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

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Abstract

We elucidated the structure of the principle factors regulating the initiation of the acute stress response in common carp: corticotrophin-releasing hormone (CRH), CRH-receptor 1 (CRH-R1) and CRH-binding protein (CRH-BP). Phylogenetic analyses reveal that these proteins are evolutionarily well conserved in vertebrates. CRH and CRH-BP expression are not co-localised in the same hypothalamic perikarya. On the contrary, CRH-BP expression is limited to the perimeter of the nucleus preopticus (NPO), but is abundant in other regions, including an area directly rostral from, and in close proximity to, the NPO. Despite the lack of co-expression, the nerve fibres projecting onto both the rostral pars distalis (rPD) as well as the large fibre bundles projecting onto the pars intermedia (PI) contain CRH as well as CRH-BP, suggesting that both ACTH release from the rPD as well as the release of PI melanotrope content is regulated via CRH and CRH-BP. Finally, we show via real-time quantitative PCR that expression of hypothalamic CRH and CRH-BP following a 24 h restraint significantly increases, whereas PD CRH-R1 expression decreases; this reflects desensitisation of the PD for hypothalamic CRH output. We conclude that these factors are actively involved in the regulation of acute stress responses in the teleost fish.

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Introduction

In fish, as in other vertebrates, corticotrophin-releasing hormone (CRH) is the dominant hypothalamic hormone controlling the stress axis (Spiess *et al.* 1981, Wendelaar Bonga 1997). Upon central registration of an imminent or ongoing disturbance of homeostasis, CRH is released from the nucleus preopticus (NPO). This evokes the sequential secretion into the circulation of adreno-corticotrophic hormone (ACTH) from the pituitary pars distalis (PD) and cortisol from the inter-renal cells of the head kidney. The stress axis of teleost fish is generally similar to that of mammals, although some striking differences exist. First, the cortisol secreting cells are found around the blood vessels of the head kidney, an organ unique to fish and an important site of haematopoiesis (Chester-Jones *et al.* 1980). Secondly, cortisol in fish

combines glucocorticoid and mineralocorticoid functions (Wendelaar Bonga 1997). It is thus not only involved in recruitment of energy to cope with stressors, but also acts to restore and maintain the hydromineral balance in concert with prolactin (fresh-water fish) and growth hormone (sea-water fish). Despite these differences, the stress axis is conserved throughout vertebrate evolution: several 'classical' peptide hormones and receptors, including CRH have been identified in teleost fish (Okawara *et al.* 1988, Olivereau & Olivereau 1988).

CRH is derived from a 160-amino-acid prepro-hormone, that is cleaved into a mature 41-amino-acid bioactive peptide (Vale *et al.* 1981). With the discovery of urotensin-I and -II (UI and UII) in teleost fish (Pearson *et al.* 1980, Lederis *et al.* 1982) followed by the identification of their mammalian orthologues urocortin-I (UcnI) and UII respectively (Vaughan *et al.* 1995, Coulouarn

et al. 1998), it became apparent that a family of CRH-like factors exists. Aided by recent whole-genome sequencing efforts, two more CRH-like family members have been identified to date, named urocortin-II and -III (UcnII and UcnIII) (Lewis *et al.* 2001, Reyes *et al.* 2001). These peptides signal through seven-helix G-protein-coupled receptors. UII appears to utilise its own exclusive receptor, but two receptors for CRH and UcnI (CRH-R1 and CRH-R2) display overlapping ligand specificities. CRH-R1 has equal affinity for both CRH and UcnI/UI in catfish (Arai *et al.* 2001) and mammals (Coste *et al.* 2002), while CRH-R2 has higher affinity for Ucn over CRH (Wei *et al.* 1998, Hsu & Hsueh 2001). Recently a third CRH receptor (CRH-R3) has been identified in catfish (*Ameiurus nebulosus*) with five-fold higher affinity for CRH over UI (Arai *et al.* 2001).

Levels of bioactive CRH in circulation are influenced by CRH-binding protein (CRH-BP) (Potter *et al.* 1991), a 322-amino-acid soluble protein structurally unrelated to the CRH receptors. The affinity of CRH-BP for CRH is higher than that of the CRH-R1 (Potter *et al.* 1991, Cortright *et al.* 1995). CRH-BP is generally considered to be an antagonist of CRH (McClennen *et al.* 1998).

Here we report on the characterisation of the hormones and receptors of the CRH system involved in the stress axis in common carp (*Cyprinus carpio*). Our data include extensive sequence and expression analyses of CRH-BP in a teleost fish. From a phylogenetic perspective it follows that the novel CRH, CRH-R1 and CRH-BP sequences described here are highly conserved in structure and are orthologous to CRH, CRH-R1 and CRH-BP of other non-teleostean vertebrates. Furthermore we assess the expression of CRH and CRH-BP at the mRNA as well as the protein level. Finally we describe the integrated regulation of hypothalamic and pituitary expression of these genes in an acute restraint-stress paradigm.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) were reared at 23 °C in recirculating u.v.-treated tap water at the De Haar Vissen facility in Wageningen, with a photoregimen of 12 h light:12 h darkness. Fish were fed pelleted dry food (Provimi, Rotterdam, The Netherlands) at a daily ration of 0·7% of their estimated body weight. R3xR8 are the hybrid offspring of a cross between fish of Hungarian (R8 strain) and Polish origin (R3 strain) (Irnazarow 1995). Carp of the same strain housed under identical conditions at the fish facilities of the Department of Animal Physiology at the University of Nijmegen were used for analyses of *in vivo* stress responses. Fish were anaesthetised with 0·2 g/l tricaine methane sulphonate (TMS) buffered with 0·4 g/l NaHCO$_3$ or with 0·1% 2-phenoxyethanol.

Cloning and sequencing

For CRH, PCR was carried out with CRH16 sense and CRH11 antisense (Table 1) primers on carp hypothalamic cDNA. The 5′ and 3′ untranslated regions (UTRs) were amplified from a phorbol 12-myristate 13-acetate (PMA)-stimulated phago-cyte cDNA library (Saeij *et al.* 2000) by the use of SK- and T7-anchored primers respectively. For CRH-R1, degenerate oligonucleotide primers (CRH-R.fw3 and CRH-R1.rv1) were designed based on mammalian, *Xenopus* and catfish CRH-R1 sequences, which yielded a 0·4 kb partial CRH-R1 sequence. The majority of remaining coding sequence was amplified by the use of a second set of primers (CRH-R1.fw4 and CRH-R1.rv2). CRH-BP was amplified by the use of degenerate oligonucleotide primers (CRH-BP.fw2 and CRH-BP.rv1) based on conserved regions from mammalian and *Xenopus* sequences, which yielded a partial sequence of 0·55 kb. The complete coding sequence and the 5′ and 3′ UTR were amplified from a λZAP cDNA library of total carp brain without pituitary gland, constructed from pooled total RNA of ten adult carp, of which five individuals were restrained for 90 min. T3- and T7-anchored primers in combination with two new sequence-specific primers (CRH-BP.fw4 and CRH-BP.rv4) were used in anchored PCR. Oligonucleotides were obtained from Eurogentec (Seraing, Belgium). PCR reactions were performed with 0·5 μl Taq DNA polymerase (Goldstar, Eurogentec) supplemented with 1·5 mM MgCl$_2$, 200 μM dNTPs and 400 μM of each primer in a final volume of 25 μl. Cycling conditions were 94 °C for 2 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min for 30 cycles and 72 °C for 10 min, by the use of a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA, USA). Products

Table 1 Primer sequences and corresponding accession numbers

Gene	Accession number	Primer	Sequence
CRH	AJ317955, AJ576243	CRH16sense	GGRGAGGARRAYTWCATCCG
		CRH11antisense	CATTTGTTCGGCTCTGGCCAT
		qCRH.fw2	CATCCGGCTCGGTAACAGAA
		qCRH.rv2	CCAACAGACGCTGCGTTAACT
CRH-R1	AJ576244	CRH-R.fw3	GAYSIIATYGGIACSTGYTGGCC
		CRH-R1.rv1	CCAAACCAGCACTTYTCATTG
		CRH-R1.fw4	CATGAGAGCAATGTGATCTGGTG
		CRH-R1.rv2	CCACICGIGCICGGATKGARTGC
		qCRH-R1.fw2	CCCTGCTGATCGCCTTCAT
		qCRH-R1.rv2	GCAGGATAAATGCTGTAATCAGGTT
CRH-BP	AJ490880, AJ490881	CRH-BP.fw2	GGCTGGATGATGAAGGGAGAGAA
		CRH-BP.rv1	CACCATTCTGACCACAGTGTTATC
		CRH-BP.fw4	AATGAAGGTTGGCTGTGATAACACT
		CRH-BP.rv4	CGCAGTAATCAGAGTAACGCTGTAC
		qCRH-BP.fw1	ACAATGATCTCAAGCGGTCCAT
		qCRH-BP.rv1	CCACCCAGAAGCTCGACAAA
UI	P01146	qUI.fw1	GCACCTGTGTCCAGCATGAA
		qUI.rv1	GGTGCTCAGCGGGATGTG
40S ribosomal protein S11	AB012087	q40S.fw1	CCGTGGGTGACATCGTTACA
		q40S.rv1	TCAGGACATTGAACCTCACTGTCT
β-actin	CCACTBA	qACT.fw1	CAACAGGGAAAAGATGACACAGATC
		qACT.rv1	GGGACAGCACAGCCTGGAT
Vector		SK	CGGCCGCTCTAGAACTAGTGGACT
		T7	TAATACGACTCACTATAGGG
		T3	CGCAATTAACCCTCACTAAAG

amplified by PCR were ligated and cloned in JM-109 cells with the pGEM-T-easy kit (Promega) according to the manufacturer's protocol. Plasmid DNA was isolated from the cloned cells by the use of the QIAprep Spin miniprep kit (Qiagen). Sequence reactions were carried out by the use of the ABI Prism Bigdye terminator cycle sequencing ready reaction kit according to the manufacturer's protocol and analysed with an ABI 377 sequencer. Signal peptide predictions were done by the use of the SignalP program v2·0 (Nielsen *et al.* 1997) at http://www.cbs.dtu.dk/services/SignalP-2·0/. Prediction of the pufferfish CRH-BP gene organisation was done with GENSCAN at http://genes.mit.edu/GENSCAN.html.

Restraint-stress paradigm

We used an acute (30 min) and a prolonged (24 h) restraint model to study the stress response. Fish ($n=8$) were housed in identical tanks ($0·6 \times 0·3 \times 0·3$ m (30 min) or $0·9 \times 0·5 \times 0·4$ m (24 h)). At $t=0$ the fish were restrained for 30 min

or 24 h, by netting. Fish were sampled at the indicated times following the initiation of stressor by rapid anaesthesia applied to their tank, followed by sampling of the anaesthetised animal. Blood was collected by puncture of the caudal vessels using a heparinised (Leo Pharmaceuticals Products, Weesp, The Netherlands) 25 Gauge needle. This method has previously been successfully employed for 'stress-free' sampling of fish (Huising *et al.* 2003). For each time point a non-stressed control group was included in the experimental design to rule out circadian or sampling effects.

Plasma hormone determination

Freshly collected heparinised blood was spun down in a cooled microcentrifuge (10 min at 10 000 r.p.m.). Plasma was taken off and stored at -20 °C until use. Cortisol was measured by RIA (Arends *et al.* 1998), by the use of a commercial antiserum (Bioclinical Services Ltd, Cardiff, UK). All constituents were in phosphate-EDTA buffer (0·05 M Na_2HPO_4, 0·01 M Na_2EDTA, 0·003 M NaN_3,

pH 7·4). Ten microlitre samples or standards in RIA buffer (phosphate-EDTA buffer containing 0·1% 8-anilio-1-naphthalene sulphonic acid and 0·1% w/v bovine γ-globulin) were incubated with 100 µl antiserum (in RIA buffer containing 0·2% normal rabbit serum) for 4 h. Samples were incubated overnight with 100 µl iodinated cortisol (approximately 1700 c.p.m./tube; ^{125}I-labelled cortisol, Amersham) and 100 µl goat anti-rabbit γ-globulin (in RIA buffer). Bound and free cortisol were separated by the addition of 1 ml ice-cold precipitation buffer (phosphate-EDTA buffer containing 2% w/v bovine serum albumin and 5% w/v polyethylene glycol). The tubes were centrifuged at 4 °C (20 min at 2000 *g*), the supernatant aspirated and counted in a gamma counter (1272 clinigamma, LKB Wallac, Turku, Finland).

RNA isolation

Organs for RNA isolation were harvested and flash-frozen on dry ice. RNA isolation was conducted by the use of Trizol (Invitrogen) according to the manufacturer's protocol. Total RNA was precipitated in ethanol, washed and dissolved in water. Concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1·5% agarose gel. RNA was stored at − 80 °C until use.

DNAse treatment and first-strand cDNA synthesis

For each sample a non-reverse transcriptase (-RT) control was included. One microlitre 10 × DNAse I reaction buffer and 1 µl DNAse I (Invitrogen, 18068–015) was added to 1 µg total RNA and incubated at room temperature for 15 min in a total volume of 10 µl. DNAse I was inactivated by the addition of 1 µl 25 mM EDTA and incubation at 65 °C for 10 min. To each sample, the following were added: 300 ng random hexamers, 1 µl 10 mM dNTP mix, 4 µl 5 × first-strand buffer, 2 µl 0·1 M dithiothreitol (DTT) and 10 U RNAse inhibitor (Invitrogen, 15518–012); the mix was then incubated for 10 min at room temperature and for an additional 2 min at 37 °C. To each positive sample (but not the -RT controls) 200 U superscript RNAse H$^{−}$ reverse transcriptase (RT; Invitrogen, 18053–017) was added and reactions were incubated for 50 min at 37 °C. All reactions were filled up with demineralised water to a total volume of 1 ml and stored at − 20 °C until further use.

Real-time quantitative PCR

Primer Express software (Applied Biosystems) was used to design primers for use in real-time quantitative PCR (RQ-PCR; Table 1). For RQ-PCR, 5 µl cDNA and forward and reverse primer (300 nM each) were added to 12·5 µl Sybr Green PCR Master Mix (Applied Biosystems) and filled up with demineralised water to a volume of 25 µl. RQ-PCR (2 min at 48 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C) was carried out on a GeneAmp 5700 sequence detection system (Applied Biosystems). Data were analysed with the ΔΔCt method (AppliedBiosystems 2001). Dual internal standards (40S and β-actin) were incorporated in all RQ-PCR experiments and results were confirmed to be very similar following standardisation to either gene. Only results standardised for 40S expression are shown.

Western blotting

Fresh aqueous homogenates of hypothalamus and pituitary gland of carp were prepared by the use of a Potter glass-to-glass homogenisation device. The water-insoluble moiety was removed by centrifugation (10 min at 15 800 *g*) and the water-soluble moiety was run under denaturing conditions on a 12·5% SDS gel. Human recombinant CRH-BP (hrCRH-BP 254–299; a generous gift of Professor Dr W Vale) was loaded as size marker. After running, the gel was blotted on a nitro-cellulose filter and blocked with PBS containing 1% casein. CRH-BP was detected by the use of a rabbit anti-human CRH-BP antiserum (No. 5144; a generous gift of Professor Dr W Vale) (Potter *et al.* 1992) at a 1:2000 dilution for 1 h followed by goat anti-rabbit Ig-HRP (1:1500, 1 h; BioRad). The signal was detected with a chemoluminescence kit (Amersham) according to the manufacturer's protocol and visualised by the use of Lumni-film chemiluminescent detection film (Roche). Controls without primary antibody were negative.

Immunohistochemistry

Tissue was fixed in Bouin (15 ml picric acid, 5 ml formol, 1 ml glacial acetic acid), dehydrated and

embedded in paraffin. Sections of 5 µm were used for immunohistochemistry. CRH was detected by the use of a rabbit anti-sheep CRH antiserum (1:1000; Incstar, Stillwater, MN, USA). CRH-BP was detected with a rabbit anti-human CRH-BP antiserum (No. 5144) at a dilution of 1:1000. Arginine vasotocin (AVT) was detected with a rabbit anti-AVT antiserum at a dilution of 1:4000 (Dierickx & Vandesande 1977). Primary antibodies were incubated overnight. Goat anti-rabbit IgG-biotin (1:200, 1 h; BioRad) was used as secondary antibody followed by amplification via the vectastain ABC amplification kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. The signal was visualised with 3-amino-6-ethylcarbazole (AEC; Sigma) as a substrate. Controls for cross-reactivity of the secondary reagents and for endogenous enzyme activity were included in all experiments and were negative.

Phylogenetic analyses

Sequences were retrieved from the Swissprot, EMBL and Genbank data bases at the SRS mirror site of the Centre of Molecular and Biomolecular Informatics (www.cmbi.kun.nl). Pufferfish (*Takifugu rubripes*) sequences were retrieved from http://www.ensembl.org/Fugu_rubripes/. Zebrafish sequences were retrieved from http://www.ensembl.org/Danio_rerio/. Multiple-sequence alignment was carried out with ClustalW at the CMBI mirror site. Phylogenetic trees were constructed on the basis of amino acid difference (*p*-distance) using the neighbour-joining method (Saitou & Nei 1987) with MEGA version 2·1 (Kumar *et al.* 2001). The reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

Statistics

All statistical analyses were carried out with SPSS software (version 10·1·0). Data were tested for normal distribution with the Shapiro–Wilk test. Homogeneity of variances was tested with the Levene test. Differences were evaluated with one-sided, one factor ANOVA. The Kruskal–Wallis *H* test was applied in cases where the distribution was not normal. If the results of the Kruskal–Wallis test were significant, the Mann–Whitney *U* test was used to determine which means differed significantly from controls.

Results

Cloning and characteristics of two carp CRH genes

A partial sequence of carp CRH was obtained in a homology cloning approach from carp hypothalamic cDNA. In the search for the corresponding full-length sequence, two highly similar sequences were found (Fig. 1). They were designated CRH1 and CRH2. The 3′ UTR contains a polyadenylation site (base pairs (bp) 918–923) and four potential instability motifs (attta; bp 615–619, 670–674, 683–687, 687–691). Both genes consist of an open reading frame encoding a 162-amino-acid protein, which encompasses a signal peptide (M^1–A^{24}), a conserved region within the cryptic peptide (R^{48}–R^{60}) and the mature peptide (S^{120}–F^{160}) based on similarity with mammalian CRH sequences. The mature peptide is flanked by a dibasic cleavage site and a potential C-terminal amidation site. The coding regions of both genes differ in 13 nucleotides, which result in 8 amino acid substitutions. One of these substitutions (L^{12} to P^{12}) is within the predicted signal peptide and one (A^{123} to P^{123}) is within the mature peptide. The remaining substitutions are situated within the pro-hormone. Both carp CRH sequences bear high (96%) to intermediate (60%) sequence identity to other teleost fish CRH sequences (Table 2). Sequence identity to mammalian CRH sequences is considerably lower (around 45%). However, sequence identity within the various conserved regions, most notably the mature peptide, is considerably higher (up to 90% identity between carp and human).

Cloning and characteristics of carp CRH-R1

Two highly similar partial cDNA sequences encoding the carp CRH-R1 were obtained from brain cDNA using a homology cloning approach. Both sequences differ at 17 nucleotide positions, but none of these result in an amino acid substitution (not shown). Overall, the sequences of teleost fish and mammals are highly similar at the amino acid level (Fig. 2). The N-terminal part of the protein, which contains the signal peptide, is the most variable part of the protein. The 7 predicted transmembrane (TM) regions are well conserved (Fig. 2). The C-terminal region of the receptor is extremely well conserved; the third
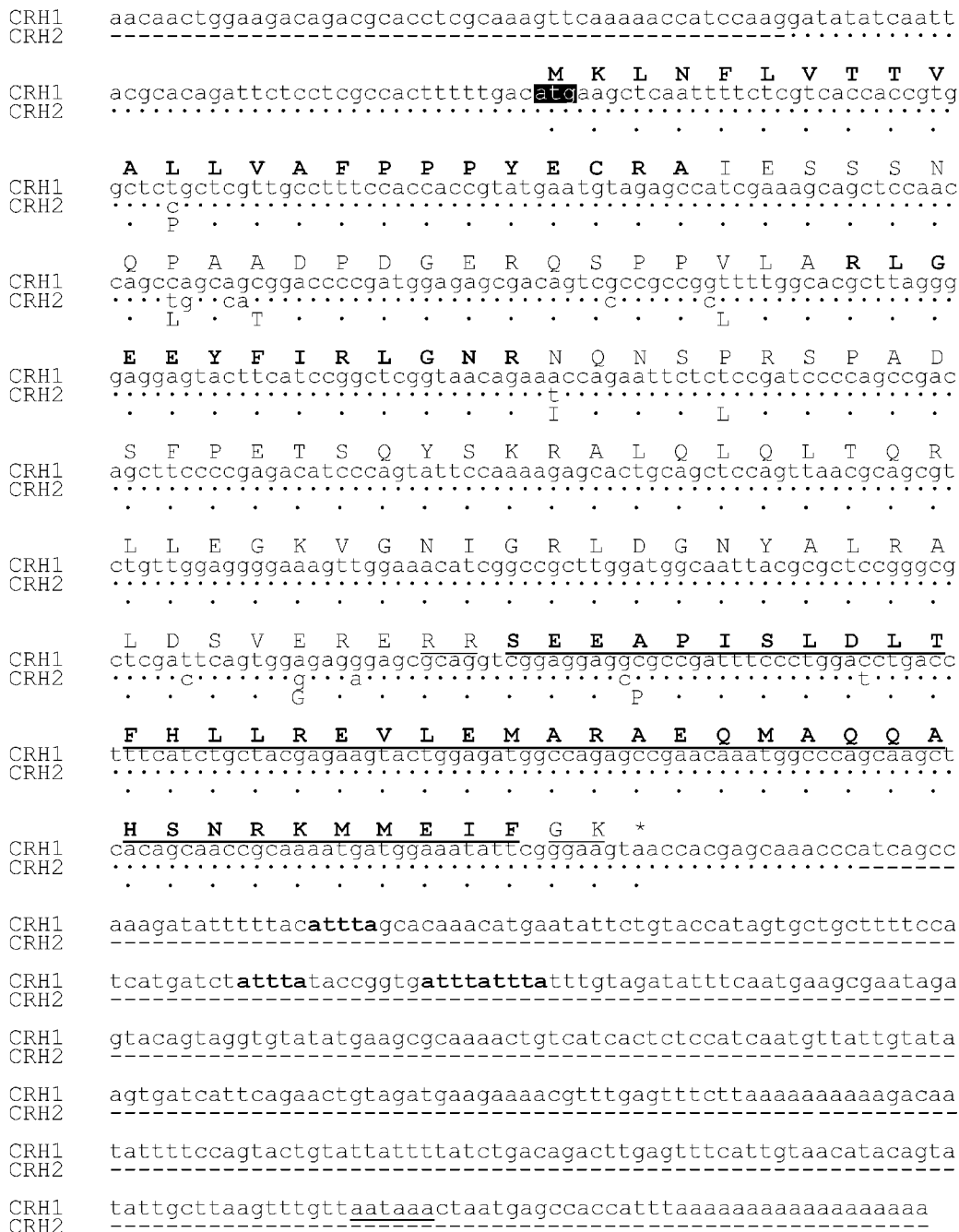
```
                  M  K  L  N  F  L  V  T  T  V
CRH1  aacaactggaagacagacgcacctcgcaaagttcaaaaaccatccaaggatatatcaatt
CRH2  -----------------------------------------·············

                        M  K  L  N  F  L  V  T  T  V
CRH1  acgcacagattctcctcgccacttttttgac[atg]aagctcaattttctcgtcaccaccgtg
CRH2  ·····················       ·  ·  ·  ·  ·  ·  ·  ·  ·

      A  L  L  V  A  F  P  P  P  Y  E  C  R  A  I  E  S  S  S  N
CRH1  gctctgctcgttgcctttccaccaccgtatgaatgtagagccatcgaaagcagctccaac
CRH2  ·····c··········································
       ·  P  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·

      Q  P  A  A  D  P  D  G  E  R  Q  S  P  P  V  L  A  R  L  G
CRH1  cagccagcagcggaccccgatggagagcgacagtcgccgccggtttttggcacgcttaggg
CRH2  ····tg··ca················c·········c········
       ·  L  ·  T  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  L  ·  ·  ·

      E  E  Y  F  I  R  L  G  N  R  N  Q  N  S  P  R  S  P  A  D
CRH1  gaggagtacttcatccggctcggtaacagaaaccagaattctctccgatccccagccgac
CRH2  ································t··········
       ·  ·  ·  ·  ·  ·  ·  ·  ·  I  ·  ·  ·  ·  L  ·  ·  ·  ·

      S  F  P  E  T  S  Q  Y  S  K  R  A  L  Q  L  Q  L  T  Q  R
CRH1  agcttccccgagacatcccagtattccaaaagagcactgcagctccagttaacgcagcgt
CRH2   ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·

      L  L  E  G  K  V  G  N  I  G  R  L  D  G  N  Y  A  L  R  A
CRH1  ctgttggaggggaaagttggaaacatcggccgcttggatggcaattacgcgctccgggcg
CRH2   ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·

      L  D  S  V  E  R  E  R  R  S  E  E  A  P  I  S  L  D  L  T
CRH1  ctcgattcagtggagagggagcgcaggtcggaggaggcgccgatttccctggacctgacc
CRH2  ·····c·······g···a···········c········t·····
       ·  ·  ·  ·  ·  G  ·  ·  ·  ·  ·  ·  P  ·  ·  ·  ·  ·  ·

      F  H  L  L  R  E  V  L  E  M  A  R  A  E  Q  M  A  Q  Q  A
CRH1  tttcatctgctacgagaagtactggagatggccagagccgaacaaatggcccagcaagct
CRH2   ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·

      H  S  N  R  K  M  M  E  I  F  G  K  *
CRH1  cacagcaaccgcaaaatgatggaaatattcgggaagtaaccacgagcaaacccatcagcc
CRH2   ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·-----

CRH1  aaagatattttttacatttagcacaaacatgaatattctgtaccatagtgctgcttttcca
CRH2  ------------------------------------------------------------

CRH1  tcatgatctatttataccggtgatttattttatttgtagatatttcaatgaagcgaataga
CRH2  ------------------------------------------------------------

CRH1  gtacagtaggtgtatatgaagcgcaaaactgtcatcactctccatcaatgttattgtata
CRH2  ------------------------------------------------------------

CRH1  agtgatcattcagaactgtagatgaagaaaacgtttgagtttcttaaaaaaaaaagacaa
CRH2  ------------------------------------------------------------

CRH1  tattttccagtactgtattatttttatctgacagacttgagtttcattgtaacatacagta
CRH2  ------------------------------------------------------------

CRH1  tattgcttaagtttgttaataaactaatgagccaccatttaaaaaaaaaaaaaaaaaaa
CRH2  ------------------------------------------------------------
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Figure 1 Nucleotide and deduced amino acid sequences of two carp CRH cDNAs. The start codon is boxed, the stop codon is indicated by the asterisks. Dots indicate identical residues, hyphens indicate gaps. Potential instability motifs are indicated in bold. A putative polyadenylation signal is underlined. The deduced amino acid sequences of CRH1 and CRH2 are displayed above and underneath the nucleotide sequences respectively. The predicted signal peptide (M^1–A^{24}) and the conserved cryptic motif within the cryptic peptide (R^{48}–R^{60}) are presented in bold capitals. The sequence of the mature peptide (S^{120}–F^{160}) is underlined and presented in bold capitals. The predicted cleavage site and the C-terminal amidation site are underlined. Accession numbers: CRH1, AJ317955; CRH2, AJ576243.

Table 2 Percentages of amino acid sequence identity for CRH sequences of various vertebrate species. The identity is given for the complete sequence and for the mature peptide (in parentheses)

	Carp 1	Carp 2	White sucker 1	White sucker 2	Tilapia	*Xenopus*	Sheep	Rat	Human
Carp 1	100								
Carp 2	96 (98)	100							
White sucker 1	91 (95)	90 (98)	100						
White sucker 2	88 (93)	85 (95)	91 (98)	100					
Tilapia	62 (76)	61 (78)	62 (83)	59 (78)	100				
Xenopus	51 (85)	49 (88)	48 (88)	49 (85)	49 (68)	100			
Sheep	48 (76)	46 (78)	48 (80)	51 (78)	46 (37)	49 (80)	100		
Rat	54 (90)	53 (93)	54 (95)	56 (93)	51 (76)	58 (93)	67 (83)	100	
Human	56 (90)	54 (93)	55 (95)	56 (93)	50 (76)	56 (93)	77 (83)	82 (100)	100

intracellular loop, presumably involved in G-protein binding, is identical in amino acid sequence in fish, amphibian, avian and mammalian sequences.

Cloning and characteristics of two carp CRH-BP genes

Two highly similar genes encoding the carp orthologues of mammalian CRH-BP were amplified from a total-brain cDNA library. They were designated CRH-BP1 and CRH-BP2 and both genes contain an open reading frame encoding a 321 amino–acid protein (Fig. 3). The 3′ UTR contains one potential instability motif (bp 1294–1298) and a polyadenylation signal (bp 1320–1325). In the 5′ UTR both genes differ in 2 small insertions and/or deletions (indels) and a nucleotide substitution. The coding regions of both genes differ at 34 additional nucleotide positions, resulting in 7 amino acid substitutions. The predicted signal peptide (M^1–R^{24}) is the most variable part of the protein, whereas the rest of the protein is better conserved (Table 3). The 10 cysteine residues implicated in the formation of 5 disulphide bonds (carp C^{62}–C^{83}, C^{106}–C^{143}, C^{185}–C^{207}, C^{239}–C^{266}, C^{279}–C^{320}) are conserved between fish, amphibian, avian and mammalian sequences (Fig. 4). In order to study the CRH-BP gene organisation in fish, we retrieved the pufferfish CRH-BP gene from its genome data base using the complete carp CRH-BP sequences. Comparison of the genomic organisation of the pufferfish CRH-BP gene with that of human revealed a well-conserved gene, consisting of 7 exons (Fig. 5). The exons comprising the pufferfish CRH-BP gene are of identical length to those of human CRH-BP, with the exception of exons 5 and 7, that both extend one triplet in the human gene. All pufferfish introns contain well-recognisable 5′ donor (GT) and 3′ splice acceptor (AG) sites.

Phylogenetic analyses

To address the relationship between carp CRH, CRH-R1 and CRH-BP and their mammalian and non-mammalian orthologues, we constructed phylogenetic trees using the neighbour-joining method. The overall topology of the CRH-family tree (Fig. 6A) shows clustering of the fish CRH sequences, with sequences from cyprinid species clustering together apart from CRH sequences from the more recent teleost species tilapia and pufferfish. The cluster of mammalian CRH sequences is separated from the fish sequences by *Xenopus* CRH. Mammalian Ucn and teleost fish UI sequences cluster together, reflecting their proposed orthology. The vertebrate CRH and UI/Ucn clusters together form one clade, supported by a bootstrap value of 99.

All vertebrate CRH-R1 sequences cluster separately from CRH-R2 (Fig. 6B). The topology of each cluster reflects the evolutionary relationship between the vertebrate classes. The carp CRH-R1 sequence clusters well within the teleost fish CRH-R1 cluster. The catfish CRH-R3 sequence (Arai *et al.* 2001) clusters separately from both the CRH-R1 and the CRH-R2 cluster.

Avian and amphibian CRH-BP cluster close to the mammalian CRH-BP sequences, whereas the evolutionarily more distantly related teleost fish sequences also cluster more distantly (Fig. 6C). The
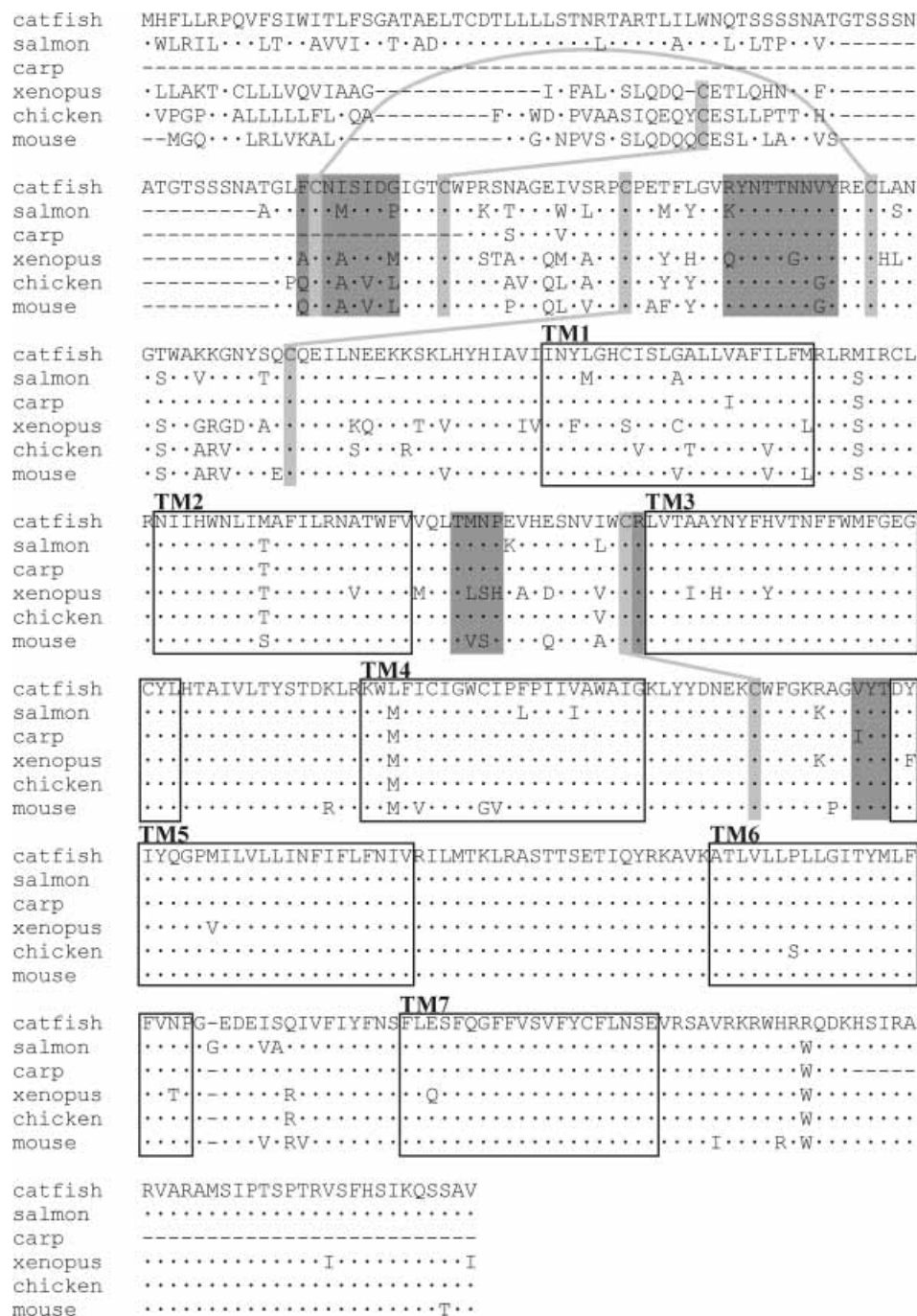
Figure 2 Amino acid alignment of CRH-R1 sequences from various vertebrate species, including carp. Dots indicate identical residues, hyphens indicate gaps. Cysteine residues involved in the formation of disulphide bonds (mouse C^{30}–C^{54}, C^{44}–C^{87}, C^{68}–C^{102}) are indicated with light shading. The residues implicated in ligand binding in the human CRH-R1 are indicated by dark shading. The seven predicted transmembrane (TM) regions are boxed. Note the exceptional conservation of the C-terminal part of the protein. Accession numbers: catfish (*Ameiurus nebulosus*), AF229359; salmon (*Oncorhynchus keta*), AJ277157; carp (*Cyprinus carpio*), AJ576244; *Xenopus* (*Xenopus laevis*), O42602; chicken (*Gallus gallus*), Q90812; mouse (*Mus musculus*), P35347.

Figure 3 Nucleotide and deduced amino acid sequences of two carp CRH-BP cDNAs. The start codon is boxed, the stop codon is indicated by the asterisk. Dots indicate identical residues, hyphens indicate gaps. Potential instability motifs are indicated in bold. A putative polyadenylation signal is underlined. The deduced amino acid sequences of CRH-BP1 and CRH-BP2 are displayed above and underneath the nucleotide sequences respectively. The predicted signal peptide is indicated in bold. Accession numbers: CRH-BP1, AJ490880; CRH-BP2, AJ490881.

Table 3 Percentages of amino acid sequence identity for CRH-BP sequences of various vertebrate species

	Carp 1	Carp 2	Pufferfish	*Xenopus*	Chicken	Sheep	Rat	Human
Carp 1	100							
Carp 2	97·8	100						
Pufferfish	68·2	68·7	100					
Xenopus	58·9	59·2	55·3	100				
Chicken	59·8	59·5	57·9	72·0	100			
Sheep	57·0	57·0	55·3	62·6	70·8	100		
Rat	58·3	58·6	55·7	63·9	71·1	77·3	100	
Human	61·7	62·0	58·5	68·2	73·9	85·1	84·5	100

very similar carp CRH-BP sequences cluster in very close proximity, as do both murine CRH-BP sequences, reflecting a recent common origin.

Expression of the CRH system

The expression of CRH, CRH-R1 and CRH-BP as well as UI was studied in the hypothalamus and pituitary gland of healthy non-stressed carp. Expression was plotted relative to the expression of ribosomal protein 40S. CRH and UI expression was detected in the hypothalamus only (Fig. 7A). CRH-R1 was expressed abundantly in the hypothalamus (Fig. 7B). Within the pituitary gland, CRH-R1 was expressed more abundantly (approximately four-fold) in the PD than in the PI. Hypothalamic CRH-BP expression was very high, with CRH-BP messengers approaching the abundance of 40S mRNA. Pituitary gene expression of CRH-BP was relatively low, with most CRH-BP expressed in the PI. None of these four genes were significantly expressed within either muscle or liver tissue (not shown). Non-reverse transcriptase controls were negative (not shown).

To study the expression of CRH-BP in conjunction with CRH at the protein level we performed immunohistochemistry. The anti-CRH-BP antiserum detected a protein of very similar size to the 37 kDa hrCRH-BP in the water-soluble fraction of homogenates from non-stressed carp hypothalamus and pituitary gland (Fig. 8). Although we cannot compare total avidity and affinity of the antiserum to hrCRH-BP and carp CRH-BP, this suggests that at least some of the epitopes recognised by the antiserum are formed by sufficiently conserved parts of the carp CRH-BP sequence. In sections of the hypothalamus of non-stressed carp, the NPO shows

prominent CRH-positive neurones. A dorsally situated magnocellular area (pars magnocellularis (NPOpmc)) was clearly distinguishable from a ventral parvocellular area (pars parvocellularis (NPOppc)) (Fig. 9A). Higher magnification of the NPOpmc on serial sections stained with haemaluin/eosin (Fig. 9B), CRH (Fig. 9C) and AVT (Fig. 9D) showed that a considerable number of the magnocellular perikarya contained either CRH or AVT and that some perikarya were positive for both hormones. Within the NPOppc a similar pattern was observed with regard to the presence of CRH and AVT (not shown). When the same area was stained on serial sections for the presence of CRH-BP, a limited number of relatively small CRH-BP[+] perikarya were observed at the caudal perimeter of the NPO (Fig. 9E). These CRH-BP[+] perikarya did not contain CRH or AVT. Abundant CRH-BP staining was observed in a cluster of cells and nerve fibres located at the floor of the hypothalamus 600 μm rostrally of the NPOppc (Fig. 9F). This area contained neither CRH nor AVT (not shown).

In the pituitary gland, CRH was most conspicuously present in the nerve fibre bundles of the pars nervosa (PN) that project to the PI melanotropes. Also, a relatively small set of nerve fibres of the rostral pars distalis (rPD) stained clearly for the presence of CRH (Fig. 10A). The proximal pars distalis (pPD) did not contain CRH immunoreactivity. A higher magnification of the rPD showed that the ACTH cells were aligned in a sheet around these fibres (Fig. 10B). The nerve fibres proper contained immunoreactive CRH in a distinct granular pattern (Fig. 10C) as well as immunoreactive CRH-BP that was distributed more evenly within the fibre (Fig. 10D). The PN also contained considerable CRH-BP immunoreactivity (see

```
carp-I    MSGTSRAQLCFLLLSVTALRGHARFLDIQDNEISPEGLLSLLS--SELKRELPEEFVYRR
carp-II   ......V....................G..............L.--..............
xenopus   .TPA..PDW.LI..FLAV...ES.YIQMR--.AAEDA.FL.N.---DF....S.GQI...
chicken   .PSAFQL.CHLV.ILLA.SK.DT.Y.EVR--DAGEDEPFL...--ED.....SAGHI...
sheep     .AP.LKL.CH.I.VCLL....ES.Y.ELR--.AVDHDPFPH.AGGASP....EG.PL...
mouse     ..PNFKL.CH.I.ILL.....ES.Y.EV.--.AAVYDP.L.F.--AN...D.A..QP...
human     ..PNFKL.CH.I.IFL.....ES.Y.ELR--.AADYDPFL.F.--AN.....AG.QP...

carp-I    ALRCLDMVAIEGQFTFTAERPQLNCAVFFIGEPSDIISIEYDSVNIDCRGGDFIKVFDGW
carp-II   ...........V................................................
xenopus   S...I..LS........Q.D....H..L.L....EEF.I...NF.....I...IL.....
chicken   S...I..LS.......DQ...H..T......EELLT....F.....Q....L........
sheep     ....V..LSLQ.......D.R..H..T...A..EEF.T.H..L.S...LR..ILQ.....
mouse     .......LSLP.......D...H..A......EEF.T.H..L.S...Q....L.......
human     .......LSLQ.......D....H..A...S..EEF.T.H..Q.S...Q....L......

carp-I    VMKGEKFPSTQDHPLPLYKRYSDYCETGVTRPIVRSSQNVAMLFFRLHQSGSSFTVTFRK
carp-II   .......................E....................................
xenopus   II.......SL....STME..T.I..D.DVGS.T.........I...VQ.P.HG..L.I..
chicken   IL.......SL....TSQ..T.F..S.AVQRSI.........I...I..P.NG..I.VK.
sheep     IL.......S......TTE..V.F.DS.LS.RSI.........I...V.EP.NG..I.VKT
mouse     IL.......S......TM...T.F..S.L..RSI.........V...V.EP.NG..I.IKT
human     IL.......S......SAE..I.F..S.LS.RSI.........I...V.EP.NG..L.IKT

carp-I    LINPFPCNVVSQTPEGSFTMIIPQQHRNCSFSIIYPVEIQIGELSLGQHNDLKR---SIL
carp-II   ............................................................
xenopus   IP.L.....I..SMN.R....T.H...........V.K.FD.T..HF.E.QLKKPPPK
chicken   SA.L.....I....S.R...V..H...........V.K.SD.I..HL.G.FLKNP.-V
sheep     EP.L.....II....N.R..LVM.H...........A.K.SD.T..HL.G.QLKKS.-A
mouse     DP.L.....I....S.R..LVV.Y..Q.........A.K.SD.T..HLHG.QLKKPA-A
human     DP.L.....I....N.K..LVV.H...........V.K.SD.T..HV.G.QLKKS.-A

carp-I    GCAGSGDFVELLGGNGMDTSKMYPMADLCYSFNGPAQMKVGCDNTVIRMVSSGKFVNRVS
carp-II   .........I..............F...........................V........
xenopus   ..GDA.........A.L.P...F.L....H..H.S....I......V.....N.I...T
chicken   ....V.........T.L.P...F.L....H..H.S....I......L......HI...T
sheep     ..G.I.........T.L.P...LLL....PLR........H..L......HI...T
mouse     ..G.T.........T.L.P...M.L....P.L......IS...A.V......HI...T
human     ..E.I.........T.L.P...T.L....P.H......V......V......H....T

carp-I    FQYRLLGHQELQQMKGNSVEDVCLRA-
carp-II   ......................RA.
xenopus   .E.NQ.D-RQ.EKKQ.....EA.FPSD
chicken   .E.YQ.DL..IENR.E..I.EF.FPGI
sheep     .E..Q.EPY..ENPN...IQEF..STL
mouse     .E..Q.EPF..ETST...IPEY..SSL
human     .E..Q.EPY..ENPN...IGEF..SGL
```

Figure 4 Amino acid alignment of CRH-BP sequences from various vertebrate species, including carp. Dots indicate identical residues, hyphens indicate gaps. Cysteine residues involved in the formation of disulphide bonds (carp C^{62}–C^{83}, C^{106}–C^{143}, C^{185}–C^{207}, C^{239}–C^{266}, C^{279}–C^{320}) are shaded. Accession numbers: carp (*Cyprinus carpio*) CRH-BP1, AJ490880; carp CRH-BP2, AJ490881; *Xenopus* (*Xenopus laevis*), Q91653; chicken (*Gallus gallus*), predicted from BU358572 and BU367671; sheep (*Ovis aries*), Q28557; mouse (*Mus musculus*), Q60571; human (*Homo sapiens*), P24387.
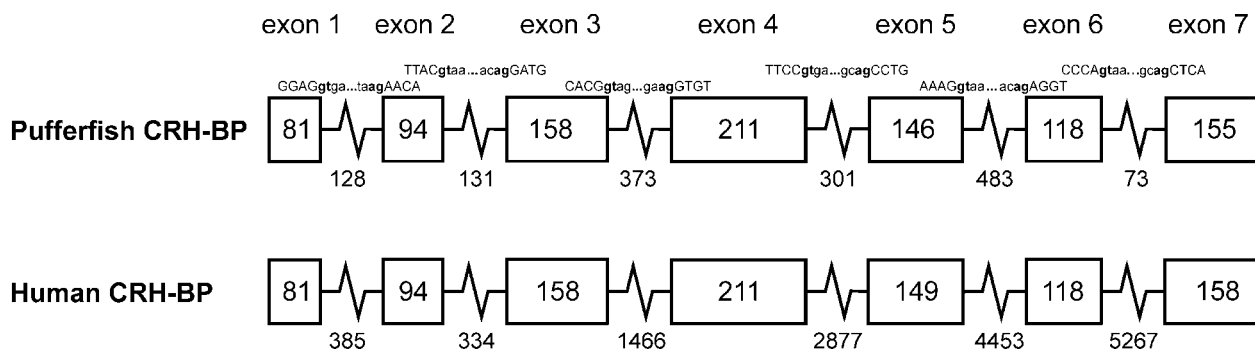
Figure 5 Comparison of the genomic organisation of the pufferfish and human CRH-BP genes. Boxes represent exons. Exon sizes are indicated within the boxes, intron sizes are given underneath the introns. The four nucleotide residues surrounding each splice site are given for the pufferfish gene, coding residues are represented by capitals. The actual splice donor and acceptor residues are indicated in bold. Accession number for the pufferfish CRH-BP gene is BN000457.

below). Controls for cross-reactivity of the secondary reagents or endogenous enzyme activity were consistently and completely negative.

Regulation of the CRH system during acute restraint stress

To assess the regulation of CRH, CRH-R1 and CRH-BP during stress, we subjected carp to either a 30 min or a 24 h period of restraint stress. In the 30 min restraint-stress paradigm two groups of fish were followed for up to 90 and 240 min respectively, to allow potential changes in gene transcription to establish. After 30 min of restraint, plasma cortisol values had risen to 100 ng/ml (Fig. 11A). Within the next 90 min they had returned to baseline. The 30 min restraint period did not have a detectable effect on hypothalamic

Figure 6 Neighbour-joining trees of vertebrate CRH-like amino acid sequences (A), CRH-R sequences (B) and CRH-BP sequences (C). Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Clusters of sequences are shaded in increasingly darker shades of grey from teleostean fish (light grey) to mammals (dark grey). Starting point for cluster formation is indicated by an open circle. Scientific names are as follows: carp, *Cyprinus carpio*; catfish, *Ameiurus nebulosus*; chicken, *Gallus gallus*; chum salmon, *Oncorhynchus keta*; cow, *Bos taurus*; golden hamster, *Mesocricetus auratus*; goldfish, *Carassius auratus*; human, *Homo sapiens*; laughing frog, *Rana ridibunda*; mouse, *Mus musculus*; pig, *Sus scrofa*; pufferfish, *Takifugu rubripes*; rainbow trout, *Oncorhynchus mykiss*; rat, *Rattus norvegicus*; rhesus macaque, *Macaca mulatta*; sheep, *Ovis aries*; spadefoot toad, *Spea hammondii*; tilapia, *Oreochromis mossambicus*; tree shrew, *Tupaia belangeri*; white sucker, *Catostomus commersoni*; *Xenopus, Xenopus laevis*; zebrafish, *danio rerio*. Accession numbers are as follows: carp CRH1, AJ317955; carp CRH2, AJ576243; carp CRH-BP1, AJ490880; carp CRH-BP2, AJ490881; carp CRH-R1, AJ576244; carp UI, P01146; carp UIIa, P04560; carp UIIg, P06580; catfish CRH-R1, AF229359; catfish CRH-R2, AF229360; catfish CRH-R3, AF229361; chicken CRH-BP, BU358572/BU367671; chicken CRH-R1, Q90812; chum salmon CRH-R1, AJ277157; chum salmon CRH-R2, CAC81754; cow CRH, AAK83231; cow CRH-R1, BAB21864; golden hamster CRH-R1, AAK59707; goldfish CRH, Q9PTS1; goldfish UI, Q9PTQ4; human CRH, P06850; human CRH-BP, P24387; human CRH-R1, P34998; human CRH-R2a, Q13324; human CRH-R2b, AAB94503; human CRH-R2g, AAC71654; human Ucn, NP_003344; human UcnII, Q96RP3; human UcnIII, Q969E3; human UIIa, NP_068835; human UIIb, O95399; laughing frog UII, P33715; mouse CRH-BP1, Q60571; mouse CRH-BP2, XP_138793; mouse CRH-R1, P35347; mouse CRH-R2, Q60748; mouse Ucn, P81615; mouse UcnII, Q99ML8; mouse UcnIII, Q924A4; mouse UII, Q9-QZQ3; pig CRH, P06296; pig UIIa, Q95J46; pig UIIb, Q95K72; pufferfish CRH, SINFRUP00000155211 (http://www.ensembl.org/Fugu_rubripes/); pufferfish CRH-BP, BN000457; rainbow trout CRH-BP, BX076908; rainbow trout UI, O93448; rat CRH, P01143; rat CRH-BP, P24388; rat CRH-R1, P35353; rat CRH-R2, P47866; rat Ucn, P55090; rat UcnII, Q91WW1; rat UII, Q9QZQ4; rhesus macaque UII, AAL55429; sheep CRH, P01142; sheep CRH-BP, Q28557; sheep CRH-R1, O62772; sheep Ucn, AAC35555; tilapia CRH, Q9I9D0; spadefoot toad CRH, AY262255; tree shrew CRH-R1, CAD19577; tree shrew CRH-R2a, CAD19578; tree shrew CRH-R2b, CAD19579; white sucker CRH1, P13241; white sucker CRH2, P25308; *Xenopus* CRH, P49188; *Xenopus* CRH-BP, Q91653; *Xenopus* CRH-R1, O42602; *Xenopus* CRH-R2, O42603; zebrafish CRH, ENSDARP00000003130 (http://www.ensembl.org/Danio_rerio/); zebrafish UI, BI844591; zebrafish CRH-BP, BQ450925/BQ615049.
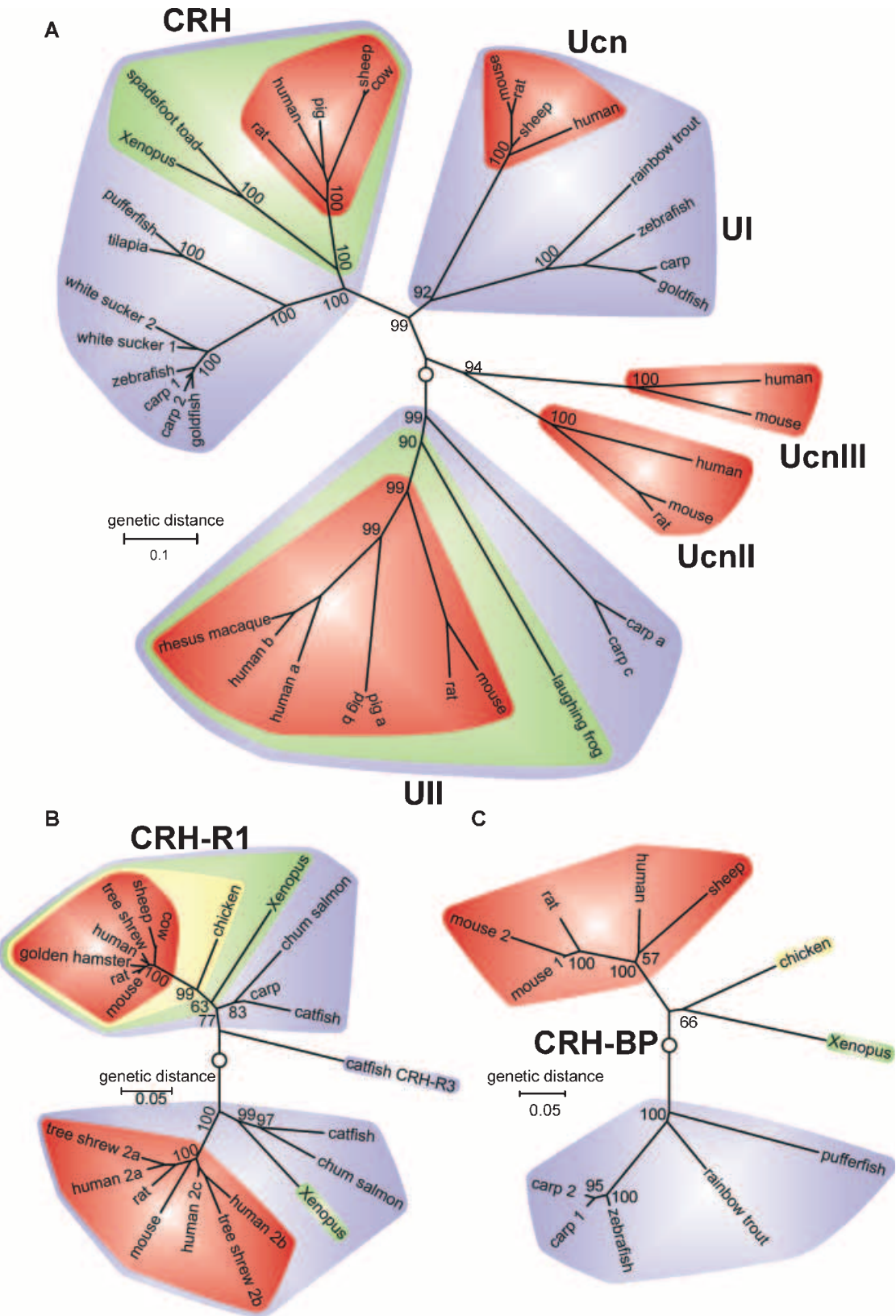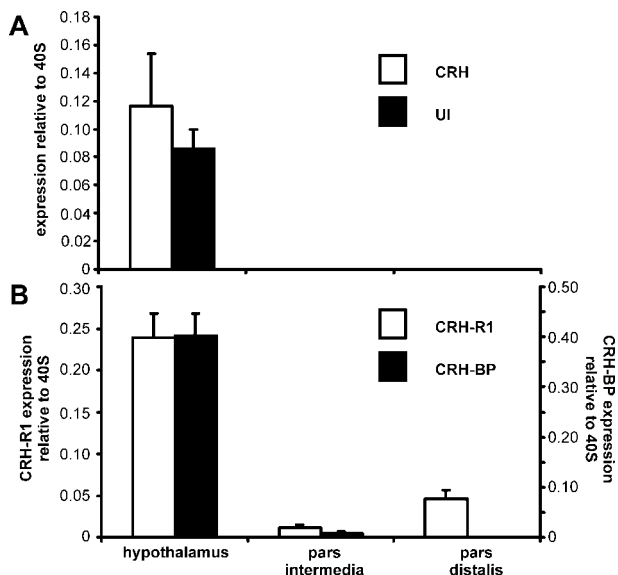
Figure 7 (A) Basal expression of CRH (open bars) and UI (filled bars) and (B) basal expression of CRH-R1 (open bars) and CRH-BP (filled bars) in various organs of carp. CRH and UI expression within the pars intermedia and pars distalis was non-detectable. Expression was assessed by RQ-PCR and is plotted relative to the expression of 40S. Error bars indicate the S.D. of five replicate measurements.

expression of CRH or CRH-BP as expression levels of both genes remained constant throughout the experiment (Fig. 11C). Following 24 h of restraint, plasma cortisol levels had reached nearly 500 ng/ml (Fig. 11B). After 24 h of restraint, hypothalamic CRH as well as CRH-BP expression had increased significantly, whereas UI expression remained unaltered (Fig. 11D). The expression of CRH-R1
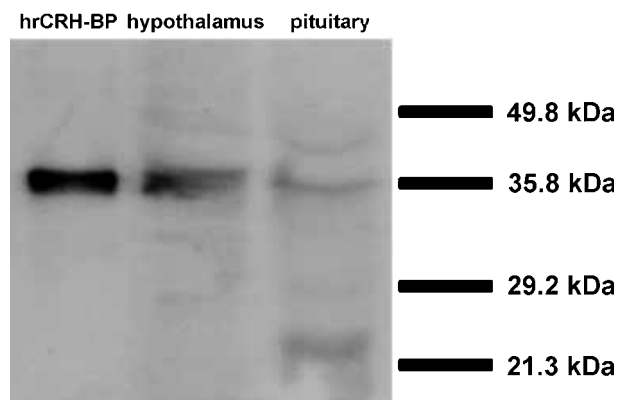
Figure 8 Western blot showing proteins of very similar size to hrCRH-BP in the water-soluble moiety of crude lysates of carp hypothalamus and pituitary gland.

within the PD showed a concomitant and significant decrease following 24 h restraint (Fig. 11E). CRH-BP expression within the PD did not change significantly.

Upon histological examination of the hypothalamic area following 24 h of restraint no differences were observed with regard to CRH, AVT or CRH-BP content (not shown). Within the PN, considerable differences were observed between non-stressed fish and fish restrained for 24 h. The PN fibre bundles of non-stressed carp contained considerable amounts of CRH (Fig. 12A) as well as CRH-BP (Fig. 12B). CRH and CRH-BP were co-localised as visualised in serial sections. Following 24 h restraint the amount of CRH contained within the PN fibres was unaltered or slightly diminished (Fig. 12C). However, the amount of immunoreactive CRH-BP contained within the PN fibre bundles was markedly reduced as shown by serial sections (Fig. 12D).

Discussion

CRH, CRH-R1 and CRH-BP, some of the principal molecules that regulate the initiation of the vertebrate stress response were sequenced in common carp. The overall amino acid identity of these proteins between fish and mammalian species is relatively high, generally around 55–60%, confirming that the signal system regulating the stress response is conserved throughout vertebrate evolution. The high degree of conservation of the CRH family of peptides earlier facilitated the discovery of several of the mammalian family members following the elucidation of their teleost fish orthologues (Vaughan *et al.* 1995, Conlon 2000). The elucidation of the complete set of CRH, CRH-R1 and CRH-BP in one teleost fish species allowed the study of the expression of CRH in relation to the expression of CRH-BP and CRH-R1, the principle pituitary gland receptor for CRH. The involvement of these molecules in the regulation of the teleost fish stress response is demonstrated in a restraint-stress paradigm.

Protein characteristics

For each of the three proteins described, two highly similar (>95% amino acid identity) genes were found; probably as a result of the teleost fish
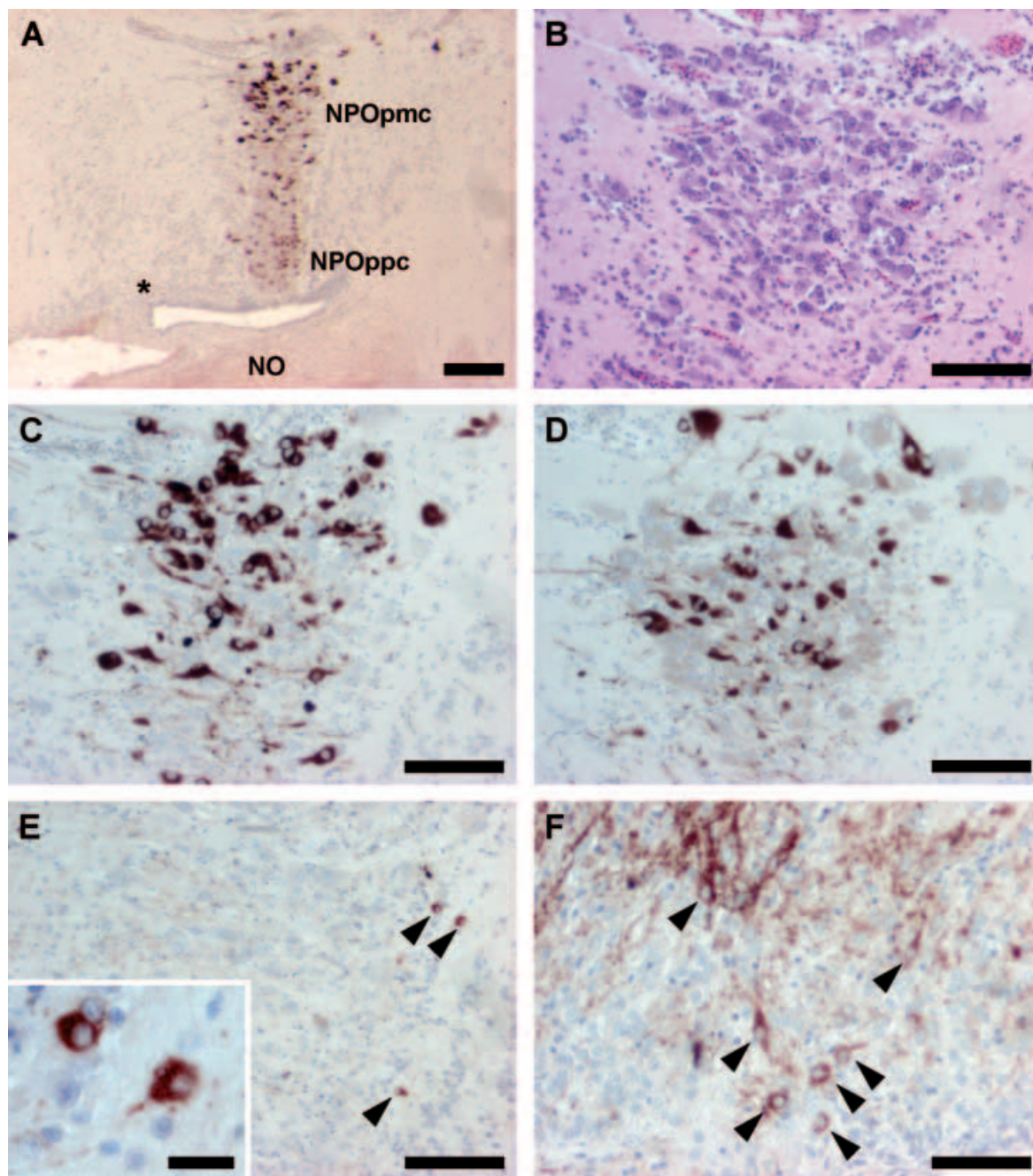
Figure 9 Serial immunohistochemistry on paramedian sagittal sections of the NPO. (A) Overview of the preoptic area, stained with an anti-CRH antiserum. (B) NPOpmc enlarged with an haemaluin-eosin stain. (C and D) The same area on serial sections stained for CRH and AVT respectively. (E) Serial section showing the presence of several small CRH-BP positive perikarya on the caudal perimeter of the NPO, at the region marked by the asterisk in panel A. The same cells are shown in enlarged in the inset. (F) Abundant CRH-BP staining in perikarya as well as neurones in the recessus opticus, just rostral from the NPO. NPOpmc, nucleus preopticus pars magnocellularis; NPOppc, nucleus preopticus pars parvocellularis; NO, nervus opticus. Arrowheads mark CRH-BP-positive perikarya. Scale bars: A, 200 μm; B–E, 100 μm, E (inset), 20 μm; F, 50 μm.
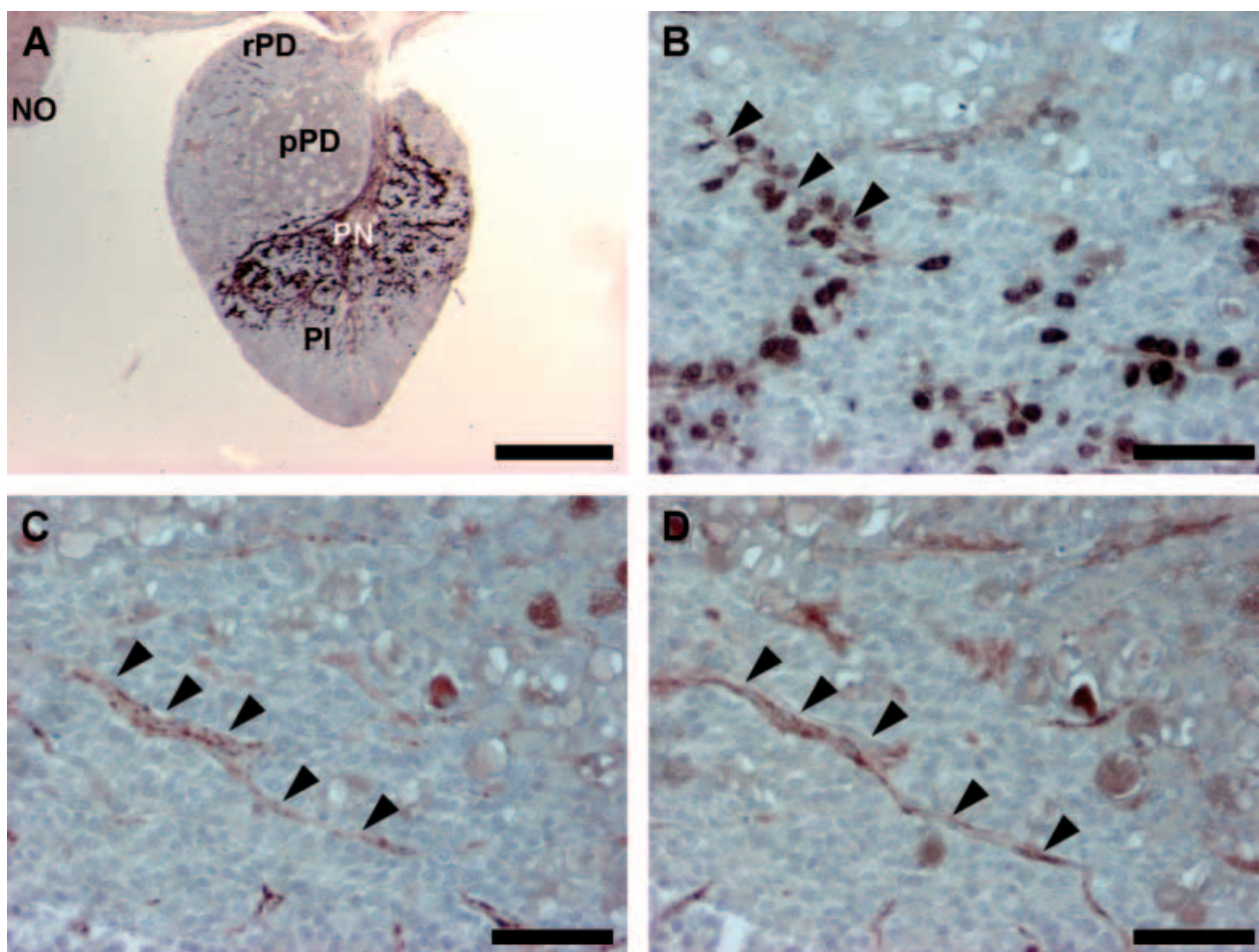
Figure 10 Serial immunohistochemistry on sagittal sections of the pituitary gland. (A) Overview of the pituitary gland, stained with an anti-CRH antiserum. Note the profound staining of the PN fibres projecting on the PI as well as the staining of the fibres within the rPD but not the pPD. (B, C and D) Details of the rPD of serial sections stained for ACTH, CRH and CRH-BP respectively. Note the alignment of ACTH-positive cells in a double row (B) surrounding a nerve fibre that is positive for both CRH (C) and CRH-BP (D). Arrowheads mark a distinct CRH/CRH-BP double-positive nerve fibre. PI, pars intermedia; PN, pars nervosa; pPD, proximal pars distalis; rPD, rostral pars distalis; NO, nervus opticus. Scale bars: A, 500 µm; B–D, 50 µm.

tetraploid genome. This is in agreement with previous findings for many other genes, including β-actin, growth hormone, prolactin and pro-opiomelanocortin, that are present in duplicate form in the carp (Larhammar & Risinger 1994, Arends *et al.* 1998). Nonetheless, any amino acid substitution observed potentially alters protein structure and consequently function. Most noteworthy is the substitution of amino acid residue P^{123} of carp CRH2 for A^{123} in CRH1; a unique substitution among all known vertebrate CRH sequences. This amino acid is not essential for binding to CRH-R1 (Rivier *et al.* 1984) or CRH-BP

(Sutton *et al.* 1995) but may yet have implications for protein structure, as proline residues are known to bend the protein backbone.

Several features of the carp sequences described here are more conserved than the overall sequence between representatives of an early (teleost fish) and a more recent (mammals) group of vertebrates. These include the mature CRH peptide as well as the majority of the extracellular CRH-R1 residues that have been implicated in CRH agonist binding (Liaw *et al.* 1997, Wille *et al.* 1999). The small sequence dissimilarities between the proposed peptide binding region of mammals and fish should be
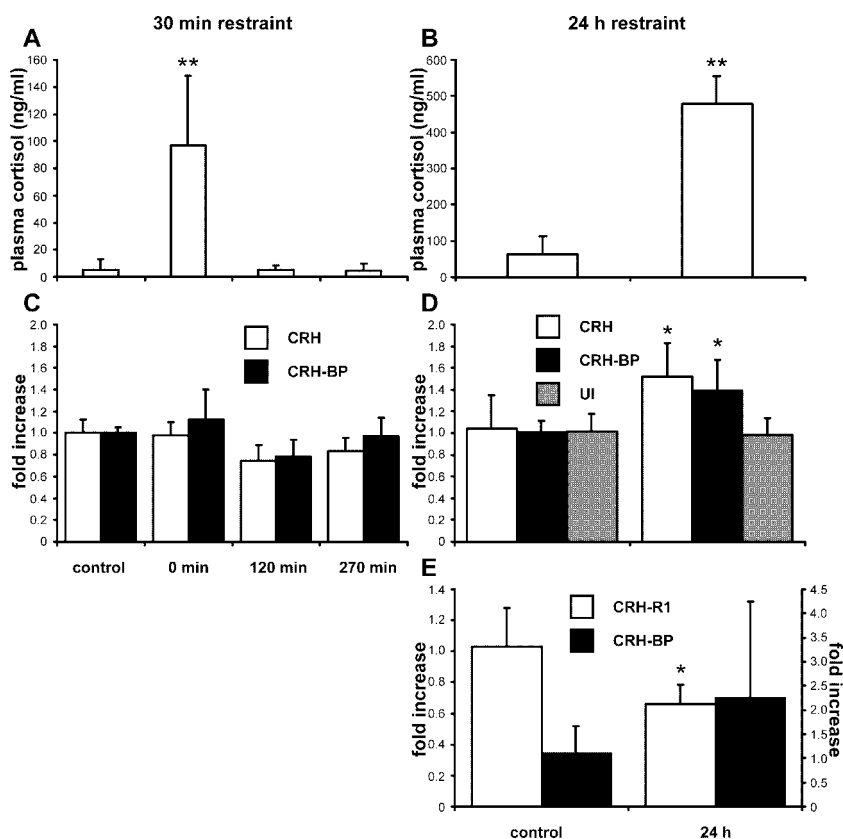
Figure 11 Changes in plasma cortisol values (A and B) and in hypothalamic and PD expression levels (C–E) during 30 min (A and C) and 24 h (B, D and E) restraint. The indicated times reflect the time passed since the initiation of restraint stress. Note the different scales of the *y* axes in panels A and B. Error bars indicate the S.D. of eight (cortisol) or five (expression) replicate measurements. Asterisks indicate significant differences from the control (**P>0·01; *P>0·05). Expression is standardised for 40S and the relative quantitation value expressed as $2^{-\Delta\Delta Ct}$.

viewed in the light of the highly conserved, but nevertheless slightly different, fish CRH sequences. Besides the conserved peptide binding regions, the carp CRH-R1 sequence contains several cysteine residues that have been suggested to form extracellular disulphide bridges (Qi *et al.* 1997, Hofmann *et al.* 2001). Finally, the complete absence of amino acid substitutions in the third intracellular loop (which is involved in G-protein coupling (Perrin & Vale 1999)) between carp and mammalian CRH-R1 sequences is additional proof of orthology of the carp CRH-R1 sequence to other vertebrate CRH-R1 sequences. Also CRH-BP is evolutionarily well conserved. In our in-silico searches we reconstituted the chicken CRH-BP sequence from two EST sequences and we retrieved CRH-BP sequences of

several teleost fish species in addition to the two carp genes described here. Both the presence and spacing of the ten cysteines forming the five consecutive disulphide bonds that determine tertiary structure were conserved in all sequences found. The orthology of fish CRH-BP to their non-teleostean vertebrate orthologues was confirmed in phylogenetic analyses that established the CRH-BP origin before the fish–tetrapod split in vertebrate evolution. This is confirmed by recent work of Seasholtz *et al.* (2002) who showed that a CRH binding factor of similar size to human CRH-BP was present in brain extracts of representatives of many vertebrate classes, including jawless fish.

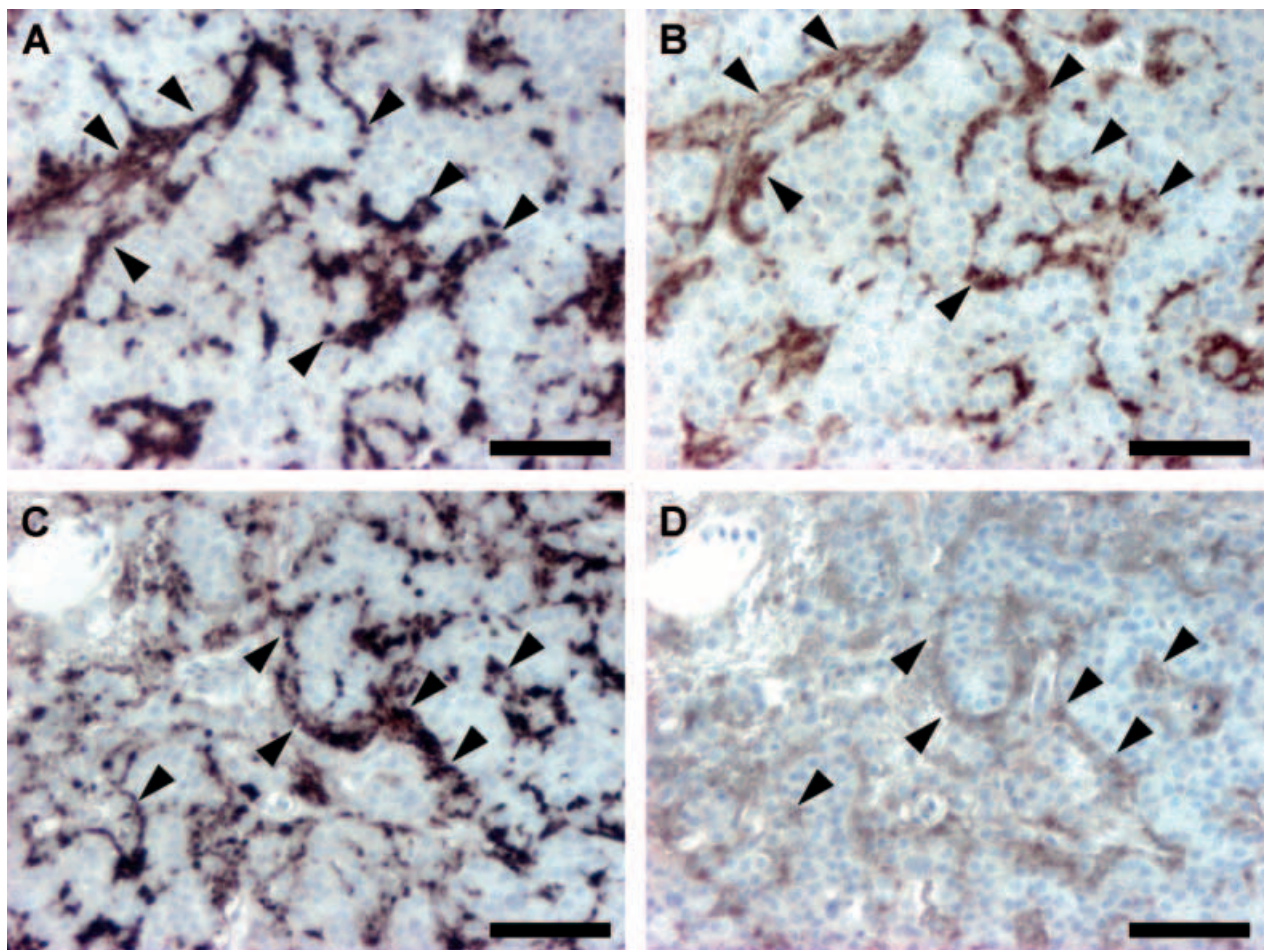Whereas CRH-BP is evolutionarily well-conserved, it bears no considerable sequence

Figure 12 Serial immunohistochemistry on saggital sections of the PI. In control carp, the nerve fibres projecting on the PI contain both CRH (A) and CRH-BP (B). Following 24 h restraint, the amount of immunoreactive CRH (C) remained unaltered or was slightly diminished, whereas immunoreactivity to CRH-BP (D) was sharply reduced. Arrowheads mark the same features within panels A and B, and C and D respectively. Immunoreactivity was assessed on serial sections of two fish per group. Scale bars, 50 μm.

similarity to any known group of proteins and might constitute an autonomous family of proteins. This implies that, unlike many soluble ligand-binding factors that structurally resemble the ligand receptor, CRH-BP is unrelated to the receptors for any of the CRH family of ligands. This partially relates to the heptahelical nature of the CRH receptors, which would radically alter their folding in solution (if they were soluble at all). Nonetheless, this implies a compelling scenario of two different, structurally unrelated, proteins with high affinity for the CRH family of ligands, which arose independently in evolution.

Innervation of the teleost pituitary gland

In contrast to mammals, most teleost fish species lack a hypothalamo-pituitary portal vessel system (Peter & Fryer 1984). Consequently, neurosecretory fibres emanating from the NPO project directly to the pituitary, and this enables a histological assessment of their target cells. Within the NPO, CRH co-localises with AVT, as has been reported earlier (Yulis & Lederis 1987). Both hormones are present in nerve fibres that project onto the pituitary and have been suggested to exert either additive or synergistic effects on ACTH secretion

(Fryer *et al.* 1985, Baker *et al.* 1996). Consistent with the original studies on the distribution of CRH in the teleost pituitary gland (Olivereau & Olivereau 1988), we find CRH-positive fibres within the rPD. These fibres are surrounded by ACTH-secreting cells, in line with the well-established role for CRH as an ACTH-releasing factor (Vale *et al.* 1981). We also observed prominent CRH immunoreactivity within the PN fibre bundles that project onto the PI. Although many previous investigations have focussed on CRH immunoreactivity within the teleost rPD, considerable CRH immunoreactivity has also been reported within the PI of several species (Yulis & Lederis 1987, Matz & Hofeldt 1999, Pepels *et al.* 2002). The large majority of the PI melanotropes innervated by these CRH-positive fibre bundles secrete α-melanocyte-stimulating hormone (α-MSH) and contain various acetylated forms of β-endorphin (van den Burg *et al.* 2001). In some species of fish CRH is capable of stimulating the secretory activity of PI melanotropes *in vitro* (Tran *et al.* 1990, Lamers *et al.* 1994, Rotllant *et al.* 2000); this concurs with early reports on the α-MSH- or β-endorphin-releasing capability of CRH in higher vertebrates (Vale *et al.* 1981, Kraicer *et al.* 1985, Verburg-Van Kemenade *et al.* 1987). Notably, transcription levels of CRH-R1 are four-fold higher in the PD compared with the PI, while ACTH-releasing cells constitute merely an estimated 10% of the PD cell mass. Given the abundance of PI melanotropes and the paucity of CRH-R1 in the PI, the regulation of PI output by CRH is probably mediated by another receptor with considerable affinity for CRH. Candidates are CRH-R2 and CRH-R3, although the latter has so far been described in only one fish species (Arai *et al.* 2001).

In teleosts, β-endorphin and α-MSH alone or in concert display corticotrophic activity in some species (Lamers *et al.* 1992, Balm *et al.* 1995). Recently, in carp, the presence of a corticotrophic factor in the pituitary PI was confirmed, but this factor was neither α-MSH nor β-endorphin, alone or in concert (van den Burg *et al.* 2003). Nevertheless, the vast quantities of CRH observed within the PN projecting onto the PI melanotropes substantiate a probable role for CRH in the regulation of the release of one or several PI corticotrophic factors *in vivo*. Alternatively, CRH (and CRH-BP) could be released to the circulation.

In Chinook salmon (*Oncorhynchus tshawytscha*), co-localisation of CRH-positive fibres and thyrotrophin (TSH)-secreting cells was reported (Matz & Hofeldt 1999). However, in carp no CRH immunoreactivity was observed in nerve fibres projecting onto the pPD, which contains the TSH-releasing cells. This is corroborated by previous reports in several species (Yulis & Lederis 1987, Pepels *et al.* 2002). The absence of direct innervation of the pPD TSH cells by CRH is remarkable and in paradox with the potent *in vitro* and *in vivo* TSH-releasing capacity of CRH in fish (De Pedro *et al.* 1995, Larsen *et al.* 1998) as well as in amphibians and birds (Denver 1988, Geris *et al.* 1996). In chicken, CRH is a potent TSH-releasing factor, although the pituitary thyrotropes do not express CRH-R1 (De Groef *et al.* 2003). Possibly the regulation of the thyroid axis is a secondary effect of CRH, potentially mediated in a paracrine way via one of the pituitary hormones that is under direct CRH control.

CRH-BP mode of action

In mammals the pituitary corticotropes constitute a prominent site of CRH-BP mRNA expression (Burrows *et al.* 1998). This is suggestive of a role for CRH-BP as a negative regulator of CRH activity during acute stress (Seasholtz *et al.* 2001), a function that is corroborated by the *in-vitro* attenuation of CRH-induced ACTH release from pituitary cells (Potter *et al.* 1991) or AtT-20 cells (Cortright *et al.* 1995). Contrary to this notion, CRH-BP immunoreactivity within the rat anterior pituitary is largely associated with secondary lysozomes and multivesicular bodies, suggesting a role for CRH-BP in the processing and degradation of CRH and/or ligand receptor complexes (Peto *et al.* 1999).

The co-localisation of CRH-BP with CRH in both the rPD as well as the PI strongly suggests that it is a key regulator of CRH activity in both parts of the pituitary. Furthermore, the localisation within fibre bundles is suggestive of an *in vivo* modulatory role in the regulation of the levels of 'free' CRH, rather than a role in protein degradation. This is substantiated by our observation of the decrease in PD CRH-R1 expression and the concomitant increase in PD CRH-BP expression following a 24 h restraint period, as both effects would reflect the desensitisation of the PD for the ACTH-releasing effects of hypothalamic CRH. Substantial

CRH-BP gene expression is observed in both parts of the carp pituitary gland, despite the lack of significant CRH-BP immunoreactivity associated with cell bodies. This suggests tight regulation at the translational level. Thus, although we observe substantial CRH-BP gene expression at the pituitary level, we are currently unable to attribute this gene expression to a particular pituitary cell type.

Although localised in the same pituitary fibre bundles, CRH-BP is not co-expressed by the CRH-positive neurones of the NPO, which project onto the pituitary (Olivereau & Olivereau 1988) but must originate from other cells. Indeed, a limited number of relatively small perikarya at the caudal perimeter of the NPO express CRH-BP and may be the source of this CRH-BP. Another hypothalamic site of considerable CRH-BP expression in many fibres, as well as perikarya, is found ventro-rostrally of and in close proximity to the NPO. Also the fibres emanating from this area potentially project onto the pituitary, although, as yet, we can not demonstrate this conclusively.

Regulation of restraint stress

In our *in vivo* experiments we could not detect altered gene expression following a 30 min restraint, but following a prolonged (24 h) restraint period, considerable differences in hypothalamic, as well as PD gene, expression were observed. This is not entirely surprising for two reasons. First, the moderate peak plasma cortisol values that had dissipated within 120 min of the onset of the stress response are indicative of a mild and transient stress response. In contrast, a 24 h restraint period resulted in very high plasma cortisol values of nearly 500 ng/ml at the termination of restraint; these values had probably persisted for the major part of the restraint period. Secondly, ACTH release by CRH is initiated within approximately 1 min of the application of a stressor (E van den Burg, unpublished observations); this implies that the initiation of the stress response depends on stored signal protein. The amounts of peptide released following a typical acute and transient stressor (e.g. 30 min restraint) are so small in comparison to the amount of stored protein, that any additional gene transcription initiated on top of the constitutive gene expression will remain undetectable. However, the persistent stimulation of the stress axis during the 24 h restraint period

may have depleted CRH and CRH-BP protein stores, necessitating the observed enhancement of hypothalamic CRH and CRH-BP gene expression to guarantee homeostasis. At the same time this observation illustrates that hypothalamic CRH-BP is directly involved in the regulation of the stress axis, despite the virtual absence of CRH-BP immunoreactivity from the NPO. Hypothalamic UI expression remained unaltered throughout both experiments, confirming that the increases in CRH and CRH-BP expression are specific.

Despite the increased hypothalamic CRH-BP gene expression, PN CRH-BP protein content had markedly dropped following 24 h of restraint. This suggests that some regulation of PI output had occurred, consistent with a role for PI corticotrope output under longer-term stress conditions (Lamers *et al.* 1994). A 24 h restraint period might have crossed the 'border' between acute stress, regulated by PD output, and chronic stress where the PI output takes over the chronic regulation of plasma cortisol levels (Lamers *et al.* 1994).

Perspectives

The observations above indicate that the regulation of pituitary gland output by CRH extends well beyond the release of ACTH from the rPD. The co-localisation of CRH-BP with CRH throughout the pituitary gland indicates a profound regulatory capacity for CRH-BP in the regulation of rPD as well as PI output. CRH and ACTH are generally regarded as the principle initiators of the acute stress response in fish, whereas thyrotrophin-releasing hormone (TRH) and α-MSH have been suggested to regulate the stress response during exposure to chronic stressors. This study indicates that, although acute and chronic stress responses are regulated by rPD and PI output respectively, CRH and CRH-BP potentially contribute to the regulation of both phases of the stress response by virtue of their presence in both parts of the pituitary gland. Now that we know the major regulatory molecules in teleost fish, we can further unravel the intricate regulation of the pituitary gland together with the ensuing stress response. To this end, teleost fish form a convenient model system, since they lack an eminentia mediana and consequently reveal many discrete regulatory pathways via the direct hypothalamic innervation of pituitary target cell populations.

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