ORIGINAL ARTICLE

Distribution and Regional Stressor–Induced Regulation of Corticotrophin–Releasing Factor Binding Protein in Rainbow Trout (*Oncorhynchus mykiss*)

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The corticotrophin-releasing factor (CRF) system plays a key role in the co-ordination of the physiological response to stress in vertebrates. Although the binding protein (BP) for CRF-related peptides, CRF-BP, is an important player in the many functions of the CRF system, the distribution of CRF-BP and the impact of stressors on its expression in fish are poorly understood. In the present study, we describe the distribution of CRF-BP in the brain and peripheral tissues of rainbow trout (*Oncorhynchus mykiss*) using a combination of real-time reverse transcriptase-polymerase chain reaction, *in situ* hybridisation and immunohistochemistry. Our results indicate a widespread and highly localised distribution of CRF-BP in the central nervous system, but do not support a significant peripheral production of the protein. Major expression sites in the brain include the area ventralis telencephali, nucleus preopticus, anterior and lateral tuberal nuclei, and the posterior region of the pituitary pars distalis. We further characterise changes in CRF-BP gene expression in three discrete brain regions after exposure to 8 h and 24 h of social stress or hypoxia. The plasma cortisol concentration in subordinate fish was much higher than in dominant fish and controls, and was indicative of a relatively severe stressor. By contrast, the increase in plasma cortisol concentration in fish exposed to hypoxia was characteristic of the response to a mild stressor. Changes in CRF-BP gene expression were only observed after 24 h of either stressor, and were region-specific. CRF-BP mRNA in the telencephalon increased in both subordinate fish and fish exposed to hypoxia, but CRF-BP in the preoptic area only increased after 24 h of hypoxia exposure. In the hypothalamus, CRF-BP mRNA levels decreased in dominant fish relative to controls after 24 h. Taken together, our results support a diverse role for CRF-BP in the central actions of the fish CRF system, but a negligible role in the peripheral functions of circulating CRF-related peptides. Furthermore, the differential changes in forebrain CRF-BP mRNA appear to occur independently of the hypothalamic-pituitary-inter-renal axis.

Key words: CRF, CRF-BP, gene expression, *in situ* hybridisation, stress.

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Corticotrophin-releasing factor (CRF) belongs to a family of related neuropeptides that regulate a variety of physiological processes associated with the maintenance of homeostasis during stress (1). In fish, other members of the CRF family include urotensin-I (UI; orthologue to tetrapod urocortin, UCN), and homologous peptides to mammalian UCN-2 and -3 (2). CRF is perhaps best known as the apex hormone of the hypothalamic-pituitary-inter-renal (HPI) axis that controls circulating levels of glucocorticoids; however,

CRF-related peptides play a diverse role in mediating an animal's response to stress (3), including cardiovascular modulation (4) and altered food intake (5). CRF-related peptides signal through a common family of receptors (6,7) and are further modulated by a shared binding protein (CRF-BP) (8).

CRF-BP is a highly conserved 37-kDa protein originally isolated from human plasma (9) and is present in all vertebrate classes (8). With a high-affinity for all CRF-related peptides (10) and a broad

distribution in the brain (11, 12), CRF-BP is likely to be an important player in the many functions of the vertebrate CRF system. Central distribution of CRF-BP in the zebrafish (12) is widespread and shares some homologous expression sites with mammals (11). Conversely, peripheral distribution varies tremendously among vertebrates. For example, most primates have a circulating CRF-BP (13) that is produced in the liver (14) and intrauterine tissues (15). *Xenopus laevis* tadpoles express CRF-BP in the tail (16) and *Cyprinus carpio* contain CRF-BP immunopositive cells in the gills and skin (17).

The first functions described for CRF-BP were to inhibit CRF signalling at its receptors. For example, CRF-BP reduced CRF-induced release of adrenocorticotrophic hormone (ACTH) in rat pituitary cultures (14), and the high levels of circulating CRF during the third trimester are attenuated by parallel high levels of CRF-BP in humans (18). More recently, high levels of CRF-BP in tadpole tails have been shown to protect the cells from the apoptotic signalling of CRF (19). Contrary to these inhibitory functions, CRF-BP is obligate for CRF signalling in certain dopaminergic neuronal populations in rats (20); thus, its functional role is likely distribution-dependent (21). Furthermore, regulation of CRF-BP by glucocorticoids and/or CRF in the brain (22, 23), plasma (24, 25), and in cultured nerve cells (26–28) implies a role for CRF-BP in the stress-induced functions of the CRF system.

Fish present an opportunity to study both conserved and unique attributes of the vertebrate CRF system. Although stressor-induced activation of the HPI-axis and changes in CRF-related peptide expression have been well studied, the impact of stress on CRF-BP expression in fish is poorly understood. Both UI and CRF circulate in fish (29, 30), and the concentrations of plasma CRF reached in stressed fish greatly exceeds any reported in mammals (30). Conversely, CRF-BP does not appear to circulate in fish (30), suggesting that it may be involved in local and tissue-specific peripheral regulation of CRF-related peptides. The extent of CRF-BP production outside the central nervous system (CNS) remains largely unknown. The present study aimed to characterise CRF-BP distribution in rainbow trout (*Oncorhynchus mykiss*) using a combination of gene and protein expression tools, and to assess its regulation during two different stress regimes in three brain regions. We measured the relative mRNA levels using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in ten regions of the CNS, twelve peripheral tissues, and in the brains of fish in dominant-subordinate pairs or exposed to hypoxia at two time points. CRF-BP location in the brain was further characterised using *in situ* hybridisation and immunohistochemistry. This research supports an important functional role for CRF-BP in the fish CRF system based on its broad central distribution and region- and time-specific responses to stressors.

Materials and methods

Animals

Juvenile rainbow trout (*O. mykiss*) of mixed sex were obtained from Rainbow Springs Trout Farm (Thamesford, ON, Canada) and housed in 650-l fibreglass tanks in the Hagen Aqualab Facility at the University of Guelph (Guelph, ON, Canada). Tanks were supplied with aerated local well water maintained at 14 °C. Fish were exposed to a 12 : 12 h light/dark cycle and fed *ad lib* once daily with commercial trout pellets (4pt Classic Sinking fish pellets; Martin Mills, Elmira, ON, Canada). The University of Guelph Animal Care Committee approved care and use of the animals, as per the principles of the Canadian Council for Animal Care.

Tissue survey of CRF-BP gene expression

Fish of either sex weighing approximately 200 g (n = 6–8) were terminally anaesthetised in 2 ml/l 2-phenoxyethanol (Sigma-Aldrich, St Louis, MO, USA) and placed on ice. The CNS was regionally dissected into ten parts: the caudal neurosecretory system (CNSS), and eight brain regions plus the pituitary after (31) (Fig. 1A). The following twelve peripheral tissues were also collected: whole blood, gill, heart, stomach, intestine, liver, spleen, head kidney, posterior kidney, adipose, skin, and skeletal muscle. All tissues were snap frozen in liquid nitrogen and stored at −80 °C until total RNA extraction.

Social stress experiment

Twenty-one pairs of juvenile trout (101.0 ± 1.9 g) were size-matched according to weight. The average difference within a pair was 1.5 ± 0.2 g,
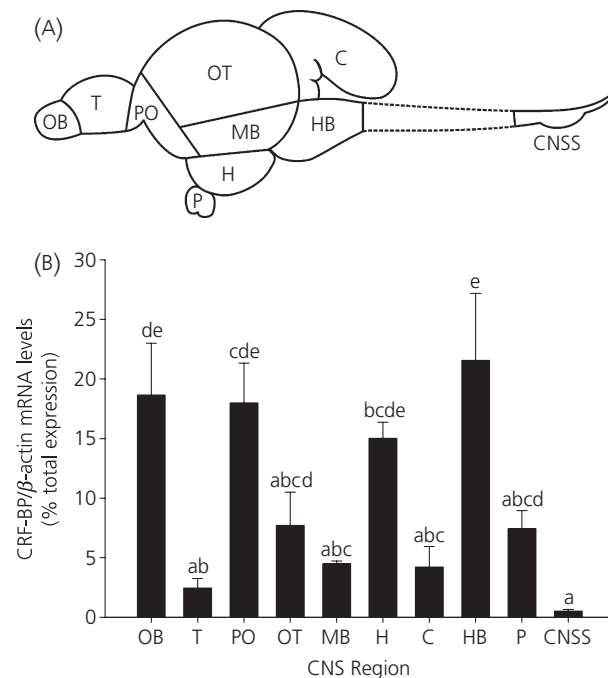
Fig. 1. Evidence for broad distribution of CRF-binding protein (CRF-BP) mRNA in the central nervous system (CNS) of rainbow trout. (A) Schematic of the trout CNS showing the ten regions assayed for CRF-BP mRNA. (B) Real-time reverse transcriptase-polymerase chain reaction amplification of CRF-BP normalised to β-actin gene expression in the CNS regions outlined in (A), expressed as per cent total expression. Bars that do not share a common letter are significantly different, as assessed with a one-way ANOVA and Tukey's test (n = 6-7; P < 0.05). C, cerebellum; CNSS, caudal neurosecretory system; H, hypothalamus; HB, hindbrain; MB, midbrain; OB, olfactory bulb; OT, optic tectum; P, pituitary (hypophysis); PO, preoptic area; T, telencephalon.

with a maximal difference of 4.4 g. Each fish in a pair was placed in 30-l aquaria on either side of an opaque divider supplied with constant system water. Fish were fed daily during a 72-h acclimation period, at the end of which the divider was removed and fish were allowed to interact for 8 h or 24 h. During the first 3 h of interaction, social behaviour was indirectly observed for three 5-min periods through an angled mirror. Points were assigned for aggressive attacks, position in the tank, and food acquisition as previously described (31) such that the dominant fish in each pair would have a higher score. In all but one pair, the score of the dominant fish was three- to 50-fold higher than the subordinate; the pair without a clear difference in behavioural score was removed from further analysis. After 8 h or 24 h of interaction, each pair was quickly and terminally anaesthetised as above. Blood was collected via caudal puncture, separated by centrifugation, and the plasma stored at −80 °C. The telencephalon, preoptic area, and hypothalamus from each fish were collected and snap frozen for subsequent RNA extraction. Additionally, six fish (162.4 ± 10.6 g) were quickly netted from the original stock tank that had not been disturbed for at least 2 weeks prior. These fish were sampled as above and served as a control group.

Hypoxia stress experiment

Fish were divided into three 125-l tanks (n = 10–11; 120.2 ± 3.2 g) and acclimated for 3 weeks during which they were fed daily. At the end of the acclimation period, fish were exposed to normoxic water (85% O_2 saturation; 8.8 mgO_2/l; control) or hypoxic water (30% O_2 saturation; 3.1 mgO_2/l) for 8 h or 24 h. The oxygen was stripped from a header tank by bubbling nitrogen through the water column, and this water was used to supply the hypoxia treatment groups. At the end of the exposure, fish were terminally anaesthetised and sampled as in the social stress experiment.

Total RNA extraction and first strand cDNA synthesis

All tissues were mechanically homogenised and total RNA was extracted using TRIzol Reagent according to manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). One microgram of total RNA was treated with DNase I and reverse transcribed to cDNA using SuperScript II RNase H- reverse transcriptase according to manufacturer's instructions (Invitrogen). Nonreverse transcribed (no-RT controls) representatives from each tissue were included during cDNA synthesis to monitor genomic contamination.

Real-time RT-PCR analysis of gene expression

Triplicates of each cDNA sample were amplified by real-time RT-PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The 20-μl reactions contained 10 μl 2X SYBR Green PCR Master Mix (Applied Biosystems), 5 μl five-fold diluted first strand cDNA template or no-RT controls, and 2.5 μl each of forward and reverse primers (0.4 μM). Manufacturer's settings were used for PCR: 10 min initial denaturation at 95 °C, followed by 40 cycles of 15 s denaturation at 94 °C and 1 min of annealing/extension at 60 °C. To verify that the amplification signal resulted from a single PCR product, a dissociation cycle from 60–90 °C was performed following the 40th cycle, and only samples with a unimodal dissociation curve and predicted melting temperature were used. No template controls were used to monitor background amplification and were consistently free of contamination. Primer pairs for real-time RT-PCR were designed using Primer Express 2.0 (Applied Biosystems) based on the complete coding sequence for rainbow trout β-actin (GenBank accession number: AJ438158) and CRF-BP (GenBank accession number: AY363677). An

exon–exon boundary, identified by homology alignments of known genomic sequences (β-actin) or the rainbow trout partial genomic sequence (CRF-BP; GenBank accession number: AY686703) was positioned in either the forward or reverse primer, respectively, to prevent co-amplification of genomic DNA. Primer pairs are listed in Table 1. No-RT controls did not amplify genomic DNA, thus confirming the efficiency of DNase I treatment and primer design. As the level of β-actin mRNA can be affected by exposure to cortisol (S. Alderman, unpublished observation), elongation factor-1α (EF-1α; GenBank accession number: AF498320) was used for normalisation in both stress experiments.

An initial survey of peripheral tissues suggested a low and inconsistent expression of CRF-BP. To ensure that the signal obtained was not a result of nonspecific PCR products, these samples were additionally analysed with the more specific TaqMan assay. Samples (n = 6 per tissue) were run in duplicate and two no-RT controls were included for each tissue. Each reaction contained 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 2.5 μl each of forward and reverse primers (9 μM), 2.5 μl of probe (2.5 μM), and 5 μl of five-fold diluted first strand cDNA template or no-RT control. With the exception of the dissociation curve, PCR conditions were as described above. Primers and probe were designed against the reverse complement of the tCRF-BP coding sequence and were generously provided by Applied Biosystems (Table 1). The probe was labelled at the 5′ end with a FAM fluorescent reporter dye, and at the 3′ end with the nonfluorescent quencher minor groove binder.

To account for differences in amplification efficiency, a standard curve was constructed for each target gene using known dilutions of pituitary or hypothalamus cDNA. Input values were obtained by fitting the average threshold cycle (C_T) value to the antilog of the standard curve. To correct for minor variations in template input and transcriptional efficiency, these input values were then normalised to the housekeeping gene β-actin (distribution survey) or EF-1α (stress experiments). To account for regional differences in β-actin expression levels, all input values were corrected to the mean input of the hypothalamus (arbitrarily chosen). All data are expressed as mean ± SEM.

Table 1. Sequences of Primer Pairs and Probe Used to Amplify β-Actin, Elongation Factor-1α and Corticotrophin-Releasing Factor Binding-Protein (CRF-BP) in Two Real-Time Reverse Transcriptase-Polymerase Chain Reaction Assays and for Synthesising the CRF-BP Riboprobe Used for *In Situ* Hybridisation.

	Sequence (5′ to 3′)
SYBR Green assay	
β-actin	F: GCC CCC CTC AAC CCC
	R: GAA GGT CTC AAA CAT AAT CTG GGT C
EF-1α	F: GGG CAA GGG CTC TTT CAA GT
	R: CGC AAT CAG CCT GAG AGG T
CRF-BP	F: GGA GGA GAC TTC ATC AAG GTG TT
	R: CTT CTC TCC CTT CAT CAC CCA G
TaqMan assay	
CRF-BP	F: GAG TAT GAC AGC GTT GAC ATC GA
	R: CAT CAC CCA GCC ATC AAA CAC
	P: TTG ATG AAG TCT CCT CCC CT
Riboprobe synthesis	
CRF-BP	F: GCT GGA CCC GAC CAG GTC AT
	R: CAG CTC CAC AAA GTC CCC TG

F, Forward primer; R, reverse primer; P, probe; EF-1α, elongation factor-1α.

Plasma cortisol measurements

The cortisol content of plasma samples was determined using a radioimmunoassay. Each 600 μl reaction contained equal volumes of plasma or standards, ^3H-cortisol (7500 cpm; 73.0 Ci/mmol; Amersham Biosciences, Piscataway, NJ, USA) and rabbit anti-cortisol antibody (cat no. F3-314; Esoterix Endocrinology, Calabasas Hills, CA, USA), all appropriately diluted in assay buffer (21.4 mM $Na_2HPO_4 \cdot 7$ H_2O; 9.3 mM $NaH_2PO_4 \cdot H_2O$; pH 7.6; 0.1% gelatin; 0.01% thimerosal; Sigma). Following overnight incubation at 4 °C, reactions were cooled on ice for 10 min then incubated a further 10 min in the presence of 200 μl dextran-coated charcoal (0.5% activated charcoal and 0.05% dextran in assay buffer; Sigma). Bound cortisol was pelleted by centrifugation and the supernatant was combined with 5 ml scintillation cocktail [667 ml toluene, 333 ml Triton X-100, 4 g 2,5-diphenyloxazole, and 0.2 g 1,4-bis(5-phenyl-2-oxazolyl)benzene; 2,2′-*p*-phenylene-bis(5-phenyloxazole); Sigma] and counted on a multi-purpose scintillation counter (LS 6500; Beckman, Fullerton, CA, USA). The antibody concentration used was adjusted to yield 35–45% binding in the absence of cold competitor, and the assay was validated with serial dilutions of trout plasma that ran parallel to the standard curve. Samples were diluted to fall within the 20–80% range of the standard curve (ten- and 100-fold for unstressed and stressed fish, respectively). Determination of plasma cortisol concentration was achieved by a three-parameter sigmoidal curve regression equation fitted to the standard curve (SigmaPlot 10.0; SPSS, Chicago, IL, USA).

In situ hybridisation

Juvenile trout of either sex weighing 80–120 g (n = 5) were terminally anaesthetised in 2 ml/l 2-phenoxyethanol then injected with 100 IU heparin in 0.9% NaCl by caudal puncture. Fish were transcardially perfused with 0.9% NaCl for 10 min, followed by 25 min with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 2 ml/min using a Miniplus 3 peristaltic pump (Gilson Inc, Middleton, WI, USA). Brains were removed intact to 4% PFA in PBS and processed for cryosectioning as previously described (12). Serial transverse 12 μm cryosections were collected every 150 μm throughout the brain. Sense and antisense digoxygenin (DIG)-labelled riboprobes were generated using a 479-bp cloned fragment of tCRF-BP and were hybridised to adjacent sections as previously described (12) (for primers, see Table 1). Identification of positively stained nuclei was performed as previously described (32).

Immunohistochemistry

Two fish were terminally anaesthetised and the hypothalamus quickly removed for overnight fixation in Bouin's fixative. Following dehydration and embedding in paraffin, serial sagittal sections (7 μm) were collected and immunostained for CRF-BP, as previously described (33), using the Vectastain ABC method (Vector Laboratories, Burlingame, CA, USA). Briefly, sections were deparaffinised in xylene and rehydrated through a graded ethanol series. Endogenous peroxidase activity was blocked in 1% H_2O_2 in methanol, and nonspecific binding sites were blocked in 5% goat serum. Rabbit anti-human CRF-BP (generously donated by W. Vale) was applied at 1 : 2000 for 1 h at room temperature, followed by goat anti-rabbit IgG-biotin (1 : 200) for 30 min. After 30 min of incubation in an avidin-biotin horseradish peroxidase (HRP) complex (Vectastain ABC reagent; Vector Laboratories), HRP was visualised using freshly prepared 0.1% 3,3′-diaminobenzidine tetrachloride in 0.1 M Tris buffer (pH 7.2) containing 0.02% H_2O_2. Controls on serial sections for endogenous protein-bound biotin and cross-reactivity of the primary and secondary antibodies were all negative. Pre-adsorption controls on serial sections were also included to confirm positive immunostaining results. Briefly, the undiluted primary antibody was incubated with recombi-

nant hCRF-BP (kindly provided by W. Vale) at a ratio of 1 : 10 (v/w) overnight at 4 °C and centrifuged for 25 min at 17 000 g. The supernatant was diluted to the working dilution of 1 : 2000 in PBS and used in place of the primary antibody in the procedure above.

Imaging

Photomicrographs were captured on a digital camera using OpenLab software (Improvision Incorporated, Lexington, KT, USA). Minor adjustments in brightness and contrast were made in Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA) to improve the quality of presentation without changing or enhancing positive staining. Line drawings were created from original photomicrographs using CorelDRAW X3 (Corel Corporation, Ottawa, Canada).

Statistical analysis

For the tissue survey of gene expression, four outliers from the CNS, one from each of olfactory bulbs, telencephalon, pituitary, and midbrain, were determined as being greater than or less than 2.0× inter-quartile range from the upper quartile or lower quartile, respectively, and were subsequently removed. A one-way ANOVA followed by Tukey's test for multiple comparisons was used to determine differences in gene expression between regions in the CNS (n = 6–7) or between treatment groups (n = 6–11), and to assess changes in plasma cortisol between treatment groups (n = 6–11). Data that did not meet the assumption of normality was log-transformed prior to analysis (Social Stress: telencephalon and hypothalamus gene expression; Hypoxia: telencephalon and preoptic area gene expression). If the transformed data still did not conform to the assumptions of ANOVA, a Kruskal–Wallis one-way ANOVA on ranks followed by a Dunn's test for multiple comparisons was performed (Social Stress: hypothalamus gene expression; Hypoxia: plasma cortisol). All analyses were performed using SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Distribution of CRF-BP in the trout central nervous system

Overall, there was a broad distribution of CRF-BP gene expression in the trout CNS (Fig. 1), with four of the ten regions accounting for over 70% of total expression (Fig. 1B). Moving caudally, these areas of highest expression were the olfactory bulbs (18.7%), preoptic area (18%), hypothalamus (15%), and hindbrain (21.5%). CRF-BP gene expression was lowest in the CNSS, which represented only 0.5% of the total CRF-BP gene expression in the CNS. Although the level of expression in the CNSS did not differ significantly from other areas of low expression (P > 0.05), it was at least five-fold lower than any other region in the CNS. The remaining five regions combined (telencephalon, optic tectum, midbrain, cerebellum, and pituitary) accounted for less than 30% of total CRF-BP gene expression, with none of the regions individually representing more than 10% of total expression.

Trout brain sections successfully hybridised the antisense DIG-labelled CRF-BP riboprobe, whereas sections applied with the sense riboprobe were consistently clear of staining. Although no CRF-BP mRNA was labelled in the olfactory bulbs, the telencephalon showed positive staining in the pars ventralis (Vv; Fig. 2A) and

pars supracommisuralis (Vs; not shown) of the area ventralis telencephali.

CRF-BP mRNA was observed in both the parvocellular and magnocellular divisions of the nucleus preopticus, with the majority of expression occurring in the pars anterior of the parvocellular division (PPa; Fig. 2B). In the optic tectum, CRF-BP mRNA was circumferentially scattered around the ventriculus mesencephali in the first proximal layer of the tecti mesencephali (stratum periventriculare; SPV; Fig 2C). Staining of the SPV occurred from the rostral tip of the tectum through to the caudal end (Fig. 3C–F). Expression of CRF-BP in the pituitary gland occurred in both the proximal pars distalis (PPD) and the pars intermedia (PI; Fig. 2D), but was absent in the rostral pars distalis. Hypothalamic expression of CRF-BP was extensive, including the lateral and anterior divisions of the tuberal nucleus (nlt and nat, respectively; Fig. 2E, F), the nucleus lateralis recessus (nrl) and the nucleus magnocellularis hypothalami (nmh; Fig. 2F). CRF-BP-like immunoreactive (ir) nerve fibres were also prominent in the hypothalamus (Fig. 4A). In the tuberal hypothalamus, vertically oriented parallel fibre tracts were observed in the anterior region (Fig. 4B). Ventral to the recessus lateralis, darkly stained granular fibres were densely packed (Fig. 4C), reflecting the abundant CRF-BP mRNA in this region (Fig. 2F). Several granular CRF-BP-like-immunoreactive fibres were also found ventral to the recessus posterior (Fig. 4D). Preabsorption of the primary antibody with hrCRF-BP completely absolved the signal on serial sections (Fig. 4E).

In the cerebellum, diffuse expression of CRF-BP was observed in the stratum granulare (not shown). In the hindbrain, the raphe nucleus showed extensive expression in the superior division (ras; Fig. 2G) and a few cells in the intermediate division (not shown). Hybridisation of CRF-BP mRNA in the hindbrain was also evident in the nucleus motorius nervi trigemini (not shown).

The distribution of CRF-BP mRNA in the trout brain is summarised in Fig. 3, with the level and plane of each section depicted in Fig. 3(A).

## Expression of CRF-BP mRNA in peripheral tissues

Real-time RT-PCR of CRF-BP in trout peripheral tissues using the SYBR Green assay suggested a very low or negligible level of template cDNA was present in these samples. We therefore employed a more specific technique, the TaqMan assay, to better detect any low level of peripheral expression. Of the 72 tissue samples assayed, only six detected CRF-BP: three gills, and one each of heart, spleen, and skin. Due to this variable and inconsistent amplification, we did not pursue further characterisation of peripheral expression.

Effect of stress on CRF-BP expression

Size-matched juvenile rainbow trout quickly and irreversibly established a dominant-subordinate ranking in all but one pair. The twenty dominant fish aggressively controlled resources whereas the twenty subordinate fish were restricted to a corner of the aquarium by the frequent aggressive attacks of the dominant fish. The plasma cortisol concentration of the subordinate fish 8 h and 24 h after social stress (405.3 ± 74.7 ng/ml and 153.7 ± 19.3 ng/ml, respectively) was significantly higher than the control (2.9 ± 0.6 ng/ml), 8-h post-stress dominant (13.7 ± 6.9 ng/ml)
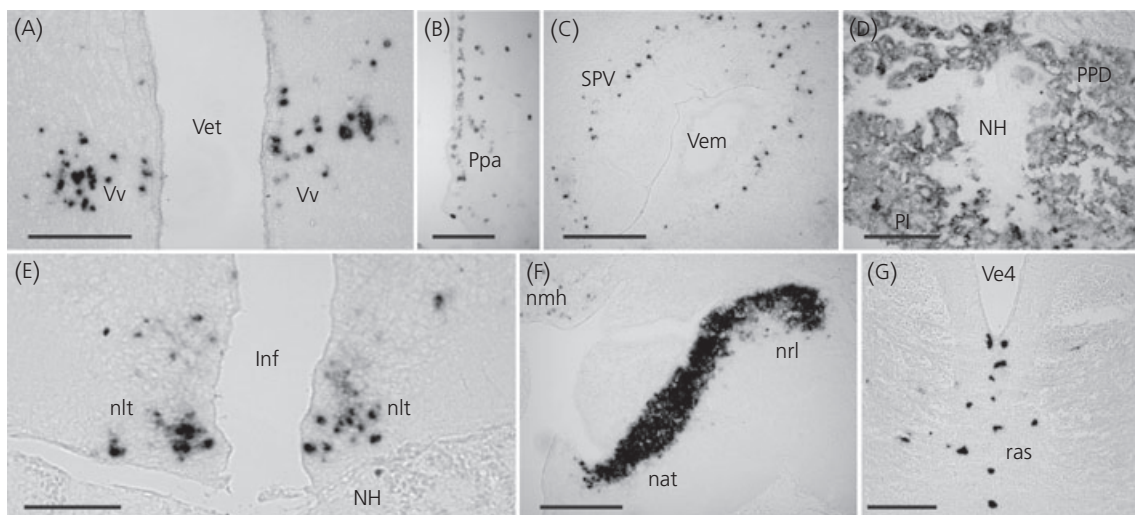
Fig. 2. CRF-binding protein (CRF-BP) mRNA localisation in the rainbow trout brain as determined by *in situ* hybridisation. (A) The pars ventralis (Vv) shows symmetrical staining on either side of the telencephalic ventricle (Vet). (B) Positively stained nuclei of the anterior parvocellular division of the nucleus preopticus (Ppa) line the third ventricle. (C) CRF-BP expression in the rostral tip of the optic tectum is circumferentially scattered around the ventriculus mesencephalon (Vem) in the stratum periventriculare (SPV). (D) Extensive staining of the pituitary occurred in both the proximal pars distalis (PPD) and pars intermedia (PI), but is obviously absent in the neurohypophysis (NH). (E) CRF-BP is expressed in the lateral tuberal nucleus (nlt) on either side of the infundibulum (Inf). (F) The most prominent staining occurred in the anterior tuberal nucleus (nat) and nucleus lateralis recessus (nrl). Several cells in the magnocellular nucleus of the hypothalamus (nmh) are also positive for CRF-BP mRNA. (G) CRF-BP expression in the superior raphe nucleus (ras) just below the fourth ventricle (Ve4). Scale bars = 300 μm, except in (E) where it equals 150 μm.
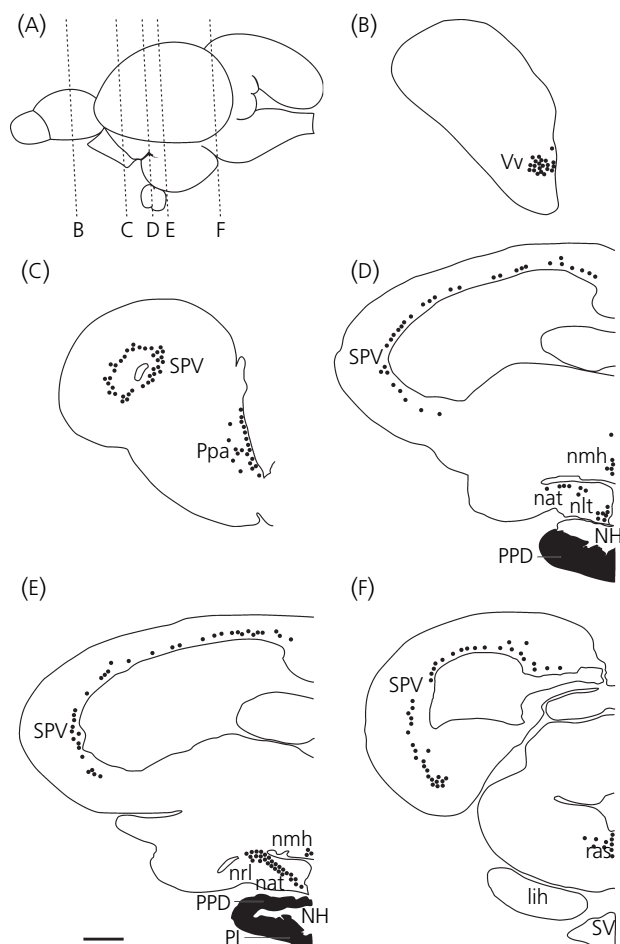
Fig. 3. Line drawings through five planes of the trout brain depicting CRF-binding protein (CRF-BP) mRNA expression (black circles). (A) Schematic of the trout brain, sagittal view, showing the sectioning level of the hemisections in (B–F). The drawings correspond approximately to the following figures in the atlas of the trout brain (32): (B) fig. 15.25, (C) fig. 15.22, (D) fig. 15.19, (E) fig. 15.18, (F) fig. 15.16. Scale bar = 600 μm. lih, lateral inferior hypothalamus; nat, anterior tuberal nucleus; NH, neurohypophysis; nlt, lateral tuberal nucleus; nmh, magnocellular nucleus of the hypothalamus; nrl, nucleus lateralis recessus; PI, pars intermedia; Ppa, anterior parvocellular division of the nucleus preopticus; PPD, proximal pars distalis; ras, superior raphe nucleus; SPV, stratum periventriculare; SV, saccus vasculosus; Vv, pars ventralis.

and 24 h-post-stress dominant (10.3 $\pm$ 1.9 ng/ml) fish groups (P < 0.001; Fig. 5A). Intra-pair differences between dominant and subordinate fish ranged from four- to over 200-fold. After 8 h of social stress, there was no difference in CRF-BP gene expression associated with social rank in any of the brain regions assessed (Fig. 5B–D). After 24 h of social stress, however, there were clear regional changes in CRF-BP mRNA levels associated with social rank. In the telencephalon, subordinate fish had nearly four-fold higher CRF-BP gene expression than any other group (Fig. 5B; P < 0.05). In the preoptic area, there was still no difference in CRF-BP mRNA levels (Fig. 5C; P > 0.05) whereas, in the hypothalamus of dominant fish, there was a significant decrease in CRF-BP

mRNA relative to control fish (Fig. 5D; P < 0.05) but not their subordinate counterparts (P > 0.05).

For hypoxia exposure, the desired O_2 level was achieved gradually over a 1-h period and maintained at 28 $\pm$ 2% saturation for the duration of the exposure. Although control fish kept to the bottom of the tanks and were comparatively active, fish exposed to hypoxia were found at the surface of the water and tended to remain very still. The plasma cortisol concentration in fish exposed to hypoxia for 8 h (7.8 $\pm$ 2.3 ng/ml) was significantly higher than control fish (4.4 $\pm$ 1.8 ng/ml; P < 0.05) but not the 24-h hypoxia-exposed fish (8.5 $\pm$ 3.5 ng/ml), and there was no significant difference between control and 24-h hypoxia-exposed fish (Fig. 6A). After 8 h of exposure to 30% hypoxia, there was no difference in CRF-BP expression relative to control fish in any of the three brain regions (Fig. 6B). After 24 h, CRF-BP mRNA in the telencephalon and preoptic area was significantly higher than the control and 8 h expression levels (P < 0.05), but remained basal in the hypothalamus (Fig. 6B). Finally, there was no effect of treatment on the gene expression levels of EF-1α in either experiment.

Discussion

The present study provides a thorough analysis of CRF-BP distribution in a teleost fish, including a survey of CRF-BP mRNA expression across the CNS and peripheral tissues. By utilising a combination of molecular techniques, we were able to detail the widespread but highly localised expression pattern of CRF-BP in the CNS of rainbow trout. Our results are supported by other studies of CRF-BP distribution (11, 12) and are in line with the known distribution and many functions of the CRF system. We further show regional- and time-dependent changes in CRF-BP gene expression in response to two unique stressors, suggesting stressor-specific responses by different CRF-BP expressing neuronal populations. Taken together, these results make a significant contribution towards understanding the distribution and function of CRF-BP in lower vertebrates.

As suggested for mammals (34), the broad distribution pattern of CRF-BP in the brain of trout is likely indicative of a role for this protein in the various endocrine and neurotransmitter functions of the equally widespread CRF-related peptides. The mechanism by which CRF-BP interacts with CRF-related peptides is likely to be reflected by its distribution. In rats, for example, the ability of CRF-BP to inhibit CRF-stimulated ACTH release (14) is reflected in the localisation of CRF-BP to corticotrophs (11), whereas the requirement of CRF-BP for CRF-stimulated firing of dopaminergic neurones in the ventral tegmental area (20) predicts a co-localisation of CRF and its binding protein in afferent fibres to this region. Indeed, throughout the rat brain, CRF and its binding protein show varying degrees of co-localisation (34), which may prescribe different functional roles for CRF-BP in different regions, as suggested by its differential subcellular localisation across the brain (35). Additionally, CRF-BP may associate with the CRF-related peptides, UCN (mammals) or UI (fish), in regions where it is not colocalised with CRF. In zebrafish, CRF-BP shows pronounced regional co-expression with CRF and comparatively little UI co-expression (12).
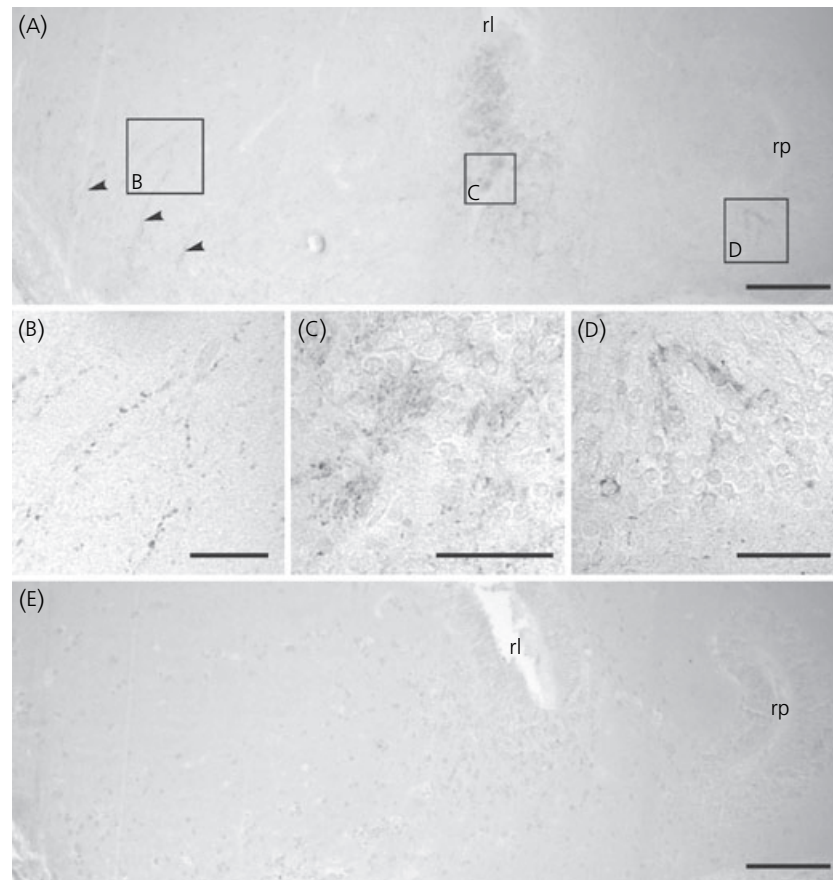
Fig. 4. Immunohistochemical localisation of CRF-binding protein (CRF-BP) in the hypothalamus. (A) Low power photomicrograph of CRF-BP-like immunoreactive nerve fibres in the hypothalamus. Image is in sagittal plane, oriented rostral–caudal. (B) Higher magnification of box B in (A) showing positively stained nerve fibres in the anterior tuberal region. (C) Higher magnification of box C in (A) showing positively stained nerve fibres in the area ventral to the recessus lateralis (rl). (D) Higher magnification of box D in (A) showing prominent CRF-BP-like immunoreactivity in the area ventral to the recessus posterior (rp). (E) Preabsorption of the primary antibody with hrCRF-BP applied to a serial section completely absolved positive staining in the regions described above. (A, E) Scale bar = 200 μm; (B–D) scale bar = 50 μm.

## CRF-BP localisation in the rainbow trout brain

Olfactory bulbs

The olfactory bulbs in rats are known to contain CRF-BP (11); however, it has yet to be localised to this region in fish. We describe the olfactory bulbs as one of four regions of highest expression in the CNS using real-time RT-PCR, however we were unable to localise the expression by *in situ* hybridisation. Indeed, this apparent discrepancy underlines the importance of complimenting these two techniques for studying unknown peptide distribution, especially when comparing expression patterns in tissues with varying signal-to-volume ratios. Assuming that CRF-BP is only expressed in a subset of the diffusely arranged neurones of the olfactory bulbs, two explanations can be provided for these inconsistent results. First, weak or scattered expression might not be detected well by our chromogenic technique and may require the additional sensitivity of fluorescent *in situ* hybridisation in order to be visualised. Second, our sectioning regime yielded less than ten cryosections through the olfactory bulbs. If CRF-BP expression is localised to a very discrete portion of the olfactory bulbs, these neurones may not have been included in the tissue sections we stained.

Telencephalon

Expression of CRF-BP in the telencephalon of rainbow trout was restricted to the area ventralis, as was true in zebrafish (12). In rats, telencephalic CRF-BP is predominantly in the amygdala (11). Due to partial eversion of the telencephalon during teleostean development, the amygdala homologue in fish is likely the medial zone of the dorsal telencephalon, which is further supported by the distribution of several neurotransmitters (36). The presence of CRF-BP in the area ventralis and absence in the area dorsalis of fish may indicate novel functions not yet described in vertebrates. CRF-related peptides have also been located in the fish telencephalon (12, 37–39), and their ability to induce anxiety and fear-related behaviours in the mammalian limbic system (40, 41) implicates the telencephalon as an important extra-hypothalamic branch of the CRF system.
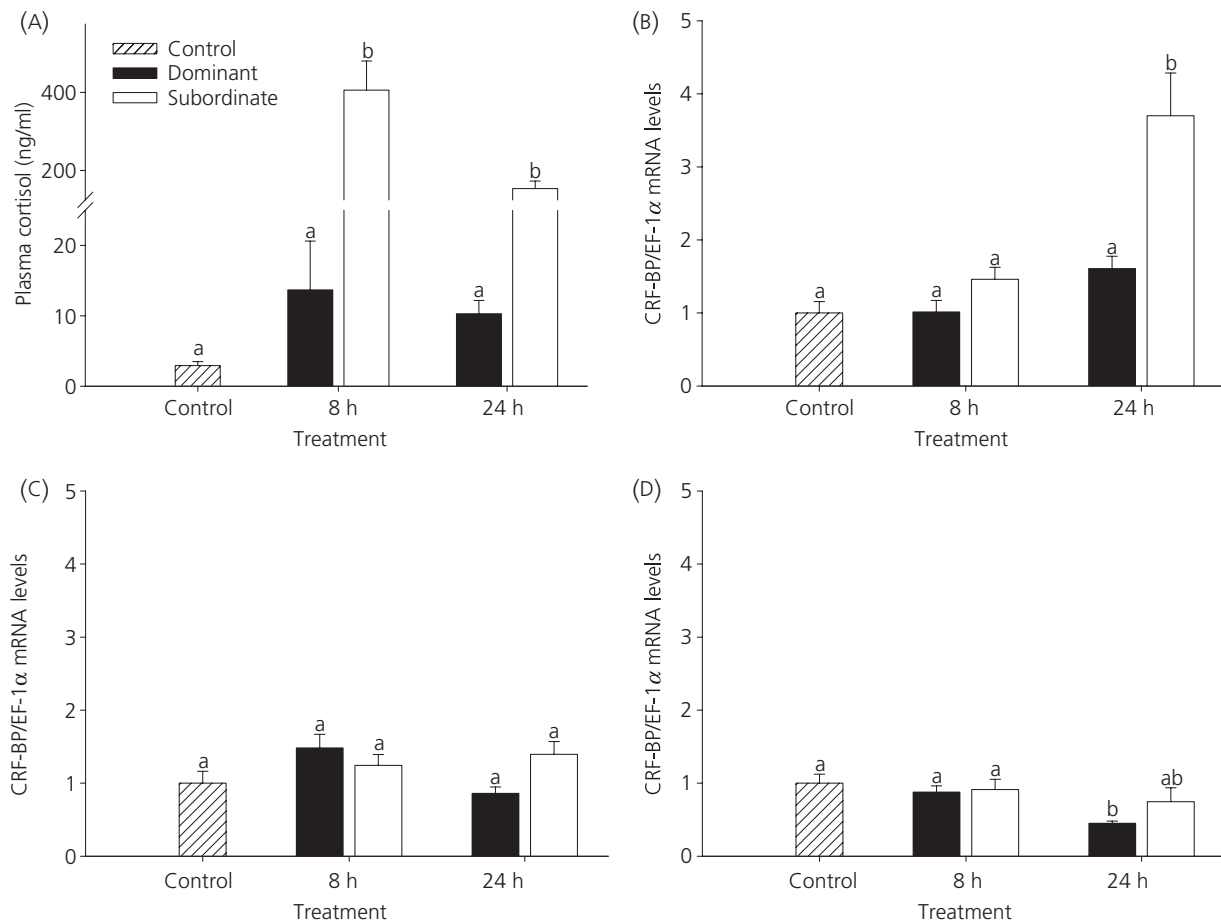
Fig. 5. Effects of social stress on (A) plasma cortisol, and CRF-binding protein (CRF-BP) mRNA expression in the (B) telencephalon, (C) preoptic area, and (D) hypothalamus of rainbow trout. Fish (n = 7–10) were size-matched according to weight and allowed to interact for 8 h or 24 h. Dominance was assigned using behavioural scoring such that dominant fish had a higher score. A control group (n = 6) was sampled at random from a stock tank for comparison. CRF-BP mRNA levels were normalised to EF-1α. Differences between control (hatched bar), dominant (black bars) and subordinate (white bars) fish were determined by a one-way ANOVA and Tukey's post-hoc test (A–C), or a Kruskal–Wallis one-way ANOVA on ranks and Dunn's post-hoc test (D; n = 6–10; P < 0.05). Bars that do not share a common letter within a given brain region are significantly different.
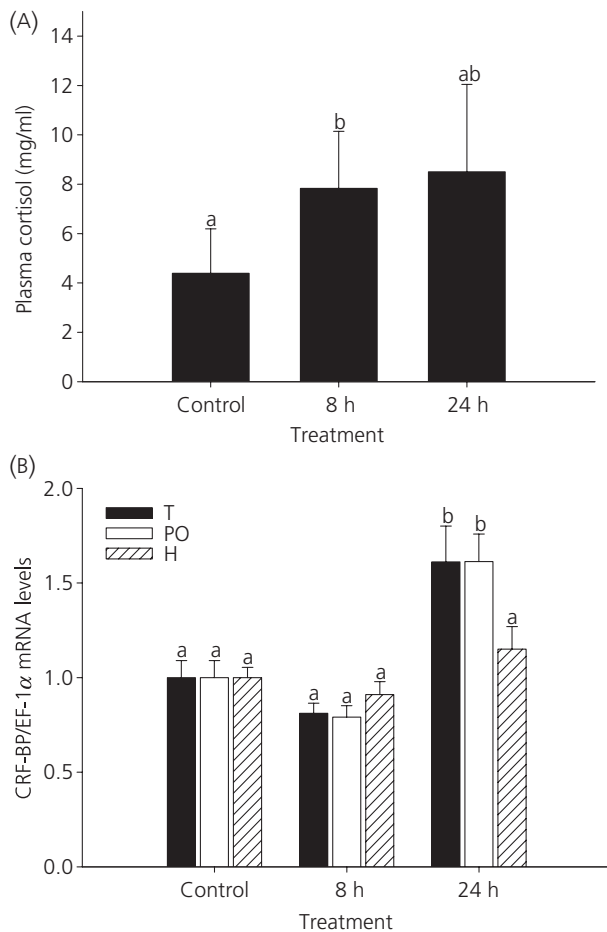
Preoptic area

The principal nucleus contained within the preoptic area is the nucleus preopticus (NPO), homologous to the paraventricular nucleus of other vertebrates. This major site of CRF production is where regulation of pituitary ACTH secretion and thus glucocorticoid regulation is initiated. We found abundant expression of CRF-BP in the preoptic area of *O. mykiss*. Although mainly localised to the PPa lining the third ventricle, some expression was found in all major divisions of the NPO. Results of CRF-BP distribution in other fish suggest possible interspecies differences in preoptic area expression. In zebrafish, for example, CRF-BP mRNA was mostly found in the PPa, occasionally in the posterior parvocellular division (PPp) and never in the magnocellular division (PM) (12). At the protein level, only a few CRF-BP-immunoreactive perikarya were observed in the NPO of the common carp (42). It should be noted, however, that all of the histological evidence for CRF-BP distribution in the NPO has been gained from unstressed fish; thus, the potential for CRF-BP expression in the various divisions of the NPO remains to be determined.

Hypothalamus

The hypothalamus proper is arguably among the most important regions of CRF-BP production in trout, and suggests a functional role for this protein in the CRF system and the neuroendocrine and autonomic control of homeostasis. Real-time RT-PCR analysis grouped the hypothalamus among those regions of highest gene expression, and *in situ* hybridisation highlighted several distinct populations of CRF-BP expression, namely nmh, nat, nlt, and nrl. CRF-BP-like-immunoreactive nerve tracts extending from the tuberal and lateral recessus nuclei reinforced the mRNA expression profile in this area, and the absence of signal in preadsorption controls implies specificity of at least some epitopes for trout CRF-BP. Huising *et al.* (42) also reported a high level of mRNA and little immunoreactivity for CRF-BP in the hypothalamus of *C. carpio*.

Several functions of the CRF-system can be attributed to hypothalamic expression. In fish, CRF-related peptides produced in the nlt are responsible for stimulating the production of ACTH in

Fig. 6. Effect of hypoxia on (A) plasma cortisol, and (B) CRF-binding protein (CRF-BP) mRNA expression in the telencephalon (T), preoptic area (PO), and hypothalamus (H) of rainbow trout after 8 h and 24 h of exposure. Differences in plasma cortisol were determined by a Kruskal–Wallis one-way ANOVA on ranks and Dunn's post-hoc test (n = 10–11; P < 0.05). CRF-BP mRNA levels were normalised to EF-1α, and differences between treatments were determined by a one-way ANOVA and Tukey's test (n = 9–11; P < 0.05). Bars that do not share a common letter within a given brain region are significantly different.

pituitary corticotrophs (43). The additional presence of CRF-BP in this nucleus (12; present study) lends support to a functional role for CRF-BP in regulating the neuroendocrine activities of the CRF-system. Stress-induced anorexia is another documented role for CRF-related peptides in the hypothalamus (5). Intracerebroventricular injections of CRF, UI, or CRF-receptor antagonists (44, 45) implicate the CRF-system in the central control of food intake in goldfish. Mammalian studies further suggest a functional role for CRF-BP in the neural control of feeding (46–48).

Optic tectum

Expression of CRF-BP occurs throughout the optic tectum in the first proximal layer, or SPV. Zebrafish also express CRF, UI, and CRF-BP in

the proximal optic tectum (12). In general, the optic tectum is responsible for processing visual input and integrating it with other sensory information. The SPV itself harbours the most numerous population of tectal neurones (32). A functional role for the CRF system in the optic tectum has been well described in amphibians, where CRF-producing neurones in the tectum are part of a circuit activated in response to a predator-like visual stimulus (49).

Hindbrain

CRF-BP expression in the hindbrain was relatively high, and transcripts were mainly concentrated in the superior and intermediate divisions of the raphe nucleus. Some CRF-BP expression in the hindbrain can also be attributed to the nucleus motorius nervi trigemini. This expression pattern mimics that observed in zebrafish (12) and rats (11), suggesting a strong evolutionary conservation for CRF-BP functions in the brainstem. Indeed, the CRF system has been implicated in locomotor activity stemming from serotonergic neurone activation in the raphe nucleus of fish (50), amphibians (51), and mammals (52, 53). In mammals, the CRF system in the raphe nucleus also affects the activity of forebrain projecting serotonergic neurones that contribute to various non-ambulatory stress-related behaviours (54). Current perspectives on the involvement of the brainstem CRF system in ambulatory and non-ambulatory stress-induced behaviours are provided elsewhere (55).

Pituitary

There are several reports of CRF-BP mRNA and protein isolated to rat corticotrophs (11, 35), where it inhibits CRF-induced release of ACTH (14, 56). By contrast, we found no evidence of CRF-BP in the rostral pars distalis of rainbow trout. This pituitary region in fish contains the corticotrophs which, as in all vertebrates, are responsive to CRF-related peptide signalling (57,58). In the present study, CRF-BP mRNA was only detected in the proximal pars distalis and pars intermedia. Interestingly, the proximal pars distalis in fish secretes thyrotrophin (TSH) and the pars intermedia secretes melanotrophin (α-MSH) in response to CRF and UI (57, 58). In carp, the majority of pituitary CRF-BP mRNA was located in the pars intermedia (42). Doyon et al. (59) observed a stress-induced increase in pituitary CRF-BP expression; however, Huising et al. (42) found no change in the level of mRNA in the carp pars distalis. Unlike other vertebrates, in fish, the hypothalamic-releasing factors that influence pituitary secretions are released directly on the pituitary cells rather than into a portal system (1); thus, involvement of CRF-BP in the ACTH-releasing actions of the CRF system could stem from co-synaptic or parasynaptic release from NPO neurones onto corticotrophs. Indeed, CRF-BP is localised with CRF and UI in the zebrafish PPa (12), and stress increases expression in the preoptic area of CRF-BP in fish (42; present study). Conversely, CRF-stimulated TSH release from the proximal pars distalis and α-MSH release from the pars intermedia could be modulated by locally produced CRF-BP.

Peripheral expression

Recently, Pepels *et al.* (30) showed a rapid and massive increase in circulating CRF following stress in tilapia, likely originating from the lateral nucleus of the area ventralis telencephali. Another source of circulating CRF and UI in fish is the CNSS (60). Despite high circulating levels of its ligands, CRF-BP is believed not to circulate in fish (30). This is in sharp contrast to the situation in primates, where elevated circulating CRF during late gestation is attenuated with parallel high plasma levels of CRF-BP produced by intrauterine tissues (61). Instead, CRF-BP may serve a paracrine modulatory role in peripheral targets of CRF-related peptides in nonmammalian species, as suggested by studies in *Xenopus* (19) and carp (17, 62). Although we were unable to conclude that CRF-BP is constitutively produced in any of the trout peripheral tissues that we assessed, we cannot exclude local production by specific cell types in some tissues. However, our results support the brain and pituitary as phylogenetically conserved expression sites of CRF-BP (63), whereas the variation in peripheral expression sites among vertebrate taxa (64) suggests a more recent incorporation of CRF-BP outside the CNS. Consequently, the functions of CRF-BP within the CNS are likely more conserved, whereas peripheral functions may be more specialised.

Regulation of CRF-BP during stress

The stress regimes used in this study were chosen for two reasons. First, they offered two extremes of HPI-axis activation to help ascertain how CRF-BP fits into our current understanding of the CRF system in fish. We have shown previously that social subordination elicits a pronounced and robust cortisol response that contrasts the slight increase observed during hypoxia exposure, and that both stressors elicit a concomitant increase in CRF-related peptide mRNA in the preoptic area (65). Second, given the nature of each stressor, their perception and integration at the level of the brain is likely to be quite different, therefore helping to elucidate any regional differences in CRF-BP functions. In rats, for example, CRF-BP mRNA increased after acute stress in the basolateral but not the central amygdalar nucleus (22).

There is currently no direct evidence to support involvement of CRF-BP in CRF-related peptide signalling activities of fish; however, the changes in CRF-BP gene expression observed in the present study and previously (59) suggest a stress-induced response by the CRF-BP gene. Of particular interest in the present study are the differing responses observed between brain regions during the same stressor, and within brain regions during different stressors. Although CRF-BP expression in the telencephalon increased after 24 h of both social and hypoxic stress, preoptic area expression only increased after 24 h of hypoxic stress, and hypothalamic expression actually decreased in dominant fish after 24 h while remaining at control levels in subordinate fish. Interestingly, increased gene expression was observed with hypoxia exposure despite only a very small increase in plasma cortisol, and preoptic area CRF-BP remained at control

levels in subordinate fish despite a very large increase in plasma cortisol, suggesting that CRF-BP can be regulated independently of the HPI-axis.

The mechanism of CRF-BP gene activation remains poorly understood, and only a partial promoter has been sequenced to date (66). Although *in vitro* studies suggest CRF-BP is modulated in neuronal tissue by numerous factors including CRF and the synthetic glucocorticoid dexamethasone (28), results obtained *in vivo* confirm CRF activation but fail to show a response to corticosterone in the rat amygdala (23). Because CRF mRNA in the preoptic area of *O. mykiss* increases over a similar time course (65) to that of telencephalic CRF-BP in the present study in response to the same stressors, and NPO neurones project to the Vv and Vs (67, 68), CRF may also regulate CRF-BP in fish. Furthermore, the distribution of glucocorticoid receptors in the brain of *O. mykiss* (69) parallel many of the key CRF-BP expressing regions we observed (e.g. Vv, NPO, nlt, nrl), thus providing an opportunity for feedback regulation by cortisol.

General conclusions

In short, CRF-BP is abundantly and broadly expressed in the CNS of rainbow trout, and its distribution suggests involvement in the many functions of the CRF system. By contrast, despite the presence of circulating CRF-related peptides in fish, the peripheral expression of CRF-BP appears to be negligible and any peripheral function of CRF-BP is likely to be highly localised. The increased expression of CRF-BP in fish under social and hypoxic stress suggests an involvement of this peptide in mediating the physiological response to various stressors; however, the increased gene expression observed in the absence of a clear cortisol response in hypoxia-exposed fish suggests that CRF-BP can be regulated independently of the HPI-axis. Further investigations into the connectivity of CRF-BP expressing neurones will enhance our understanding of the neural circuitry controlling stress physiology. Promoter sequencing combined with *in vitro* and *in vivo* studies of CRF-BP gene regulation will help clarify the activation and feedback regulating mechanisms of the CRF-system.

References

1 Flik G, Klaren PHM, Van den Burg EH, Metz JR, Huising MO. CRF and stress in fish. *Gen Comp Endocrinol* 2006; **146**: 36–44.

2 Chang CL, Hsu SY. Ancient evolution of stress-regulating peptides in vertebrates. *Peptides* 2004; **25**: 1681–1688.

3 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.

4 Le Mével JC, Mimassi N, Lancien F, Mabin D, Conlon JM. Cardiovascular actions of the stress-related neurohormonal peptides, corticotropin-releasing factor and urotensin-I in the trout *Oncorhynchus mykiss. Gen Comp Endocrinol* 2006; **146**: 56–61.

5 Bernier NJ. The corticotropin-releasing factor system as a mediator of the appetite-suppressing effects of stress in fish. *Gen Comp Endocrinol* 2006; **146**: 45–55.

6 Arai M, Assil IQ, Abou-Samra AB. Characterization of three corticotropin-releasing factor receptors in catfish: a novel third receptor is predominantly expressed in pituitary and urophysis. *Endocrinology* 2001; **142**: 446–454.

7 Pohl S, Darlison MG, Clarke WC, Lederis K, Richter D. Cloning and functional pharmacology of two corticotropin-releasing factor receptors from a teleost fish. *Eur J Pharmacol* 2001; **430**: 193–202.

8 Seasholtz AF, Valverde RA, Denver RJ. Corticotropin-releasing hormone-binding protein: biochemistry and function from fishes to mammals. *J Endocrinol* 2002; **175**: 89–97.

9 Behan D, Linton E, Lowry P. Isolation of the human plasma corticotrophin-releasing factor-binding protein. *J Endocrinol* 1989; **122**: 23–31.

10 Sutton S, Behan D, Lahrichi S, Kaiser R, Corrigan A, Lowry P, Potter E, Perrin M, Rivier J, Vale W. Ligand requirements of the human corticotropin-releasing factor-binding protein. *Endocrinology* 1995; **136**: 1097–1102.

11 Potter E, Behan DP, Linton EA, Lowry PJ, Sawchenko PE, Vale WW. The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF. *Proc Natl Acad Sci USA* 1992; **89**: 4192–4196.

12 Alderman SL, Bernier NJ. Localization of corticotropin-releasing factor, urotensin I, and CRF-binding protein gene expression in the brain of the zebrafish, *Danio rerio. J Comp Neurol* 2007; **502**: 783–793.

13 Bowman ME, Lopata A, Jaffe RB, Golos TG, Wickings J, Smith R. Corticotropin-releasing hormone-binding protein in primates. *Am J Primatol* 2001; **53**: 123–130.

14 Potter E, Behan DP, Fischer WH, Linton EA, Lowry PJ, Vale WW. Cloning and characterization of the cDNAs for human and rat corticotropin releasing factor-binding proteins. *Nature* 1991; **349**: 423–426.

15 Petraglia F, Potter E, Cameron VA, Sutton S, Behan DP, Woods RJ, Sawchenko PE, Lowry PJ, Vale W. Corticotropin-releasing factor-binding protein is produced by human placenta and intrauterine tissues. *J Clin Endocrinol Metab* 1993; **77**: 919–924.

16 Valverde RA, Seasholtz AF, Cortright DN, Denver RJ. Biochemical characterization and expression analysis of the *Xenopus laevis* corticotropin-releasing hormone binding protein. *Mol Cell Endocrinol* 2001; **173**: 29–40.

17 Mazon AF, Verburg-van Kemenade BM, Flik G, Huising MO. Corticotropin-releasing hormone-receptor 1 (CRH-R1) and CRH-binding protein (CRH-BP) are expressed in the gills and skin of common carp *Cyprinus carpio* L. and respond to acute stress and infection. *J Exp Biol* 2006; **209**: 510–517.

18 Petraglia F, Florio P, Benedetto C, Gallo C, Woods RJ, Genazzani AR, Lowry PJ. High levels of corticotropin-releasing factor (CRF) are inversely correlated with low levels of maternal CRF-binding protein in pregnant women with pregnancy-induced hypertension. *J Clin Endocrinol Metab* 1996; **81**: 852–856.

19 Boorse GC, Kholdani CA, Seasholtz AF, Denver RJ. Corticotropin-releasing factor is cytoprotective in Xenopus tadpole tail: coordination of ligand, receptor, and binding protein in tail muscle cell survival. *Endocrinology* 2006; **147**: 1498–1507.

20 Ungless MA, Singh V, Crowder TL, Yaka R, Ron D, Bonci A. Corticotropin-releasing factor requires CRF binding protein to potentiate NMDA receptors via CRF receptor 2 in dopamine neurons. *Neuron* 2003; **39**: 401–407.

21 Chan RK, Vale WW, Sawchenko PE. Paradoxical activational effects of a corticotropin-releasing factor-binding protein 'ligand inhibitor' in rat brain. *Neuroscience* 2000; **101**: 115–129.

22 Herringa RJ, Nanda SA, Hsu DT, Roseboom PH, Kalin NH. The effects of acute stress on the regulation of central and basolateral amygdala CRF-binding protein gene expression. *Brain Res Mol Brain Res* 2004; **131**: 17–25.

23 Herringa RJ, Mackenrodt DB, Barlow JD, Roseboom PH, Nanda SA, Kalin NH. Corticotropin-releasing factor (CRF), but not corticosterone, increases basolateral amygdala CRF-binding protein. *Brain Res* 2006; **1083**: 21–28.

24 Kasckow JW, Lupien SJ, Behan DP, Welge J, Hauger RJ. Circulating human corticotropin-releasing factor-binding protein levels following cortisol infusions. *Life Sci* 2001; **69**: 133–142.

25 Ohmori N, Suda T, Sato Y, Kasagi Y, Tozawa H, Dobashi I, Demura H. Corticotropin-releasing factor-binding protein concentrations in plasma of patients with hypothalamic-pituitary-adrenal disorders. *Endocr J* 1994; **41**: 553–558.

26 Behan DP, Maciejewski D, Chalmers D, De Souza EB. Corticotropin releasing factor binding protein (CRF-BP) is expressed in neuronal and astrocytic cells. *Brain Res* 1995; **698**: 259–264.

27 Maciejewski D, Crowe PD, De Souza EB, Behan DP. Regulation of corticotropin-releasing factor-binding protein expression in cultured rat astrocytes. *J Pharmacol Exp Ther* 1996; **278**: 455–461.

28 Kasckow JW, Regmi A, Seasholtz AF, Mulchahey JJ. Regulation of corticotropin-releasing factor-binding protein expression in amygdalar neuronal cultures. *J Neuroendocrinol* 1999; **11**: 959–966.

29 Suess U, Lawrence J, Ko D, Lederis K. Radioimmunoassays for fish tail neuropeptides: I. Development of assay and measurement of immunoreactive urotensin I in *Catostomus commersoni* brain, pituitary, and plasma. *J Pharmacol Methods* 1986; **15**: 335–346.

30 Pepels PP, Van Helvoort H, Wendelaar Bonga SE, Balm PH. Corticotropin-releasing hormone in the teleost stress response: rapid appearance of the peptide in plasma of tilapia (*Oreochromis mossambicus*). *J Endocrinol* 2004; **180**: 425–438.

31 Doyon C, Gilmour KM, Trudeau VL, Moon TW. Corticotropin-releasing factor and neuropeptide Y mRNA levels are elevated in the preoptic area of socially subordinate rainbow trout. *Gen Comp Endocrinol* 2003; **133**: 260–271.

32 Meek J, Nieuwenhuys R. Holosteans and teleosts. In: Nieuwenhuys R, ten Donkelaar H, Nicholson C, eds. *The Central Nervous System of Vertebrates.* Berlin: Springer-Verlag, 1998: 759–937.

33 Raine JC, Leatherland JF. Morphological and functional development of the thyroid tissue in rainbow trout (*Oncorhynchus mykiss*) embryos. *Cell Tissue Res* 2000; **301**: 235–244.

34 Behan DP, De Souza EB, Lowry PJ, Potter E, Sawchenko P, Vale WW. Corticotropin releasing factor (CRF) binding protein: a novel regulator of CRF and related peptides. *Front Neuroendocrinol* 1995; **16**: 362–382.

35 Peto CA, Arias C, Vale WW, Sawchenko PE. Ultrastructural localization of the corticotropin-releasing factor-binding protein in rat brain and pituitary. *J Comp Neurol* 1999; **413**: 241–254.

36 Wullimann MF, Mueller T. Teleostean and mammalian forebrains contrasted: evidence from genes to behavior. *J Comp Neurol* 2004; **475**: 143–162.

37 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 goldfish brain. *Gen Comp Endocrinol* 1999; **116**: 461–477.

38 Zupanc GK, Horschke I, Lovejoy DA. Corticotropin releasing factor in the brain of the gymnotiform fish, *Apteronotus leptorhynchus*: immunohistochemical studies combined with neuronal tract tracing. *Gen Comp Endocrinol* 1999; **114**: 349–364.

39 Pepels PP, Meek J, Wendelaar Bonga SE, Balm PH. Distribution and quantification of corticotropin-releasing hormone (CRH) in the brain of the teleost fish *Oreochromis mossambicus* (tilapia). *J Comp Neurol* 2002; **453**: 247–268.

40 Koob GF, Heinrichs SC. A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. *Brain Res* 1999; **848**: 141–152.

41 Contarino A, Heinrichs SC, Gold LH. Understanding corticotropin releasing factor neurobiology: contributions from mutant mice. *Neuropeptides* 1999; **33**: 1–12.

42 Huising MO, Metz JR, van Schooten C, Taverne-Thiele AJ, Hermsen T, Verburg-van Kemenade BM, Flik G. Structural characterisation of a cyprinid (*Cyprinus carpio* L.) CRH, CRH-BP and CRH-R1, and the role of these proteins in the acute stress response. *J Mol Endocrinol* 2004; **32**: 627–648.

43 Lederis K, Fryer JN, Okawara Y, Schronrock CHR, Richter D. Corticotropin-releasing factors acting on the fish pituitary: experimental and molecular analysis. In: Sherwood NM, Hew CL, eds. *Fish Physiology*. San Diego, CA: Academic Press, Inc., 1994: 67–100.

44 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.

45 Bernier NJ, Peter RE. Appetite-suppressing effects of urotensin I and corticotropin-releasing hormone in goldfish (*Carassius auratus*). *Neuroendocrinology* 2001; **73**: 248–260.

46 Heinrichs SC, Lapsansky J, Behan DP, Chan RK, Sawchenko PE, Lorang M, Ling N, Vale WW, De Souza EB. Corticotropin-releasing factor-binding protein ligand inhibitor blunts excessive weight gain in genetically obese Zucker rats and rats during nicotine withdrawal. *Proc Natl Acad Sci USA* 1996; **93**: 15475–15480.

47 Lovejoy DA, Aubry JM, Turnbull A, Sutton S, Potter E, Yehling J, Rivier C, Vale WW. Ectopic expression of the CRF-binding protein: minor impact on HPA axis regulation but induction of sexually dimorphic weight gain. *J Neuroendocrinol* 1998; **10**: 483–491.

48 Heinrichs SC, Li DL, Iyengar S. Corticotropin-releasing factor (CRF) or CRF binding-protein ligand inhibitor administration suppresses food intake in mice and elevates body temperature in rats. *Brain Res* 2001; **900**: 177–185.

49 Carr JA. Novel effects of CRF on visuomotor behavior and autonomic function in anuran amphibians. *Gen Comp Endocrinol* 2006; **146**: 28–35.

50 Clements S, Moore FL, Schreck CB. Evidence that acute serotonergic activation potentiates the locomotor-stimulating effects of corticotropin-releasing hormone in juvenile chinook salmon (*Oncorhynchus tshawytscha*). *Horm Behav* 2003; **43**: 214–221.

51 Lowry CA, Rose JD, Moore FL. Corticotropin-releasing factor enhances locomotion and medullary neuronal firing in an amphibian. *Horm Behav* 1996; **30**: 50–59.

52 Kirby LG, Rice KC, Valentino RJ. Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. *Neuropsychopharmacology* 2000; **22**: 148–162.

53 Lowry CA, Rodda JE, Lightman SL, Ingram CD. Corticotropin-releasing factor increases in vitro firing rates of serotonergic neurons in the rat dorsal raphe nucleus: evidence for activation of a topographically organized mesolimbocortical serotonergic system. *J Neurosci* 2000; **20**: 7728–7736.

54 Forster GL, Feng N, Watt MJ, Korzan WJ, Mouw NJ, Summers CH, Renner KJ. Corticotropin-releasing factor in the dorsal raphe elicits temporally distinct serotonergic responses in the limbic system in relation to fear behavior. *Neuroscience* 2006; **141**: 1047–1055.

55 Lowry CA, Moore FL. Regulation of behavioral responses by corticotropin-releasing factor. *Gen Comp Endocrinol* 2006; **146**: 19–27.

56 Cortright DN, Nicoletti A, Seasholtz AF. Molecular and biochemical characterization of the mouse brain corticotropin-releasing hormone-binding protein. *Mol Cell Endocrinol* 1995; **111**: 147–157.

57 Tran TN, Fryer JN, Lederis K, Vaudry H. CRF, urotensin I, and sauvagine stimulate the release of POMC-derived peptides from goldfish neurointermediate lobe cells. *Gen Comp Endocrinol* 1990; **78**: 351–360.

58 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.

59 Doyon C, Trudeau VL, Moon TW. Stress elevates corticotropin-releasing factor (CRF) and CRF-binding protein mRNA levels in rainbow trout (*Oncorhynchus mykiss*). *J Endocrinol* 2005; **186**: 123–130.

60 Craig PM, Al-Timimi H, Bernier NJ. Differential increase in forebrain and caudal neurosecretory system corticotropin-releasing factor and urotensin I gene expression associated with seawater transfer in rainbow trout. *Endocrinology* 2005; **146**: 3851–3860.

61 Petraglia F, Florio P, Gallo R, Salvestroni C, Lombardo M, Genazzani AD, Di Carlo C, Stomati M, D'Ambrogio G, Artini PG. Corticotropin-releasing factor-binding protein: origins and possible functions. *Horm Res* 1996; **45**: 187–191.

62 Huising MO, van der Aa LM, Metz JR, de Fatima Mazon A, Kemenade BM, Flik G. Corticotropin-releasing factor (CRF) and CRF-binding protein expression in and release from the head kidney of common carp: evolutionary conservation of the adrenal CRF system. *J Endocrinol* 2007; **193**: 349–357.

63 Westphal NJ, Seasholtz AF. CRH-BP: the regulation and function of a phylogenetically conserved binding protein. *Front Biosci* 2006; **11**: 1878–1891.

64 Boorse GC, Denver RJ. Widespread tissue distribution and diverse functions of corticotropin-releasing factor and related peptides. *Gen Comp Endocrinol* 2006; **146**: 9–18.

65 Bernier NJ, Alderman SL, Bristow EN. Heads or tails? Stressor-specific expression of corticotropin-releasing factor and urotensin I in the preoptic area and caudal neurosecretory system of rainbow trout. *J Endocrinol* 2008; **196**: 639–652.

66 Behan DP, Potter E, Lewis KA, Jenkins NA, Copeland N, Lowry PJ, Vale WW. Cloning and structure of the human corticotrophin releasing factor-binding protein gene (CRH-BP). *Genomics* 1993; **16**: 63–68.

67 Wong CJ. Connections of the basal forebrain of the weakly electric fish, *Eigenmannia virescens*. *J Comp Neurol* 1997; **389**: 49–64.

68 Rink E, Wullimann MF. Connections of the ventral telencephalon (subpallium) in the zebrafish (*Danio rerio*). *Brain Res* 2004; **1011**: 206–220.

69 Teitsma CA, Anglade I, Toutirais G, Munoz-Cueto JA, Saligaut D, Ducouret B, Kah O. Immunohistochemical localization of glucocorticoid receptors in the forebrain of the rainbow trout (*Oncorhynchus mykiss*). *J Comp Neurol* 1998; **401**: 395–410.