Corticotropin-releasing hormone in the teleost stress response: rapid appearance of the peptide in plasma of tilapia (*Oreochromis mossambicus*)

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Abstract

High concentrations (up to 600 pg/ml) of corticotropin-releasing hormone (CRH) were detected in plasma of the teleost fish *Oreochromis mossambicus* (tilapia) when screening peripheral tissues of tilapia exposed to stress. Notably, the plasma CRH response to stressors in tilapia is much more pronounced than that in higher vertebrates, such as rats. After characterisation by RIA, by spiking plasma with synthetic tilapia CRH and by methanol–acid extraction, it is concluded that the immunoreactive (ir) material in plasma represents tilapia CRH_{1–41}. Results indicate that a CRH-binding protein is absent in tilapia plasma.

Unstressed fish had plasma CRH levels under the limit of detection (<2 pg/ml), but following capture stress plasma CRH levels (170–300 pg/ml) as well as plasma cortisol levels (120 ng/ml) increased rapidly to plateau levels, which were reached after approximately 5 min. Tilapia CRH_{1–41} tested at concentrations between 10^{-11} and 10^{-7} M *in vitro* did not stimulate the cortisol release from interrenal tissue. Also pretreatment of interrenal tissue with 10^{-9} M CRH did not sensitise the cortisol-producing cells to a subsequent ACTH challenge. Forty-

eight hours of net confinement or 48 h of cortisol treatment abolished the plasma CRH response and cortisol response to capture stress. The rapidity of the plasma CRH response and its inhibition after 48 h of stress or cortisol treatment point to release by central nervous tissue. Therefore the distribution of glucocorticoid receptors (GRs) in the brain and pituitary of tilapia was investigated. Main GR-ir cell clusters were found in the medial part (Dm) and posterior part of the dorsal telencephalon, in the preoptic region, in the inferior lobe of the hypothalamus and in the cerebellum.

We conclude from comparison of CRH brain contents of unstressed and stressed fish that plasma CRH was released by CRH-ir cells located in the lateral part of the ventral telencephalon (VI), and suggest that the cortisol feedback on CRH release by VI is mainly exerted via the forebrain Dm region. We propose that CRH is mobilised during stress to fulfil peripheral functions, such as the regulation of circulating leukocytes or of cardiac output, as CRH receptors have been reported in these organs for fish species.

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Introduction

The physiological role of circulating corticotropin-releasing hormone (CRH) in mammals is generally considered to be restricted to defined periods, e.g. the third trimester in human pregnancy (McLean *et al.* 1995). Under normal conditions human plasma CRH levels are low (less than 20 pg/ml; Linton *et al.* 1995, McLean *et al.* 1995, Catalan *et al.* 1998), and near the detection limit of methods available. Although lower vertebrates share many characteristics with mammals with respect to the neuro-endocrine stress response (Wendelaar Bonga 1997), the role of CRH may be more versatile in lower vertebrates as it has been suggested that an evolutionarily old CRH system triggers migratory behaviour in some fish species and metamorphosis in amphibians (Denver 1997).

The present study further investigates the involvement of CRH in the regulation of the hypothalamus–pituitary–interrenal axis in fish. Previously, our group has shown in the teleost fish tilapia (*Oreochromis mossambicus*) that during acute stress plasma cortisol concentrations increase extremely rapidly, in the absence of a preceding plasma adrenocorticotropin (ACTH) surge (Balm *et al.* 1994). Subsequently, the cDNA sequence of the tilapia CRH (tCRH) preprohormone demonstrated that more variation exists between vertebrate CRH sequences than previously recognised (van Enckevort *et al.* 2000), which together with the remarkably wide distribution of CRH in non-hypophysiotrophic brain centres of tilapia indicated that the role of CRH in this species may indeed be broader than previously thought (Pepels *et al.* 2002a,b).

A question unresolved in lower vertebrates is the presence of circulating CRH. Suess *et al.* (1986) reported in the white sucker fish (*Catostomus commersoni*) circulating levels of urotensin-I (U-I), another member of the CRH-like family of peptides. *In vitro*, U-I stimulated the release of cortisol from the interrenal tissue located in the head-kidneys of saltwater- but not freshwater-adapted flounder (*Platichthys flesus*) (Kelsall & Balment 1998).

We here report CRH immunoreactivity (ir) in the plasma of tilapia, and we tested whether tCRH can act directly on the *in vitro* cortisol release from the head-kidneys. Subsequently we investigated whether an acute stressor or a chronic stressor (48 h) affected the release of CRH into the blood. The brain area from which CRH is released into the circulation was determined by comparing the CRH content of brain regions before and after stress. Finally, to investigate whether cortisol affected the plasma CRH release via receptors in the brain, fish were treated with cortisol and the distribution of glucocorticoid receptors (GRs) in the brain was mapped.

Materials and Methods

Animals

Tilapia (*O. mossambicus*), between 1 and 2 years old, from our laboratory stock were kept in groups ($n=6$ or 7) of mixed sex in 100-litre aquaria filled with tap water (24°C) in a 12 h light : 12 h darkness regime. Fish were fed twice a day (0930 and 1530 h) with Tetramin tropical fish food and were left undisturbed for 6 weeks before the start of the experiments. Fish were last fed on the day prior to killing. Sampling of the fish started between 1030 and 1130 h. In most cases, fish were captured sequentially using a fishnet (Balm *et al.* 1994, Pepels *et al.* 2002b). The research was approved by the Institution's Animal Care and the National Animal Ethical Committee and conformed to the guidelines of the UFAW (Universities Federation for Animal Welfare).

Plasma and tissue preparation

Blood was collected from the caudal vessels into ice-cold glass capillaries containing Na_2EDTA and aprotinin (1.5 mg Na_2EDTA and 3000 kIU aprotinin/ml blood) (Serva, Heidelberg, Germany). From fish weighing more than 100 g blood was taken with a syringe containing Na_2EDTA and aprotinin. In both cases blood was taken from the caudal vessels at a position anterior to the tenth caudal vertebra. Plasma was obtained by centrifugation at 10 000 g for 5 min at 4°C , and frozen at -20°C until RIA analysis for CRH or cortisol. In one case the cell fraction (from 1 ml blood) was resuspended in a mixture (1:3 v/v) of methanol and 0.1 M HCl (containing 250 000 kIU aprotinin (Bayer) and 6 mM ascorbic acid),

incubated for 15 min at 0°C and then vortexed for 1 min. Following centrifugation the supernatant was vacuum dried and resuspended in RIA buffer. This procedure was repeated once. Both supernatants/extracts were analysed for CRH as described by Pepels *et al.* (2002b).

Pituitaries and brains were removed from the skull and immediately frozen and kept at -20°C until further micro-dissection. Brain micro-dissection was carried out on an ice-cooled Petri dish using $\times 5$ binocular magnification as described previously (Pepels *et al.* 2002b). For the present study, the telencephalon was transected into an anterior part containing the anterior-lateral subdivisions of the dorsal telencephalon (Da and Dla), and a posterior part containing the lateral part of the ventral telencephalon (VI) (Pepels *et al.* 2002a). The caudal end of the olfactory bulbs was used as a transection landmark. Brain tissue homogenisation and analysis for CRH were carried out according to Pepels *et al.* (2002a,b).

Effects of acute and confinement stress (experiments 1 and 2)

Experiment 1 investigated whether CRH could be measured in plasma of stressed fish. Five male tilapia fish from different mixed-sex tanks were captured. Fish were individually isolated in small transparent tanks (25 cm length, 15 cm depth, 15 cm height; containing 2 litre of water) for 12 min, after which period blood was sampled.

Experiment 2 studied the stress response elicited by sequentially capturing fish ('acute stress') from a group (Balm *et al.* 1994, Quabius *et al.* 1997) and the modulation of this response by previous chronic stress (48 h net confinement). Six fish from a tank were captured at -48 h and stressed by confinement in a small net. At $t=0$ h tilapia from a control group ($n=6$) and from the confined group were sampled sequentially in alternating order at time intervals 0, 2, 5, 8, 11 and 14 min. This experiment was repeated twice, yielding $n=3$ per sampling time point.

Characterisation of plasma CRH (experiment 3)

The CRH content of plasma (or of brain or pituitary homogenates) was determined by a RIA previously validated for tCRH (Pepels *et al.* 2002b). This RIA uses synthetic tCRH as a standard and the level of detection is 2 pg/tube. Standard curves made in RIA buffer or in a mixture (1:3 v/v) of methanol and 0.1 M HCl (containing 250 000 kIU aprotinin (Bayer) and 6 mM ascorbic acid) yielded a similar sensitivity and ED_{50} . The efficiency of CRH extraction from the plasma according to the method of Linton *et al.* (1995) was determined as follows. Plasma samples ($n=6$) obtained from a fish group ($n=6$, weight 61 ± 2 g) sampled over a period of 12 min, were each divided into two aliquots, one of which was measured directly and the other one was extracted by adding three times the volume of ice-cold methanol

(Merck, Darmstadt, Germany). After incubation on ice for 15 min, vortexing for 1 min and centrifugation (10 000 g, 5 min, 4 °C) the supernatant was dried under vacuum and resuspended in RIA buffer to achieve its original volume.

A plasma pool from control fish from experiment 2 that were caught between 8 and 14 min was used in the CRH RIA to construct a dilution curve. The plasma from fish caught prior to 8 min from experiment 2 was used to assess the recovery of synthetic tCRH added to the plasma (spikes of 45 or 75 pg tCRH). Further dilution curves were constructed using telencephalic brain tissues and pituitaries from undisturbed fish ($n=3$), which were homogenised, methanol extracted and pooled as described previously (Pepels *et al.* 2002b).

To investigate whether U-I interfered in the plasma measurement, the influence of an abundant amount (50 ng) of white sucker fish (*C. commersoni*) U-I on the CRH signal was tested.

Plasma CRH levels in undisturbed fish (experiment 4)

In order to obtain plasma samples from undisturbed fish, one fish was quickly caught from each of three control tanks within 1 min. This was repeated on the next two alternate days to obtain plasma from nine undisturbed fish (37 ± 2 g). Previously this procedure was shown to yield plasma samples containing low plasma cortisol levels (Quabius *et al.* 1997). As CRH levels turned out to be near the detection level in these samples (25 µl) we quantified the amount of CRH in 1 ml methanol-extracted plasma from undisturbed tilapia. This extract was vacuum dried and resuspended in 100 µl mixture of methanol and HCl (3:1 v/v) containing ascorbic acid (6 mM) and aprotinin (Bayer: 250 000 kIU/l).

In vitro cortisol release/superfusion of headkidneys (experiment 5)

Headkidney tissues (containing the cortisol-producing interrenal cells) were removed from the fish immediately following sacrifice ($n=24$, 43 ± 1 g). Tissue from one fish was placed in each superfusion chamber (volume 650 µl) and superfused with superfusion medium (Balm *et al.* 1994), which contained 6 mM ascorbic acid. After the cortisol release had reached a steady state headkidneys were superfused for 30 min (240–270 min) with superfusion medium containing 0 M ($n=6$), 10^{-7} M ($n=6$), 10^{-9} M ($n=6$) or 10^{-11} M ($n=6$) of synthetic tCRH_{1–41} (synthesised by Dr J Rivier, Salk Institute, La Jolla, CA, USA). The headkidneys which had received control medium (0 M) or 10^{-9} M CRH were subsequently (300–315 min) pulsed with 10^{-10} M human ACTH (Peninsula, Merseyside, UK). This sub-optimal concentration of ACTH was chosen as the use of higher concentrations of ACTH could mask the possible effect of CRH due to an exhaustion of intracellular second

messenger pools or enzymes needed for cortisol synthesis. The application of the ACTH challenge 30 min after the CRH pulse was based on previous *in vivo* results during and after acute stress caused by capture. First CRH (this study), and then, more than 15 min later, plasma ACTH levels will increase (Balm *et al.* 1994). Superfusion fractions were collected and stored at -20 °C until cortisol analysis by RIA as described previously (Balm *et al.* 1994). From three additional tilapia (± 400 g body weight) head-kidneys, weighing 205 mg on average, were homogenised in methanol and HCl (3:1 v/v) containing ascorbic acid (6 mM) and aprotinin (Bayer: 250 000 kIU/l) and further treated for analysis in the CRH RIA as described previously (Pepels *et al.* 2002b).

Cortisol treatment (experiment 6)

Tilapia (group size seven) were fed for 48 h with Tetramin food, either sprayed with ethanol alone (control tank) or with cortisol-containing ethanol (Balm *et al.* 1994). The fish received a daily food ration of 1.5% of their body weight, thereby receiving a dose of 30 mg cortisol/kg body weight. At 48 h, sampling started by catching control and cortisol-treated fish in alternating sequence at $t=0$ and thereafter at each time interval: 2, 5, 8, 11, 14 and 18 min. Blood was immediately sampled after catching. This experiment was repeated eight times (in two cases one additional control group was sampled), yielding $n=11$ controls and $n=9$ cortisol-treated groups. Plasma samples from fish (from all the trials, total number of fish=140, weight 34 ± 1 g) were analysed for CRH and cortisol. The pituitary homogenates were also analysed for CRH. From six out of nine experiments, the brains of fish numbers 1, 2, 6 and 7 were dissected, homogenised and analysed for CRH to investigate whether stress affected the CRH content of brain regions within 14–18 min.

Immunohistochemistry

Sections for GR and CRH immunostaining were from brains which have also been used to construct a tilapia brain atlas (Pepels *et al.* 2002a). Staining procedures were done directly after sectioning of freshly fixed brains. GR-ir was performed using sections adjacent to the ones used to construct the tilapia CRH brain atlas and GR-positive cells were plotted in chart drawings of this tilapia brain atlas. The fish (± 30 g) had been perfused with 0.9% saline for 1 min and then for 10 min with Bouin's fluid. Brains had been sectioned (transversally or sagittally, 10 µm sections) and every 15th section was mounted and stained for GR-ir and the adjacent sections for α -melanocyte-stimulating hormone (α -MSH) (Antibody code L9; Pepels *et al.* 2004). The GR antibody has previously been used to study GR in tilapia (Dang *et al.* 2000) and was a generous gift from Dr B Ducouret (Endocrinologie Moléculaire de la Reproduction, University of Rennes, France).

Figure 1 (A) Effect of capture (acute stress) on plasma CRH levels of control fish (black bars), $n=3$, and 48 h-confined fish (open bars), $n=3$. (B) CRH RIA standard curve and displacement of radiolabelled tracer from the antibody by serial dilutions of a neat plasma pool obtained from stressed tilapia (8–14 min acute capture stress, same fish as in (A)) and dilutions of pituitary and telencephalon extracts (methanol–acid extraction). At each sampling time point the control group was compared with the confined group. P values <0.05 , <0.02 and <0.01 are represented as *, **, and *** respectively. Means $\pm$ S.E.M.

This antibody is directed against the ab-domain of the trout GR1 receptor (Tujague *et al.* 1998, Bury *et al.* 2003). A section in this ab-domain shares a high degree of homology with the corresponding region of both *Haplochromis burtoni* GRs. Although to date no complete GR sequences are known in tilapia (Tagawa *et al.* 1997), *H. burtoni* and tilapia amino acid sequences are 99% identical (A K Greenwood, unpublished observation). The GR antibody was diluted 2500 times and sections were further processed and treated as described Pepels *et al.* (2002a) with the only exception that the avidin-biotin complex (Vectastain ABC Reagent; Vector Laboratories, Burlingame, CA, USA) was used at a dilution of 1:200. Omission of the primary antibody abolished the immunoreactivity signal. The α -MSH antibody was 1000 times diluted. No ABC enhancer was used but otherwise the same procedure as for GR was used.

The distance from the rostral tip of the olfactory bulb is represented at each brain level (see Fig. 4) and corresponds to the levels represented in the tilapia CRH brain atlas of Pepels *et al.* (2002a).

Statistics

All data are represented as means $\pm$ S.E.M. Differences between groups and treatments of experiments 2 and 6

were analysed with the two-tailed Student's t -test (unpaired) and $P < 0.05\%$ was accepted as the level of significance. $P < 0.05$, < 0.02 and < 0.01 are represented as *, **, and *** respectively. Plateau levels of plasma CRH or plasma cortisol (experiments 2 and 6) were calculated by taking the mean plasma hormone level of tilapia caught in the time period 5–14 min per tank. In experiment 5 the two-tailed Student's t -test was used pair-wise to test differences between prepulse cortisol release rates with maximally stimulated release.

Results

Plasma of fish that were kept in isolation for 12 min contained 590 ± 109 pg/ml ($n=5$; experiment 1) of CRH-like immunoreactivity. In experiment 2, sequentially capturing fish from a group resulted in a time-dependent increase in plasma CRH-like immunoreactivity (Fig. 1A). In fish caught first, CRH levels were low and in most cases below the level of detection. Plateau levels of plasma CRH (302 ± 54 pg/ml) in control fish caught between 5 and 14 min were higher than the CRH plasma levels (36 ± 14 pg/ml) in 48 h confined fish ($P < 0.001$), in which a plasma CRH response was absent (Fig. 1A).

Table 1 Effects of CRH alone or in combination with 10^{-10} M ACTH on headkidney cortisol release ($n=6$). Values represent maximally stimulated release minus prepulse release (pg/min per g body weight). Prepulse release at 240 min was 0.38 ± 0.06 ($n=24$) and prepulse release at 290 min was 0.17 ± 0.04 ($n=12$)

	Molarity of CRH			
	0	10^{-11}	10^{-9}	10^{-7}
CRH 240–270 min	-0.06 ± 0.05	0.17 ± 0.17	0.04 ± 0.03	0.08 ± 0.15
ACTH 300–315 min	$3.77 \pm 1.45^*$	ND	$4.31 \pm 1.92^*$	ND

ND=not determined.

*Maximally stimulated release significantly different from prepulse release rates.

During characterisation, supernatants obtained from the blood cell fraction of acutely stressed fish from experiment 2 did not contain detectable amounts of CRH. Serial dilutions of neat (unextracted) plasma of these fish displaced radiolabelled tCRH from the antibody in a parallel fashion to the curves obtained with dilutions of synthetic tCRH_{1–41} (standard) and to the curve obtained with serial dilutions of tilapia pituitary or telencephalic brain tissue homogenates (Fig. 1B). Spikes of synthetic tCRH (45, 75 pg) added to neat plasma (pooled plasma samples of unstressed tilapia) were recovered with an efficiency of 95 and 104% respectively. Plasma samples ($n=6$) from a fish group sampled over a period between 1 and 12 min containing CRH in concentrations ranging from 2 to 173 pg/ml had similar amounts of CRH with or without methanol extraction ($98.9 \pm 6.7\%$).

In experiment 3, addition of 50 ng U-I to the standard and plasma samples did not affect displacement of tracer from the antibody as, first, the measured value of CRH in plasma samples to which 50 ng U-I was added was indistinguishable ($105 \pm 15\%$) from that measured in the absence of U-I. Secondly, in both the presence or absence of 50 ng U-I, the plasma dilution curves ran parallel to the standard curve and yielded similar ED₅₀ values (not shown). In both cases a similar amount of tCRH was needed to displace 50% of the bound radiolabel (ED₅₀).

In experiment 4, a dilution curve of a methanol-extracted plasma pool could not be distinguished from the curve obtained with dilutions of the neat plasma pool. The plasma cortisol and CRH levels in the undisturbed fish ($n=9$) caught were 4 ± 1 ng/ml and 5 ± 5 pg/ml respectively, with eight out of nine fish below the level of detection. Even in the equivalent of 1 ml plasma from unstressed tilapia (which was methanol extracted and vacuum dried to obtain a small volume for RIA measurement) the CRH level was under the limits of detection.

tCRH tested *in vitro* at concentrations of 10^{-11} , 10^{-9} and 10^{-7} M did not stimulate the release of cortisol from tilapia headkidney (experiment 5; Table 1). There was no difference in ACTH-stimulated (10^{-10} M) cortisol release rates between headkidneys which beforehand had received 0 M or 10^{-9} M CRH (Table 1). Headkidney

homogenates from undisturbed fish ($n=3$) contained 52 ± 11 pg/headkidney of CRH-ir.

In an attempt to mimic the effects induced by 48 h of confinement stress, fish were treated with cortisol (experiment 6; Figs 2 and 3). In the control fish (Fig. 2A) highest plasma CRH levels were measured in the sampling time period between 10 and 12 min, after which period the CRH concentration decreased ($P < 0.001$; $t=12$ vs $t=18$ min). No differences in plasma CRH plateau levels between males or females were detected (males 173 ± 26 pg/ml; females 164 ± 29 pg/ml), nor between females in different reproductive stages (late egg stage females 150 ± 49 pg/ml vs early egg stage 169 ± 34 pg/ml). Forty-eight hours of cortisol feeding inhibited the increase in plasma CRH levels during the period of 4–12 min after the start of capture (Fig. 2A). In control fish, plasma CRH plateau levels were 168 ± 19 pg/ml (Fig. 2A) and cortisol levels reached plateau levels of 118 ± 16 ng/ml (Fig. 2B). In the 48 h cortisol-fed fish (Fig. 2) the plasma CRH response (plateau levels of 70 ± 10 pg/ml) was inhibited ($P < 0.001$ in comparison with control value) and the plasma cortisol response (plateau levels of 9 ± 2 ng/ml) was absent ($P < 0.001$ in comparison with control value).

Comparison of the CRH brain content (experiment 6; Fig. 3A) of control fish caught in the first 2 min ($n=12$) with that of control fish caught between 14 and 18 min ($n=12$), revealed that the latter fish had more CRH ($P < 0.05$) in the anterior part of the telencephalon (containing the DI region), but less CRH ($P < 0.05$) in the posterior part of the telencephalon (containing the VI region). Both effects were absent in cortisol-pretreated fish (Fig. 3B). No effects of capture order in both control and cortisol-pretreated fish were observed in other brain regions (diencephalon, tectum and rhombencephalon; Fig. 3A and B).

Immunohistochemistry

To investigate whether the inhibitory effect of cortisol on CRH release could be mediated via GRs, the GR-ir cells and cell clusters in the brain were localised. The

Figure 2 Effect of capture (acute stress) on plasma CRH levels (A) or on plasma cortisol levels (B) of control fish (black bars), $n=11$, or cortisol-fed fish (open bars), $n=9$. At each sampling time point the control group was compared with the cortisol-pretreated groups. P values <0.05 and <0.01 are represented as * and *** respectively. Means $\pm$ S.E.M.

GR-ir-positive staining was detected in the cytoplasm of cells. The brain of tilapia contained five major ir-cell clusters, three of which were located in the telencephalon, one in the diencephalon and one in the cerebellum. Together these cell clusters contained approximately 75% of the total number of GR-ir cells found in the brain. All other GR-ir areas mentioned contained less cells than found in the cell clusters and these few cells were often scattered throughout the areas.

The GR-positive cell clusters are presented in a rostro-caudal direction. The first cluster located in the dorsal subdivision of the medial part of the dorsal telencephalon (Dmd; Fig. 4: 1250 and 1550 μ m, Fig. 5A and E) consisted of a large number of large round cells (11 μ m), some of which were situated close to the ependyma (Fig. 4: 1250 and 1550 μ m). Colocalisation of GR and CRH was found only in a few large pyriform cells (12 μ m) located dorsal from the ventrolateral telencephalon (Fig. 4: 1550 μ m, Fig. 5B and C). The CRH-ir cells located in the ventrolateral telencephalon (Vl: Fig. 4: 1250 μ m; Fig. 5C) were not GR-ir (Fig. 5B). The ventral telencephalon was conspicuously innervated by blood capillaries (Fig. 5A). In particular the Vl region was very rich in capillaries, which were often located near the CRH-ir cells (Fig. 5D). Some

GR-ir cells were also found in the supracommissural part of the ventral telencephalon, including Vs (Fig. 4: 1550 μ m, Fig. 5A) and Vd.

The second GR-ir cell cluster consisted of round cells located in the posterior part of the dorsal telencephalon (Dp; Fig. 4: 2150 μ m).

The third GR-ir cell cluster was found in the preoptic region. The medial part of the anterior preoptic region (Pam; Fig. 4: 1550 μ m) contained intensely stained parvocellular cells and the magnocellular preoptic nucleus (Pmc) contained intensely stained magnocellular cells (Fig. 4: 2150 and 2300 μ m, Fig. 5G). In the diencephalon small GR-ir cells (5 μ m) were found in the lateral and medial part of the nucleus tuberis lateralis (nltl, nltm; Fig. 4: 2300 μ m, Fig. 5F) and in the posterior nucleus tuberis (npt; Fig. 4: 3050 μ m). Close to the border of the nltm a few large GR-ir cells were present. Colocalisation of CRH-ir and GR-ir was observed in some parvocellular cells in the preoptic nucleus (npo) and the lateral tuberal nucleus (nlt) (not shown). The small cells found in the lateral and medial nucleus recessus lateralis were weakly stained (Fig. 4: 3050 μ m).

A fourth cell cluster was situated in the central parts of the inferior lobe of the hypothalamus (LIH; Fig. 4:

Figure 3 The CRH content of different brain regions of control fish (left panel), $n=12$, and of cortisol-fed fish (right panel), $n=12$. The CRH content of brain regions from the first captured fish (between 0 and 2 min) are depicted in open bars and the CRH content of the last captured fish (between 14 and 18 min) are depicted in black bars. P values < 0.05 are represented as *. Means $\pm$ S.E.M. Rhomb, throbencephalon; Dien, diencephalon.

3050 μm), but also many neurons located near the ependyma of the inferior lobe were GR-ir-positive (Fig. 4: 2300 and 3050 μm). The tertiary gustatory nucleus (TGN; Fig. 4: 3050 μm), the central posterior thalamic nucleus (CP; Fig. 4: 3050 μm) and cells in the periventricular layer of the tectum mesencephali (tect: Fig. 4: 3050 μm) contained a few GR-ir cells, the latter of which were weakly stained.

In the cerebellum a fifth cell cluster consisting of Purkinje cells (ccP) was located in the lateral, ventral and medial fields (Fig. 4: 5150 μm). In the rhombencephalon some cells in the lateral and posterior part of the secondary gustatory nucleus (ngs) and in the reticular formation (ret) were GR-ir (Fig. 4: 5150 μm).

In the pituitary gland, GR-ir cells were found in the proximal pars distalis (Ppd; Fig. 4: 2150 μm), in the pars intermedia (Pi; Fig. 4: 2300 μm , Fig. 5I), but relatively few cells in the rostral pars distalis were GR-ir. From comparison of adjacent sections stained for GR or α -MSH it appeared that subpopulations of the melanotrophs and the somatotactin (SL) cells (i.e. α -MSH-negative cells) were GR-ir (Fig. 5H and I).

Discussion

The high concentrations of plasma CRH in the teleost fish *O. mossambicus* (tilapia) after exposure to an acute stressor

represent the first report of plasma CRH levels in lower vertebrates. It should be noted that the increase of CRH in peripheral plasma from stressed tilapia (up to 600 pg/ml) is dramatic and much more pronounced than in rats (from 8 pg/ml in controls up to 18 pg/ml after ether-laparotomy or water immersion stress; Nishioka *et al.* 1993), or in humans under pathophysiological conditions, such as major depression (23 vs 9 pg/ml in healthy persons (Catalan *et al.* 1998)). Highest plasma CRH levels have been reported in peripheral plasma from pregnant women during their third trimester of pregnancy (± 1 –1.5 ng/ml; Linton *et al.* 1995, McLean *et al.* 1995) and in pituitary portal blood (± 200 pg/ml; Hauger *et al.* 1994). Interestingly, teleost fish do not possess a hypothalamic–pituitary portal system, but hypothalamic neuropeptidergic neurons directly innervate proopiomelanocortin-producing target cells in the pituitary.

Previously we have shown that when tilapia are sampled sequentially from a group, high levels of plasma cortisol are rapidly reached within 5 min, without a preceding rise in plasma ACTH (Balm *et al.* 1994). In the present study maximal plasma CRH levels were also rapidly reached within 5 min, and as plasma CRH levels already decreased after 14 min, we suggest that the rapid onset and decrease of the CRH response in tilapia indicates a rapidly released discrete pulse of CRH. The rapid decrease in plasma CRH peak in tilapia is consistent with the short half lives of many vertebrate neuropeptides including mammalian

CRH, which has a half life of about 20 min in plasma (Stalla *et al.* 1986).

We conclude that the immunoreactive material in plasma from stressed fish represented tCRH₁₋₄₁, as firstly dilution curves made from neat or methanol-extracted tilapia plasma ran parallel. Secondly, CRH levels were similar in neat or extracted plasma in an RIA previously validated for tCRH (Pepels *et al.* 2002b). Thirdly, exogenous CRH added to neat or extracted plasma yielded similar CRH values. In human plasma devoid of CRH, the existence of a CRH-binding protein (CRH-BP) was noticed when an unknown factor displaced radiolabelled CRH from the antibody in the RIA (Orth & Mount 1987). Methanol extracts CRH, but not its binding protein (Linton *et al.* 1995). Since in our study no radiolabelled tCRH was displaced from the antibody by plasma from unstressed tilapia, tilapia plasma probably does not contain a CRH-BP. This is in line with the absence of CRH-BP in the plasma of *Xenopus laevis*, and with the suggestion made by Kemp *et al.* (1998) that plasma BP has been recruited relatively recently in evolution to fulfil a peripheral function, i.e. binding of placental CRH in primates. Chemical crosslinking studies with radiolabelled *X. laevis* CRH have suggested the presence of BP activity in brain tissue of birds, reptiles and fish such as tilapia (Seasholtz *et al.* 2002). Consistently, we previously demonstrated that CRH extraction from tilapia brain homogenates with methanol greatly increased the recovery when compared with acid extraction (Pepels *et al.* 2002b).

Although this study is the first to measure high levels of circulating CRH, previously circulating U-I levels of 80 pg/ml have been reported in the white sucker fish *C. commersoni* (Suess *et al.* 1986). U-I, a member of the CRF-like family of peptides, is synthesised in teleost fish by neuroendocrine cells located in the caudal part of the spinal cord (Lovejoy & Balment 1999) and in minor quantities by neurons in the brain (Suess *et al.* 1986, Bernier *et al.* 1999). Interference of plasma U-I can be discounted in our RIA (Pepels *et al.* 2002b), for which previously the crossreactivity with U-I was determined to be less than 0.5%. Possible contamination during blood sampling was minimised by taking blood from the caudal

vessels anterior to the tenth terminal vertebrae. The U-I-secreting Dahlgren cells are located in the spinal cord in the area of the fifth caudal vertebrae (Fridberg *et al.* 1966). Importantly, addition of 50 ng U-I to plasma of stressed tilapia did not alter the plasma CRH-ir values measured ($105 \pm 15\%$).

The inhibition of the plasma CRH response after the cortisol treatment in our study suggests that this hormone is involved in the inhibition of the plasma CRH response observed after 48 h of confinement. Also in rats, treatment with dexamethasone reversibly decreased (from 13 to 8 pg/ml) the plasma CRH levels (Sumitomo *et al.* 1987, Yokoe *et al.* 1988). As in these studies the plasma CRH alterations were reflected in hypothalamic and pituitary CRH levels, the authors strongly suggested that the main source of plasma CRH was the hypothalamic paraventricular nucleus (pvn) neurons with their terminals in the median eminence.

Source of CRH and cortisol feedback

In fish, some circulating neuropeptides, such as melanocortin-stimulating hormone and thyrotropin releasing hormone, are released from the neural part of the pituitary. However, we exclude the pituitary as a source of plasma CRH as no time-dependent changes were found in pituitary CRH content during capture. In the case that plasma CRH originated from the neural part of the pituitary gland, a detectable reduction of about 170 pg of pituitary CRH content would have occurred (3% of the body weight is plasma in tilapia (Okimoto *et al.* 1994)).

The rapidity of the CRH response in tilapia points to control by central nervous tissue. Previously we have shown that the largest population of CRH-ir cells in the brain of tilapia consists of non-hypophysiotrophic neurons, located in the lateral part of the ventral telencephalon (VI). These cells have major projections to the anterior part of the laterodorsal telencephalon (Dla), but also locally in the VI area CRH-ir terminals were observed (Pepels *et al.* 2002a). On the basis of the rapid decrease in CRH content of the posterior telencephalon, including VI, during capture stress we propose the VI as the source for circulating

Figure 4 Rostro-caudal series of transverse brain sections illustrating the distribution of GR-ir cells in the brain of tilapia (scale bar=500 µm). The distance from the rostral tip of the olfactory bulb is indicated in µm for each level. These levels correspond to levels and sections previously described in a tilapia brain atlas (see Figure 1E, F, H, I, K, L and N in Pepels *et al.* 2002a). Abbreviations are: ca, commissura anterior; cc gran, granular layer of the corpus cerebelli; cc mol, molecular layer of the corpus cerebelli; ccP, Purkinje cell layer of the corpus cerebelli; CP, central posterior thalamic nucleus; Da, pars dorsalis telencephali pars anterior; Dd, pars dorsalis telencephali pars dorsalis; Dl, pars dorsalis telencephali pars lateralis; Dla, anterior subdivision of Dl; Dmd, dorsal subdivision of the pars dorsalis telencephali pars medialis; Dp, pars dorsalis telencephali pars posterior; eg, eminentia granularis; LIH, lobus inferior hypothalami; ltv, lateral telencephalic veins; ms, meningeal surface; ngs, nucleus gustatorius secundarius; NLLa, anterior lateral line nerve; nltl, lateral part of the nucleus lateralis tuberis; nltm, medial part of the nucleus lateralis tuberis; npt, nucleus posterior tuberis; nrl, nucleus recessi lateralis; nrlm, medial part of nrl; Pam, medial part of the preoptic region pars anterior; Pi, pars intermedia of the pituitary gland; Pn, pars nervosa of the pituitary gland; Ppd, proximal pars distalis of the pituitary gland; Pmc, magnocellular preoptic nucleus; Ppm, medial part of the preoptic region; ret, reticular formation; rl, recessus lateralis; rp, recessus posterior; tect, tectum mesencephali; TGN, tertiary gustatory nucleus; TO, tractus opticus; V, nervus trigemius; Vd, area ventralis telencephali pars dorsalis; Ve, ventricle; VII, nervus facialis; VI, area ventralis telencephali pars lateralis; Vs, area ventralis telencephali pars supracommissuralis.

CRH in tilapia. This is corroborated by three further findings. First, the decrease in CRH content after 14–18 min (175 pg) was similar to the estimated amount of CRH which appeared in the plasma (170 pg). Secondly, cortisol pretreatment concurrently abolished both the plasma CRH elevation and the drop in the posterior telencephalic CRH content. Thirdly, we showed that the ventral telencephalon of tilapia displays a strikingly high blood vascularisation, which confirms previous observations in other fish (Weiger *et al.* 1998, Butler 2000). In particular the ventrolateral telencephalic area in which the CRH-ir cells are located in tilapia contains a plexus of blood capillaries, which are drained via the lateral telencephalic vein (Weiger *et al.* 1998).

To examine routes via which the VI might receive inhibitory cortisol input, the distribution of GR in the brain was studied. The overall distribution pattern in tilapia is in line with the *in situ* hybridisation and immunohistochemical results in trout (Teitsma *et al.* 1997, 1998), which also demonstrated predominant GR-ir cell clusters in the dorsal telencephalon, in the npo, in the nlt, in the inferior lobe of the hypothalamus (LIH) and in the cerebellum.

Recently Greenwood *et al.* (2003) characterised three corticosteroid receptors in *H. burtoni*: GR1, GR2 and a mineralocorticoid receptor (MR). Two GRs and a MR have also been reported in rainbow trout (Bury *et al.* 2003), but to date in tilapia only one of these receptors has been partially cloned (Tagawa *et al.* 1997), most likely the tilapia equivalent of the *Haplochromis* GR1 (Greenwood *et al.* 2003). As the antibody used in the present study was raised against the ab-domain of trout GR1 (Tujague *et al.* 1998, Bury *et al.* 2003) equivalent it is unlikely that it detected a putative tilapia MR given the low homology between the ab-domains of GR and MR in both *H. burtoni* and trout. Although in contrast to the DNA-binding domain the GR ab-domain varies between species, the N-terminal region of the latter domain appears more conserved. As discussed by Greenwood *et al.* (2003) and Bury *et al.* (2003), GR gene duplication probably has occurred throughout the teleost lineage, in which case our results include cells expressing GR1 and those expressing GR2.

The cytoplasmic location of the GR signal in our fish corroborates results in gill tissue of tilapia, rainbow trout and Atlantic salmon (Dang *et al.* 2000), but contrasts with results in trout (Teitsma *et al.* 1998), which may relate to the immunohistochemical methodology used or to physiological characteristics (developmental stage) of the fish used. Notably, during spawning migration of Kokanee salmon, Carruth *et al.* (2000) observed a shift of the GR signal from the cytoplasm to the nucleus.

Despite the overall similarity in GR brain distribution between trout and tilapia there were some species differences. The second and third gustatory centres were GR-positive in tilapia but not in trout. This difference may relate to the regulation of feeding behaviour during mouthbreeding of eggs, unique for cichlids. In tilapia the second and third gustatory centres project into the Dmd and receive input from the vagal lobe (Yoshimoto *et al.* 1998); the latter has been related to the cessation of feeding behaviour and inhibition of the swallowing reflex, which are critical for the survival of the eggs and larvae during mouthbreeding (Pepels *et al.* 2002a). The posterior part of the vagal lobe of tilapia, but not of salmonids, contains many CRH-ir fibres and terminals (Pepels *et al.* 2002a). The inhibitory role of CRH on feeding in fish has been demonstrated by intracerebro-ventricular CRH administration (De-Pedro *et al.* 1993).

In tilapia, as in trout, colocalisation of CRH and GR was observed in cells in the npo and nlt, which substantiate previous observations that cortisol feedback can be exerted at the level of the hypothalamus (Fryer & Peter 1977) and nucleus preopticus (Fryer & Peter 1977, Olivereau & Olivereau 1988, Bernier *et al.* 1999) in fish. Additionally in tilapia very few cells dorsal to VI were GR-positive, but it is unlikely that the cortisol effects observed were directed on these cells. Also in mammals cortisol feedback is exerted indirectly. The pvn is moderately rich in GR receptors, but basal levels of corticosterone first act at the hippocampus, which tonically inhibits the pvn (reviewed by De Kloet *et al.* 1998). In tilapia a telencephalic structure via which cortisol feedback to the VI can be exerted is the medial part of the dorsal telencephalon, because the Dm contains a large group of GR-ir cells, and Dm has efferents to VI (Murakami *et al.* 1983). Interestingly, Dm not

Figure 5 Micrographs of brain and pituitary sections. White arrowheads indicate blood capillaries and asterisks indicate lateral telencephalic veins. (A) A transverse overview through the right telencephalon at the level of the VI region showing a GR-ir cell cluster in Dmd, scattered GR-ir cells dorsal to VI, GR-ir cells in Vs, a plexus of blood capillaries in VI and the lateral telencephalic veins. (B and C) Comparison of adjacent lateral sagittal sections of the telencephalon showing CRH-ir cells in VI (C), but no GR-ir cells (B) are present in the posterior part of VI. GR-ir cells are located dorsally from VI (B). Very few CRH-ir cells showed GR-ir as shown by the arrow in (B and C). Left and right side of each photograph represents the posterior and anterior side respectively. (D) A transverse section through the right telencephalon showing the plexus of blood capillaries (arrowheads) close to the CRH-ir cells and CRH-ir terminals in VI. The plexus is drained via the lateral telencephalic veins. (E) A transverse section showing GR-ir in the cytoplasm of large round perikarya situated in the Dmd region of the telencephalon. (F) A transverse section showing small pyriform GR-ir cells in the lateral and medial part of the nucleus tuberis lateralis. (G) A transverse section through the pre-optic region showing GR-ir in magnocellular and parvocellular cells. (H and I) Comparison of adjacent sagittal pituitary sections showing α -MSH-ir cells (H) and GR-ir cells (I). Arrows indicate α -MSH-ir cells in the pars intermedia (H) that are GR-positive (white arrows) or GR-negative (black arrows) in the adjacent section (I) Black arrowheads indicate GR-positive cells that are not α -MSH-positive. See Fig. 4 legend for abbreviations.

only processes gustatory information but also acoustico-lateral signals from the lateral line system, an important sensory warning and orientation system for fish (Meek & Nieuwenhuys 1998). Efferents of the Dm may either directly inhibit CRH-positive cells in the VI or may inhibit the noradrenergic input that is present in the ventral telencephalon of fish (Meek *et al.* 1993). Recently we showed that in tilapia *in vitro* telencephalic CRH release is stimulated by noradrenaline (Pepels *et al.* 2004), which is consistent with the possibility that *in vivo* release from the VI during stress is under adrenergic control.

The relatively low numbers of GR-ir cells that were found in the rostral pars distalis (Rpd) of the pituitary gland is related to the low number of corticotrophs present in the Rpd and is also in line with the very low innervation of the Rpd by CRH-ir terminals (Pepels *et al.* 2002a). Also in trout the corticotrophs are GR-positive (Teitsma *et al.* 1998). The identity of the GR-ir cells in the Ppd of the pituitary, which contains gonadotrophs, thyrotrophs and somatotrophs, is unclear but in trout both types of gonadotrophs display GR-ir (Teitsma *et al.* 1999). In the pars intermedia of the pituitary of tilapia subpopulations of the SL cells and of the melanotrophs contained GR, which supports previous physiological studies in teleost fish. Cortisol administered *in vivo* or *in vitro* (Balm *et al.* 1993) inhibited the CRH-stimulated *in vitro* α -MSH release from tilapia pars intermedia lobes. Induction of GR expression in rat MSH cells by stressors has been shown by Antakly *et al.* (1985). The release of SL, a member of the growth hormone/prolactin family unique for teleosts (Wendelaar Bonga 1997), is also influenced by stressors (Rand-Weaver *et al.* 1993).

Function

It is evident that plasma CRH is involved in the regulation of stress-related peripheral processes as unstressed tilapia had undetected CRH levels and plasma CRH levels increased after acute stress, whereas this increase was absent after chronic stress of 48 h or after cortisol pretreatment. Peripheral actions of plasma CRH are consistent with the expression of CRH receptors in heart, gills (Pohl *et al.* 2001) and spleen (Arai *et al.* 2001) of fish. We have no evidence for direct actions of CRH or of CRH in combination with a subsequent ACTH pulse on the interrenal cells, but CRH could regulate circulating leukocytes during stress, as CRH stimulates activated peripheral leukocytes of channel catfish (*Ictalurus punctatus*) *in vitro* to secrete ACTH-ir (Arnold & Rice 2000). CRH concentrations as low as 50 pg/ml modulate interleukin-1 β production by cultures of peripheral human blood monocytes (Pereda *et al.* 1995). Also circulating CRH may regulate cardiac output during stress. In the catfish *Ameiurus nebulosus* (Arai *et al.* 2001) and in salmon *Oncorhynchus keta* (Pohl *et al.* 2001), CRH-R2 are most abundantly expressed peripherally in the atrium. In

contrast to mammals the fish CRF-R1 and R2 receptors display similar affinities to U-I and to CRH (Arai *et al.* 2001). As reported plasma levels of U-I in fish do not exceed 80 pg/ml (Suess *et al.* 1986), CRH-R2 in the atrium could transduce the plasma CRH signal (600 pg/ml) during stress. Haemodynamic actions of CRH have also been reported to increase corticosteroid release (van Oers *et al.* 1992). In this case the peptide forms part of a neuroendocrine circuit involving CRH-producing cells in the adrenal gland. Activation of this circuit increases bloodflow, which in turn leads to elevated corticosteroid release (van Oers *et al.* 1992). Notably the headkidneys of unstressed tilapia contained CRH, which strongly suggests that the peptide is locally produced in this organ, as in these fish plasma CRH levels were below the level of detection.

In tilapia (Pepels *et al.* 2002a) and in other teleosts species (Lovejoy & Balment 1999) descending spinal CRH-ir projections appear to be absent in contrast to the mammalian brain where descending CRH-ir projections into the medulla and the spinal cord have been described. These eventually terminate upon cell groups that innervate smooth and cardiac muscle, various glands and body viscera (Sawchenko & Swanson 1985). Possibly in teleosts some autonomic CRH actions during stress are regulated by circulating CRH, whereas the descending neural CRH projection evolved later during evolution.

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