-related peptides [18]. Since the 41-amino acid sequences of goldfish CRH and r/hCRH differ by only three nonpolar amino acid substitutions [30], the anorectic actions of i.c.v. r/hCRH in goldfish are likely to reflect the effects of the homologous peptide. In mammals, the higher affinity of UI for the CRH_2 receptor with respect to CRH [20] has been proposed as a potential mechanism to account for the differential appetite-suppressing effects of i.c.v. UI and CRH [16]. Similarly, although very little is known about the molecular structure and pharmacological properties of the CRH receptors in fish [44, 45], our results suggest that the CRH receptor subtype responsible for mediating the anorectic effects of CRH-related peptides in goldfish may be selective for different CRH-related ligands.

As previously observed in goldfish [25] and mammals [46], relative to an injection of saline alone, i.c.v. administration of CRH increased plasma cortisol levels in this study. Similar to the effects of i.c.v. infusions of urocortin in rats [6], central injections of UI also activated the HPI axis in goldfish. In contrast to the differential effects of CRH-related peptides in inhibiting food intake (see above), both UI and CRH i.c.v. injections increased plasma cortisol levels with equal potency. These results may seem at odds with the observation that sucker UI has a significantly greater ACTH-releasing activity than either sucker CRH, r/hCRH or oCRH in superfused goldfish anterior pituitary cell columns [44]. However, evidence from studies on rats suggests that i.c.v. injections of CRH stimulate pituitary ACTH release indirectly via an activation of CRH neurons in the paraventricular nucleus [47]. Therefore, since the effects of UI and CRH i.c.v. injections on the neuronal activity of the hypophysiotropic tract of fish are not known, we cannot dismiss the possibility that i.c.v. injections of either UI or CRH stimulate the HPI axis through a common neuronal pathway. Nonetheless, the increases in plasma cortisol levels elicited by central injections UI and CRH, and their partial reversal by α-helical $CRH_{(9–41)}$ coadministration, suggest that both peptides may be involved in the hypothalamic regulation of the HPI axis in goldfish. Whether the distinct CRH and UI neuronal populations of the goldfish brain differentially regulate the HPI axis [44], and whether endogenous CRH and UI are released in response to different stimuli, remains to be established.

As a means to investigate the potential effects of endogenous UI and CRH in the regulation of food intake, we treated goldfish with either RU-486 or metyrapone to remove the negative feedback action of cortisol and stimulate the activity of the CRH- and UI-synthesizing neurons of the brain. Accordingly, we observed that i.p. administration of RU-486 and metyrapone both led to a chronic increase in CRH and UI gene expression in the telencephalon-preoptic and hypothalamic brain regions of the goldfish brain, respectively. As indicated by their effects on the circulating levels of plasma cortisol, the i.p. implants of RU-486 and metyrapone stimulated the activity of CRH- and UI-synthesizing neurons via different modes of action. As previously observed in fish [48], metyrapone, an inhibitor of 11β-hydroxylase enzymatic activity [49], blocked the negative glucocorticoid feedback mechanism by inhibiting cortisol synthesis. On the other hand, RU-486, a potent glucocorticoid receptor antagonist in both fish and mammals [50, 51], led to a temporary cortisol hypersecretion by counteracting the negative feedback action of cortisol on the HPI axis [30].

The RU-486- and metyrapone-elicited regional differences in the regulation of CRH and UI gene expression observed in this study concur with our earlier finding that i.p. RU-486 treatment increases CRH gene expression in the telencephalon-preoptic region without affecting the CRH mRNA levels in the hypothalamus [30]. Similarly, immunohistochemical studies have previously shown that i.p. metyrapone treatment specifically enhances the secretory activity of UI neurons in the nucleus lateral tuberis of the goldfish hypothalamus [28] and the activity of CRH neurons in the nucleus preopticus of goldfish, catfish and eels [29, 52, 53]. So, while the genes for UI and CRH are broadly expressed in the brain of goldfish [30], available evidence suggests that only specific brain sites may be affected by manipulations of glucocorticoid sta-

tus. Furthermore, since RU-486 implants induce a marked elevation in plasma cortisol, our results suggest that the CRH neurons of the hypothalamus and the UI neurons of the telencephalon-preoptic brain region may not behave as hypophysiotropic neurons involved in the control of the HPI axis in goldfish. Although our data suggest that the RU-486 and metyrapone i.p. treatments stimulated CRH gene expression in the telencephalon-preoptic brain region to a greater extent than UI gene expression in the hypothalamus, comparisons between different brain regions may mask more dynamic changes taking place at the level of specific nuclei. Independently of potential differences in the glucocorticoid-mediated regulation of UI and CRH gene expression in different regions of the goldfish brain, our results clearly demonstrate that counteracting the negative feedback action of cortisol leads to an increase in CRH and UI gene expression in goldfish.

Parallel to their stimulatory effects on CRH and UI gene expression in the goldfish brain, RU-486 and metyrapone i.p. implants chronically decreased food intake in a dose-dependent manner in this study. This is in agreement with the finding of De Pedro et al. [25], who reported that i.p. injection of metyrapone acutely reduced food intake in goldfish. Similarly, the ingestive behavior of rats is inhibited following treatment with metyrapone, RU-486 or adrenalectomy [9, 13, 54]. Since these treatments are known to stimulate UI (as shown in this study) and CRH gene expression (as shown in this study) [55, 56], an increase in endogenous CRH-related peptide synthesis may seem a likely explanation to account for the anorectic effects of these treatments. Accordingly, the ability of the CRH receptor antagonist α-helical $CRH_{(9–41)}$ to attenuate the reduction in food intake associated with the RU-486 and metyrapone i.p. implants suggests an involvement of endogenous CRH-related peptides in mediating the anorectic effects of these treatments in goldfish. Because α-helical $CRH_{(9–41)}$ blocks the anorectic effects elicited by both UI and CRH i.c.v. injections in goldfish, the individual contribution of UI and CRH to the overall regulation of food intake following RU-486 and metyrapone treatment cannot be determined with the current experimental design. Furthermore, investigations are needed to determine whether the CRH and UI neurons involved in the control of food intake are the same as those involved in the control of the HPI axis.

Among the fish given an i.c.v. implant of α-helical $CRH_{(9–41)}$, those treated with either RU-486 or metyrapone experienced a greater reduction in food intake than the fish that received a sham i.p. implant. Therefore, although a part of the reduction in food intake elicited by the RU-486 and metyrapone treatments may be mediated by endogenous CRH-related peptides, our results also suggest that CRH- and UI-independent pathways may account in part for the anorectic effects associated with the loss of negative feedback by glucocorticoids. In mammals, glucocorticoids, by enhancing various elements of different orexigenic pathways, can increase appetite independently of CRH [13]. Therefore, although the role of cortisol in the control of food intake in fish has yet to be clearly defined and the potential interaction of cortisol with orexigenic pathways has not been explored, our results raise the possibility that cortisol may be involved in fish appetite regulation independently of CRH and UI.

Findings from this study also suggest a role for endogenous CRH-related peptides in goldfish in mediating the attenuation of food intake induced by stress. Relative to undisturbed controls, fish given an i.c.v. injection of saline were characterized by a significant reduction in food intake and an increase in plasma cortisol. Since elevated plasma cortisol levels are considered a reliable indicator of stress in fish [57], our data suggest that the combined anesthetic and i.c.v. procedure used in this study elicited a stress response. Similarly, while i.p. administration of sham implants did not affect resting plasma cortisol levels and basal food intake, the chronic appetite reduction observed in fish given the combined i.c.v. and i.p. sham implants suggests that the i.c.v. placement of cocoa butter implants was stressful. The ability of the antagonist α-helical $CRH_{(9–41)}$ to reverse the hypophagic effects associated with an i.c.v. injection of saline and an i.p. implant of cocoa butter, suggests that endogenous ligands for CRH receptors play a functional role in mediating the impact of stress on appetite. While similar results have been observed in rats [5, 7], and a reduced appetite is often mentioned as a characteristic of the stress response in fish [58], our results provide original evidence that endogenous CRH-related peptides play a role in stress-induced anorexia in nonmammalian vertebrates.

Available evidence suggests that CRH-related peptides may be involved in a wide variety of appetite-regulating pathways. In goldfish, adrenergic and dopaminergic receptor blockade experiments suggest that CRH may mediate its anorexic effect via interactions with central catecholaminergic pathways [25, 59]. CRH may also mediate part of the anorectic effect of serotonin in goldfish, as α-helical $CRH_{(9–41)}$ pretreatment partially prevents the appetite-suppressing effect of serotonin [60]. In mammals, CRH inhibits feeding induced by neuropeptide Y and

mediates at least a part of the anorexigenic effects of bombesin, leptin and possibly serotonin [2, 61]. While it remains to be seen whether both UI and urocortin interact as extensively as CRH with other appetite-regulating pathways, the greater potency of UI and urocortin as anorectic agents than CRH suggests that they may.

In summary, results from the present study show that both UI and CRH are potent anorexic peptides in the brain of goldfish and suggest that endogenous CRH-related peptides play a physiological role in the control of food intake. Moreover, our findings demonstrate that CRH-related peptides are involved in mediating the anorexic effects of stress in fish. Although i.c.v. injections of UI are more potent than injections of CRH in reducing appetite, whether this difference reflects a differential role for CRH-related peptides in the regulation of food intake in fish awaits future investigations. Overall, the regulation of food intake in fish, as in mammals, is under multifactorial control involving a complex network of orexigenic and anorexigenic neuronal pathways [62]. Evidence presented in this paper implicates endogenous CRH-related peptides as important agents in the neuronal network responsible for the regulation of appetite in fish.

Acknowledgments

Dr. J.E. Rivier is gratefully acknowledged for providing us with c/gUI. This research was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) research grants to R.E.P. N.J.B. was the recipient of an NSERC postdoctoral fellowship.

References

1 Glowa JR, Barrett JE, Russell J, Gold PW: Effects of corticotropin releasing hormone on appetitive behaviors. Peptides 1992;13:609–621.
2 Heinrichs SC, Richard D: The role of corticotropin-releasing factor and urocortin in the modulation of ingestive behavior. Neuropeptides 1999;33:350–359.
3 Britton DR, Koob GF, Rivier J, Vale W: Intraventricular corticotropin-releasing factor enhances behavioral effects of novelty. Life Sci 1982;31:363–367.
4 Krahn DD, Gosnell BA, Levine AS, Morley JE: Behavioral effects of corticotropin-releasing factor: Localization and characterization of central effects. Brain Res 1988;443:63–69.
5 Krahn DD, Gosnell BA, Grace M, Levine AS: CRF antagonist partially reverses CRF- and stress-induced effects on feeding. Brain Res Bull 1986;17:285–289.
6 Smagin GN, Howell LA, Ryan DH, De Souza EB, Harris RBS: The role of CRF_2 receptors in corticotropin-releasing factor- and urocortin-induced anorexia. Neuroreport 1998;9:1601–1606.
7 Smagin GN, Howell LA, Redmann S Jr, Ryan DH, Harris RBS: Prevention of stress-induced weight loss by third ventricle CRF receptor antagonist. Am J Physiol 1999;276:R1461–R1468.
8 Rothwell NJ: Central effects of CRF on metabolism and energy balance. Neurosci Biobehav Rev 1990;14:263–271.
9 Trocki O, Baer DJ, Castonguay TW: Comparison of effects of adrenalectomy and RU-486 in rats given a choice of maintenance diet and fat supplement. Am J Physiol 1995;269:R708–R719.
10 Seeley RJ, Matson CA, Chavez M, Woods SC, Dallman MF, Schwartz MW: Behavioral, endorince, and hypothalamic responses to involuntary overfeeding. Am J Physiol 1996;271:R819–R823.
11 Schwartz MW, Seeley RJ: Neuroendocrine responses to starvation and weight loss. N Engl J Med 1997;336:1802–1811.
12 Weninger SC, Muglia LJ, Jacobson L, Majzoub JA: CRH-deficient mice have a normal anorectic response to chronic stress. Regul Pept 1999;84:69–74.
13 Jacobson L: Glucocorticoid replacement, but not corticotropin-releasing hormone deficiency, prevents adrenalectomy-induced anorexia in mice. Endocrinology 1999;140:310–317.
14 Britton DR, Hoffman DK, Lederis K, Rivier J: A comparison of the behavioral effects of CRF, sauvagine and urotensin I. Brain Res 1984;304:201–205.
15 Negri L, Noviello L, Noviello V: Effects of sauvagine, urotensin I and CRF on food intake in rats. Peptides 1985;6(suppl 3):53–57.
16 Spina M, Merlo-Pich E, Chan KW, Basso AM, Rivier J, Vale W, Koob GF: Appetite-suppressing effects of urocortin, a CRF-related neuropeptide. Science 1996;273:1561–1564.
17 Improta G: Evolutionary aspects in the peripheral peptidergic signals: CRF-like peptides and modulation of G.I. functions. Adv Exp Med Biol 1991;298:75–83.
18 Jones DNC, Kortekaas R, Slade PD, Middlemiss DN, Hagan JJ: The behavioural effects of corticotropin-releasing factor-related peptides in rats. Psychopharmacology (Berl) 1998;138:124–132.
19 Benoit SC, Thiele TE, Heinrichs SC, Rushing PA, Blake KA, Steeley RJ: Comparison of central administration of corticotropin-releasing hormone and urocortin on food intake, conditioned taste aversion, and *c-Fos* expression. Peptides 2000;21:345–351.
20 Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnball AV, Lovejoy D, Rivier C, Rivier J, Sawchenko PE, Vale W: Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. Nature 1995;378:287–292.
21 Lovejoy DA, Balment RJ: Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates. Gen Comp Endocrinol 1999;115:1–22.
22 De Pedro N, Alonso-Gomez AL, Gancedo B, Delgado MJ, Alonso-Bedate M: Role of corticotropin-releasing factor (CRF) as a food intake regulator in goldfish. Physiol Behav 1993;53:517–520.
23 Corpas I, Gancedo B, Alonso-Gomez AL, Delgado MJ, Alonso-Bedate M: Food intake inhibition and metamorphosis stimulation by sheep corticotropin-releasing hormone (CRF) administration in *Rana perezi*. Belg J Zool 1991;121:132–133.
24 Denbow DM, Snapir N, Furuse M: Inhibition of food intake by CRF in chickens. Physiol Behav 1999;66:645–649.
25 De Pedro N, Alonso-Gomez AL, Gancedo B, Valenciano AI, Delgado MJ, Alonso-Bedate M: Effect of α-helical-$CRF_{(9–41)}$ on feeding in goldfish: Involvement of cortisol and catecholamines. Behav Neurosci 1997;111:398–403.
26 Montecucchi PC, Henschen A: Amino acid composition and sequence analysis of sauvagine, a new peptide from the skin of *Phyllomedusa sauvagei*. Int J Pept Protein Res 1981;18:113–120.
27 Lederis K, Letter A, McMaster D, Moore G, Schlesinger D: Complete amino acid sequence of urotensin I, a hypotensive and corticotropin-releasing neuropeptide from *Catostomus*. Science 1982;218:162–165.

28 Fryer JN: Neuropeptides regulating the activity of goldfish corticotropes and melanotropes. Fish Physiol Biochem 1989;7:21–27.
29 Olivereau M, Olivereau J: Effect of pharmacological adrenalectomy on corticotropin-releasing factor-like and arginine vasotocin immunoreactivities in the brain and pituitary of the eel: Immunocytochemical study. Gen Comp Endocrinol 1990;80:199–215.
30 Bernier NJ, Lin X, Peter RE: Differential expression of corticotropin-releasing factor (CRF) and urotensin I precursor genes, and evidence of CRF gene expression regulated by cortisol in godlfish brain. Gen Comp Endocrinol 1999;116:461–477.
31 Burnstock G: Saline for fresh-water fish. J Physiol 1958;141:35–45.
32 Peter RE, Gill VE: A stereotaxic atlas and technique for forebrain nuclei of the goldfish, *Carassius auratus*. J Comp Neurol 1975;159:69–101.
33 Pankhurst NW, Stacey NE, Peter RE: An evaluation of techniques for the administration of 17β-estradiol to teleosts. Aquaculture 1986;52: 145–155.
34 Fryer JN, Peter RE: Hypothalamic control of ACTH secretion in goldfish: III. Hypothalamic cortisol implant studies. Gen Comp Endocrinol 1977;33:215–225.
35 Brown T, Mackey K: Analysis of RNA by Northern and slot blot hybridization; in Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds): Current Protocols in Molecular Biology. New York, John Wiley & Sons, 1987, vol 1, pp 4.9.1–4.9.16.
36 Church GM, Gilbert W: Genomic sequencing. Proc Natl Acad Sci USA 1984;81:1991–1995.
37 Fryer J, Lederis K, Rivier J: Urotensin I, a CRF-like neuropeptide, stimulates ACTH release from the teleost pituitary. Endocrinology 1983;113:2308–2310.
38 Larsen DA, Swanson P, Dickey JT, Rivier J, Dickhoff WW: In vitro thyrotropin-releasing activity of corticotropin-releasing hormone-family peptides in coho salmon, *Oncorhynchus kisutch*. Gen Comp Endocrinol 1998;109:276–285.
39 Dautzenberg FM, Dietrich K, Palchaudhuri MR, Spiess J: Identification of two corticotropin-releasing factor receptors from *Xenopus laevis* with high ligand selectivity: Unusual pharmacology of the type 1 receptor. J Neurochem 1997;69:1640–1649.
40 Asakawa A, Inui A, Ueno N, Makino S, Fujino MA, Kasuga M: Urocortin reduces food intake and gastric emptying in lean and *ob/ob* obese mice. Gastroenterology 1999;116:1287–1292.
41 Lederis K, Frey JN, Yulis CR: The fish neuropeptide urotensin I: Its physiology and pharmacology. Peptides 1985;6(suppl 3):353–361.
42 Platzack B, Schaffert C, Hazon N, Conlon JM: Cardiovascular actions of dogfish urotensin I in the dogfish, *Scyliorhinus canicula*. Gen Comp Endocrinol 1998;109:269–275.
43 Kelsall CJ, Balment RJ: Native urotensins influence cortisol secretion and plasma cortisol concentration in the euryhaline flounder, *Platichthys flesus*. Gen Comp Endocrinol 1998; 112:210–219.
44 Lederis K, Fryer JN, Okawara Y, Schonrock C, Richter D: Corticotropin-releasing factors acting on the fish pituitary: Experimental and molecular analysis; in Sherwood NM, Hew CL (eds): Fish Physiology. New York, Academic Press, 1994, vol XIII, Molecular Endocrinology of Fish, pp 67–100.
45 Darlison MG, Greten FR, Pohl S, Stuhmer T, Kreienkamp H-J, Richter D: Opioid and corticotropin-releasing factor receptors from lower vertebrates; in Kawashima S, Kikuyama S (eds): Advances in Comparative Endocrinology: Proceedings of the XIIIth International Congress of Comparative Endocrinology. Bologna, Monduzzi Editore, 1997, vol 1, pp 545–550.
46 Rock JP, Oldfield EH, Schulte HM, Gold PW, Kornblith PL, Loriaux L, Chrousos GP: Corticotropin releasing factor administered into the ventricular CSF stimulates the pituitary-adrenal axis. Brain Res 1984;323:365–368.
47 Parkes D, Rivest S, Lee S, Rivier C, Vale W: Corticotropin-releasing factor activates *c-fos*, NGFI-B, and corticotropin-releasing factor gene expression within the paraventricular nucleus of the rat hypothalamus. Mol Endocrinol 1993;7:1357–1367.
48 Bennett RO, Rhodes RC: Evaluation of oral administration of cortisol and metyrapone: The effects on serum cortisol in rainbow trout (*Salmo gairdneri*). Comp Biochem Physiol A 1986;83:727–730.
49 Carballeira A, Cheng SC, Fishman LM: Sites of metyrapone inhibition of steroid biosynthesis by rat adrenal mitochondria. Acta Endocrinol (Copenh) 1974;76:703–711.
50 Lee LEJ, Bols NC: The corticosteroid receptor and the action of various steroids in rainbow trout fibroblasts. Gen Comp Endocrinol 1989; 74:85–95.
51 Healy DL, Chrousos GP, Schulte HM, Gold PW, Hodgen GD: Increased adrenocorticotropin, cortisol, and arginine vasopressin secretion in primates after the antiglucocorticoid steroid RU 486: Dose-response relationships. J Clin Endocrinol Metab 1985;60:1–4.
52 Fryer JN, Boudreault-Châteauvert C: Cytological evidence for activation of neuroendocrine cells in the parvocellular preoptic nucleus of the goldfish hypothalamus following pharmacological adrenalectomy. Cell Tissue Res 1981; 218:129–140.
53 Jain MR, Khan FA, Rama Krishna NS, Subhedar N: Intracranial metyrapone stimulates CRF-ACTH axis in the teleost, *Clarias batrachus:* Possible role of neurosteroids. Neuroreport 1994;5:2093–2096.
54 Sillence MN, Rodway RG: Effects of metyrapone and etomidate on adrenal function and growth rate in female rats. J Endocrinol 1987; 113:473–478.
55 Young WS, Mezey E, Siegel RE: Quantitative in situ hybridization histochemistry reveals increased levels of corticotropin-releasing factor mRNA after adrenalectomy in rats. Neuroscience Lett 1986;70:198–203.
56 Herman JP, Schafer MK-H, Thompson RC, Watson SJ: Rapid regulation of corticotropin-releasing hormone gene transcription in vivo. Mol Endocrinol 1992;6:1061–1069.
57 Barton BA, Iwama GK: Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu Rev Fish Dis 1991;1:3–26.
58 Wendelaar Bonga SE: The stress response in fish. Physiol Rev 1997;77:591–625.
59 De Pedro N, Delgado MJ, Pinillos ML, Alonso-Bedate M: α_1-Adrenergic and dopaminergic receptors are involved in the anoretic effect of corticotropin-releasing factor in goldfish. Life Sci 1998;62:1801–1808.
60 De Pedro N, Pinillos ML, Valenciano AI, Alonso-Bedate M, Delgado MJ: Inhibitory effect of serotonin on feeding behavior in goldfish: Involvement of CRF. Peptides 1998;19:505–511.
61 Kent P, Anisman H, Merali Z: Are bombesin-like peptides involved in the mediation of stress response? Life Sci 1998;62:103–114.
62 Lin X, Volkoff H, Narnaware Y, Bernier NJ, Peyon P, Peter RE: Brain regulation of feeding behavior and food intake in fish. Comp Biochem Physiol A Mol Integr Physiol 2000;126: 415–434.