Corticosteroid receptors involved in stress regulation in common carp, *Cyprinus carpio*

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Abstract

In higher vertebrates, mineralo- (aldosterone) and glucocorticoids (cortisol/corticosterone) exert their multiple actions via specific transcription factors, glucocorticoid (GR) and mineralocorticoid (MR) receptors. Teleostean fishes lack aldosterone and mineral regulatory processes seem under dominant control by cortisol. Despite the absence of the classical mineralocorticoid aldosterone, teleostean fishes do have an MR with cortisol and possibly 11-deoxycorticosterone (DOC) (as alternative for aldosterone) as predominant ligands. We studied corticoid receptors in common carp (*Cyprinus carpio* L). Through homology cloning and bioinformatic analysis, we found duplicated GR genes and a single MR gene. The GR genes likely result from a major genomic duplication event in the teleostean lineage; we propose that the gene for a second MR was lost. Transactivation studies show that the carp GRs and MR have comparable affinity for cortisol; the MR has significantly higher sensitivity to DOC, and this favours a role for DOC as MR ligand in fish physiology. mRNA of the GRs and the MR is expressed in forebrain (in pallial areas homologous to mammalian hippocampus), corticotrophin-releasing hormone (CRH) cells in the pre-optic nucleus (NPO) and pituitary pars distalis ACTH cells, three key neural/endocrine components of the stress axis. After exposure to prolonged and strong (not to mild acute) stressors, mRNA levels of both GRs and MR become down-regulated in the brain, but not in the NPO CRH cells or pituitary ACTH cells. Our data predicts a function in stress physiology for all CRs and suggest telencephalon as a first line cortisol target in stress.

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Introduction

The adrenal cortex of mammals produces cortisol (or corticosterone) as glucocorticoid and aldosterone as mineralocorticoid. The function of these steroids is ultimately specified by the transcription factors (glucocorticoid and mineralocorticoid (MR) receptors) that mediate their actions in the diverse targets and that define which genes will be activated or repressed. Interrenal cells of teleostean fishes produce cortisol as the major steroid; in addition, deoxycorticosterone (DOC; a possible side product of progesterone conversion by 21-hydroxylase activity in cortisol pathway) is found in fish plasma (Sturm *et al*. 2005). Aldosterone is an evolutionary more recent steroid (Bridgham *et al*. 2006), believed to be absent in teleostean fishes (Balment & Henderson 1987).

In all vertebrates, glucocorticosteroids play a key regulatory role in stress responses, growth and general metabolism, reproduction and immunity (Mommsen *et al*. 1999); and in terrestrial vertebrates, a specific subtask in mineral regulation is given to aldosterone. In fish, cortisol is intimately involved in the regulation of water and mineral balance (Gilmour 2005). At least two notions come to mind at the basis of a fundamentally different corticoid endocrinology in fishes: i) cortisol, the main corticosteroid exerts receptor-defined gluco- or mineralocorticoid actions and thus the regulation of water and mineral balance in fishes is not necessarily controlled by a mineralocorticoid and ii) the poorly studied DOC could, via a MR, act as a mineralocorticoid in fishes.

The corticoid receptors are promiscuous for ligands; cortisol is bound both by GR and MRs. The evolution of multiple corticosteroid receptors and their signalling pathways in vertebrates was extensively reviewed (Bridgham *et al*. 2006, Prunet *et al*. 2006, Baker *et al*. 2007, Bury & Sturm 2007). An ancestral corticosteroid receptor (AncCR) is assumed to have been an effective receptor for cortisol; the AncCR may further have transmitted a DOC signal in the ancestors of fishes. Duplication of the AncCR gene led to separate GR and MR species over 450 million years ago; it is assumed that the MR retained an ancestral phenotype and that the GR lost sensitivity for aldosterone (Bridgham *et al*. 2006) in favour of cortisol. A second major genomic duplication event took

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place in teleostean fishes (not in tetrapods) and gave rise to further diversification of the corticosteroid receptor family. In the extant teleostean fishes studied so far, two different GR genes and one MR gene are found (Greenwood *et al.* 2003, Bury & Sturm 2007). The interesting picture arises in fish in that a single ligand (cortisol) may steer three different receptors; therefore leading to diversification of receptors rather than of ligands.

Physiological research on the role of these fish receptors (combined) in stress and osmoregulation is as yet scarce. Although extensive research effort has been put in aspects of stress handling, smoltification and reproduction, and the effects of increased cortisol levels on GR expression (Maule & Schreck 1991, Basu *et al.* 2003), only a few studies focussed on both GR and MR activities either simultaneously (Greenwood *et al.* 2003) or within the same species (Bury *et al.* 2003, Sturm *et al.* 2005). A role for MR activity in fish osmoregulation seems likely (Gilmour 2005, Sturm *et al.* 2005, Prunet *et al.* 2006), yet other MR functionality, for instance in brain function, or stress axis regulation requires far more research.

The endocrine stress axis is a pivotal and phylogenetically ancient regulatory system, key in adaptation of vertebrates to their dynamic environment (Wendelaar Bonga 1997) as well as in stress handling. The teleostean hypothalamic–pituitary–interrenal (HPI) axis is comparable with the mammalian stress axis (Wendelaar Bonga 1997, Mommsen *et al.* 1999), a result of convergent evolution. Stressful sensory information is conveyed to the hypothalamic pre-optic area (NPO) and results in release of corticotrophin-releasing hormone (CRH). NPO CRH cells in fish project directly to the rostral pars distalis adrenocorticotrophic hormone (ACTH)-producing cells. ACTH released into the general circulation will then activate interrenal cells of the head kidney to produce and secrete cortisol. This cortisol will redistribute energy flows to deal with the stressor(s) and by doing so guarantees homeostasis (Wendelaar Bonga 1997).

In mammals, both GR and MR are involved in the regulation of cortisol release and coping with stress (De Kloet *et al.* 1998). MR activity governs a tonic hippocampal inhibitory control over the hypothalamus–pituitary–adrenal (HPA) axis; GR activity exerts a negative feedback on higher brain centres under conditions of high cortisol (De Kloet *et al.* 1998, Reul *et al.* 2000). Indeed, high cortisol levels, via GR activity, inhibit CRH and ACTH release from hypothalamus and pituitary gland respectively, to counteract the stress imposed (De Kloet *et al.* 1998). In fish, where such negative feedback has been described (Wendelaar Bonga 1997, Mommsen *et al.* 1999), neither the receptors involved are known nor are the signals precisely defined (i.e. is it cortisol, DOC or may be both).

We therefore investigated which corticosteroid receptors were present in common carp and where these receptors were located in the stress axis and focussed on CRH and ACTH cells and higher brain centres (telencephalic pallial neurons). Next, we determined mRNA expression of the three receptors (GR1, GR2 and MR) and sensitivity for cortisol by transactivation analysis to assign the possible differential functionality under basal or stress conditions (i.e. when plasma cortisol is low and high). Finally, we showed that corticosteroid mRNA expression in the stress axis could be manipulated by a chronic stress paradigm suggesting the involvement of all three receptors in stress axis regulation.

Materials and Methods

Animals

Common carp (*Cyprinus carpio* L.) were kept at 23 °C in recirculating u.v.-treated tap water at 'De Haar Vissen' in Wageningen. Fish were fed with dry food pellets (Promivi, Rotterdam, The Netherlands) at a daily maintenance ration of 0·7% of their estimated body weight. The cross 'R3×R8' is offspring of Hungarian (R8) and Polish (R3) strains (Irnazarow 1995). Experimental repeats were performed with fish from different batches of eggs. All experiments were performed according to national legislation and were approved by the institutional Ethical Committee.

Identification of GR1, GR2 and MR genes

We screened the Ensembl zebrafish genome database with sequences of mammalian GR and MR genes, using the basic local alignment search tool (BLAST) algorithm. This screen revealed one GR gene and one MR gene. These genes were incorporated in separate multiple sequence alignments, using CLUSTALW (Chenna *et al.* 2003); for both the GR and MR, genes of several species were used. Primers were designed in regions of high amino acid identity. We obtained partial cDNA sequences from a λZAP cDNA library of carp brain. The corresponding full-length sequences were obtained by RACE (Invitrogen). PCR was carried out as described previously (Huising *et al.* 2004) and the sequences were determined from both strands.

Probe synthesis

Digoxygenine (DIG)-labelled carp GR1, GR2 and MR probes (Table 1) were synthesised from a PGEMTeasy (Promega) vector with the respective inserts by the use of a DIG RNA-labelling kit according to the manufacturer's instructions (Roche). The GR1 vector contained a 474 base pairs insert, the GR2 vector a 509 base pairs insert and the MR vector a 827 base pairs insert (primers shown in Table 1). Proper probe labelling was confirmed by the northern blotting. Probes were constructed in the AB domain, the least conserved region, to avoid cross-reactivity between probes (Fig. 1).

Tissue and section preparation

Nine-month-old carp (150–200 g) was anaesthetised with 0·2 g/l tricaine methane sulfonate (TMS) (Cresent Research Chemicals, Phoenix, AZ, USA) buffered with 0·4 g/l $NaHCO_3$ (Merck). Blood was collected by puncture of the caudal vessels

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Table 1 Primers used to generate *in situ* hybridisation probes

Gene	Sequence	Amplicon length (bp)
GR1	FW: 5′-AGC-ATC-CAG-GCC-CCT-GAC-AC-3′	474
	RV: 5′-AGG-ACC-ACC-CAT-CCC-TGA-CAT-CTG-3′	
GR2	FW: 5′-CCT-TCA-GTG-GAC-TCC-CTG-ATT-G-3′	509
	RV: 5′-GAA-GTG-GTG-ACG-CCG-CAG-ATG-TTA-A-3′	
MR	FW: 5′-CAY-YGT-GGG-GTC-ACC-TCC-AC-3′	827
	RV: 5′-TCC-CTT-GCG-CTC-CAA-TCT-GG-3′	

using a heparinised (Leo Pharmaceuticals Products, Ltd, Weesp, The Netherlands) syringe fitted with a 21 gauge needle. Next, fish were killed by spinal transsection, and organs and tissues for RNA extraction were carefully removed, snap frozen in dry ice or liquid N_2, and stored at −80 °C. Organs for *in situ* hybridisation were removed and fixed overnight in 4% freshly prepared paraformaldehyde (PFA) in PBS. Next, the tissues were transferred to 1·5% agarose in 15% sucrose in PBS, snap frozen in liquid N_2 and stored at −80 °C. Serial 7 μm cryostat sections were made (Frigocut 2800, Reichert-Jung) and mounted on poly-L-lysine coated microscope slides (BDH Laboratory Supplies, Poole, UK).

In situ *hybridisation*

In situ hybridisation was adapted from Engelsma *et al.* (2001) and included a proteinase K (5 μg/ml) (Promega) treatment for 10 min at 37 °C to improve probe accessibility and a 10-min exposure to 0·25% acetic acid anhydride in 0·1 M triethanolamine (TEA) to stop RNAse activity. Probes (sense 0·5 ng/μl and antisense 1·5 ng/μl) were dissolved in Hyb^+ buffer with 2% blocking reagent (Roche Applied Science) and denatured for 5 min at 80 °C, added to every glass slide, covered with parafilm and incubated overnight at 55 °C. Hyb^+ buffer was replaced with 4×SSCT (4×SCC with 0·01% Tween 20) buffer and sections were rinsed (2×15 min) in 4×SSCT buffer at 60 °C. An RNAse treatment with 10 ug/ml RNAseA (Qiagen) for 30 min at 37 °C removed unbound RNA fragments. Next, slides were washed (2× with 2×SSCT, for 5 min) at 60 °C, once with 1× SSCT for 10 min at 60 °C, once with 0·5×SSCT for 10 min at 60 °C and finally once with 0·1×SSCT for 30 min during which the samples were allowed to cool to room temperature. Washing and colour reaction were performed as described previously (Engelsma *et al.* 2001).

Immunohistochemistry

The tissue on glass slides was first fixed in 4% PFA in PBS for 15 min. The slides were washed once with PBST for 5 min and once with aquadest for 5 min. Subsequently, they were incubated 10 min with methanol plus 0·3% H_2O_2, after which the slides were rinsed twice for 10 min in PBST. Subsequently, non-specific antigenic sites were blocked with 10% normal goat serum (NGS) in PBS for 30 min. The slides were incubated overnight with polyclonal antiserum against GH (1:4000) or ACTH (1:2000) in PBS with 10% NGS. The next day the slides were rinsed twice for 10 min in PBST and incubated for 1 h with goat anti-rabbit secondary antibody at a dilution of 1:200.

Imaging

Pictures were taken with Zeiss Axiovert tv 135 microscope with a 5·0 Q-imaging colour camera and Leitz orthoplan cool snap colour camera (Roper Scientific). The pictures were edited (cropped and background colour compensation) using Adobe Photoshop.

Stress experiments

Restraint stress Prolonged restraint (24 h) was given by netting the fish and suspending the nets with the fish in the tanks (Huising *et al.* 2004). After 24 h, the experimental group was transferred all at once to a tank with 0·2 g/l TMS, resulting in rapid (<1 min) and deep anaesthesia prior to blood sampling and killing. A control group was housed in an identical tank but left undisturbed. Control fish were sampled following rapid netting and anaesthesia, immediately before sampling of the experimental group.

Cold water stress Fish were netted and transferred from a tank with 23 °C water to an identical tank with 10 °C water, and left there for 15 min, after which they were returned to their original tank. This transfer was repeated thrice a day for 3 days. On day 4, the fish were transferred once more and sampled 30 min after return to their original (warm) tank. For sampling, fish of a tank were all at once transferred to a tank with 0·2 g/l TMS, resulting in rapid anaesthesia. Sham-treated fish were housed in identical tanks and transferred as mentioned above, but to tanks with 23 °C water. Control fish were housed in identical tanks and left undisturbed. The sham and control fish were sampled just before sampling of the experimental fish.

Physiological parameters and plasma hormone determination

Freshly collected, heparinised blood was centrifuged for 10 min at 2000 ***g*** at 4 °C, after which plasma was transferred to a new

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                  10        20        30        40        50        60
                   |         |         |         |         |         |
GR1       MD--------SGQKR--------SSNNGENLT---------LGDCIERGFVPDIG-----
GR2       MDQG---GLTNGAKRD----DHLNTLDYSNSP---------VEGILRSGIQSAMP-----
MR        METKRYQSYREGANAENKLAQMPNTMDYCCSAEEHLTNSDMLMDNVNSSNAPNMPSVCKD
          *:         .* :          .: :   .        : . :. .  . :

                  70        80        90       100       110       120
                   |         |         |         |         |         |
GR1       -----VNVSALN-----------TSKDFSNGQSGSDAQRNLSLADPSLLGRNTQEPAVKA
GR2       -----VAPTSLVPQPNP--LMQPVSGDVPNGLSNSPTLEEHTTSVSSTLGIFGEDSELKM
MR        NNFKTTETTMLRVNQNQPLLFPSFNNSFQNRKSETDS-KELSKTVAESMGLYMNAAREAD
               .  : *             . ..  *  * : : .: : : .. :*   : .

                 130       140       150       160       170       180
                   |         |         |         |         |         |
GR1       FKPFRM-----QHQQKVKEPLNIGENFSLLDESIADLNRGS-------SIQAPDTFTMKM
GR2       VGKEQR-----AHQHQTLGAFTLGDSFSSLEASIADLNSTSPSVDSLIGGMDPNLFPLKT
MR        FGFSQQGTAGGQGSPQKLYPLSGRANEDSQSRTTGSPKMKAPPASFPPGAQLPNGRPQEC
          .   :        . :  .:.   . .  . : .. :  :      .   *:  . :

                 190       200       210       220       230       240
                   |         |         |         |         |         |
GR1       EQFSPMEKDRLDFP---SYGHMDKELDS-NERVIGDNTIDILKDLDLPDSLSDLNELYVA
GR2       EEYSLMDKGDMDLDQ-DSFGPIGKDGDVDNHKLFSDNTLDLLQDFELDGSPSDF---YGA
MR        AVVSASVPSAMAATLSCSTDGSGPMSSPTGHNMVSSTTSPTFFDSDCPSLASTHTNLIQG
             *    . :      * .  .   .  ...:....*   : * :  .  *        .

                 250       260       270       280       290       300
                   |         |         |         |         |         |
GR1       DEAA------FLSSLAVDDALLGESNFLKD-----------TSPVVTGN----SAACANV
GR2       DDP-------FLSTISEDALLGDLPTITER-----------DSKVAVNG----ATTTPST
MR        QHTSPNTCSPVKSSVVGSPPLASPLSVIKSPVSSPHSIGSVSSPLSCNTNMRSSVSSPTT
          :..       . *::  .  * .  .. :             * :  .    :.:  ...

                 310       320       330       340       350       360
                   |         |         |         |         |         |
GR1       NGMGKR-------QQMVEASVNIKTEKDAD------------------------------
GR2       SGIN----------TVTVSLPTVKVEKDS-------------------------------
MR        YGGNTSNIRPSISSPPTVGSMTMSSPRNSSRGFSVSSPPSGLGLVQNDVNSPESREHDFK
           * .            . .  .:.  :::

                 370       380       390       400       410       420
                   |         |         |         |         |         |
GR1       ---------------FIQLCTPGVIKQETER-----RSYCQMSGMGGPHSG---------
GR2       ---------------IIQLCTPGVIKQENNGG----TKYCQASLHSTPIN----------
MR        AFEFPKVENVDGEIFNIGLDAMGVAKYIKNEPGTDYRSMCLGSSKSAMPHSPFITHIKTE
                          * * : ** *  .:       . *  *  .

                 430       440       450       460       470       480
                   |         |         |         |         |         |
GR1       -----PTTLGDMGGQGYHYGAN---TASAVSLPDQKPPFGLF----SPLPTLSDGWVRGN
GR2       -----ICGVTTSVGQSFLIGTSP-STAAVSQQKDQKPVFNVY----TPVTSSEDGWGRGY
MR        PNREVTCSNLQFVEPQHSLGCFPSTETTYLSLRDNIDEYSLSGILGPPVLSLNGNYEPGV
                          .  *      ::  .  *:  :.:     .*: : ...:  *

                 490       500       510       520       530       540
                   |         |         |         |         |         |
GR1       --GYGDPSGMQR-ANETVLP----------------------------STYPYSRPEASA
GR2       --GFGNASEMQQRASESFSKN---------------------------YTSPYARPEDST
MR        FPNNGLPKGIKQETSDGSYYQENNNVPTSAIVGVNSGGHSFHYQIGAQGTMSFSRHNLRD
            . * .. ::: :.:                              * .::* :

                 550       560       570       580       590       600
                   |         |         |         |         |         |
GR1       SSS---------------------------------------------SGSVKPGGNTHK
GR2       ATS---------------------------------------------SAAGKSG--THK
MR        QTNPLLNLISPVTGLMETWKTRPGLSQGPLSARGDGYPGSVCLTENMESASVRHTSSTAK
           :.                                            *.:  :    * *
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Figure 1 (*continued*)

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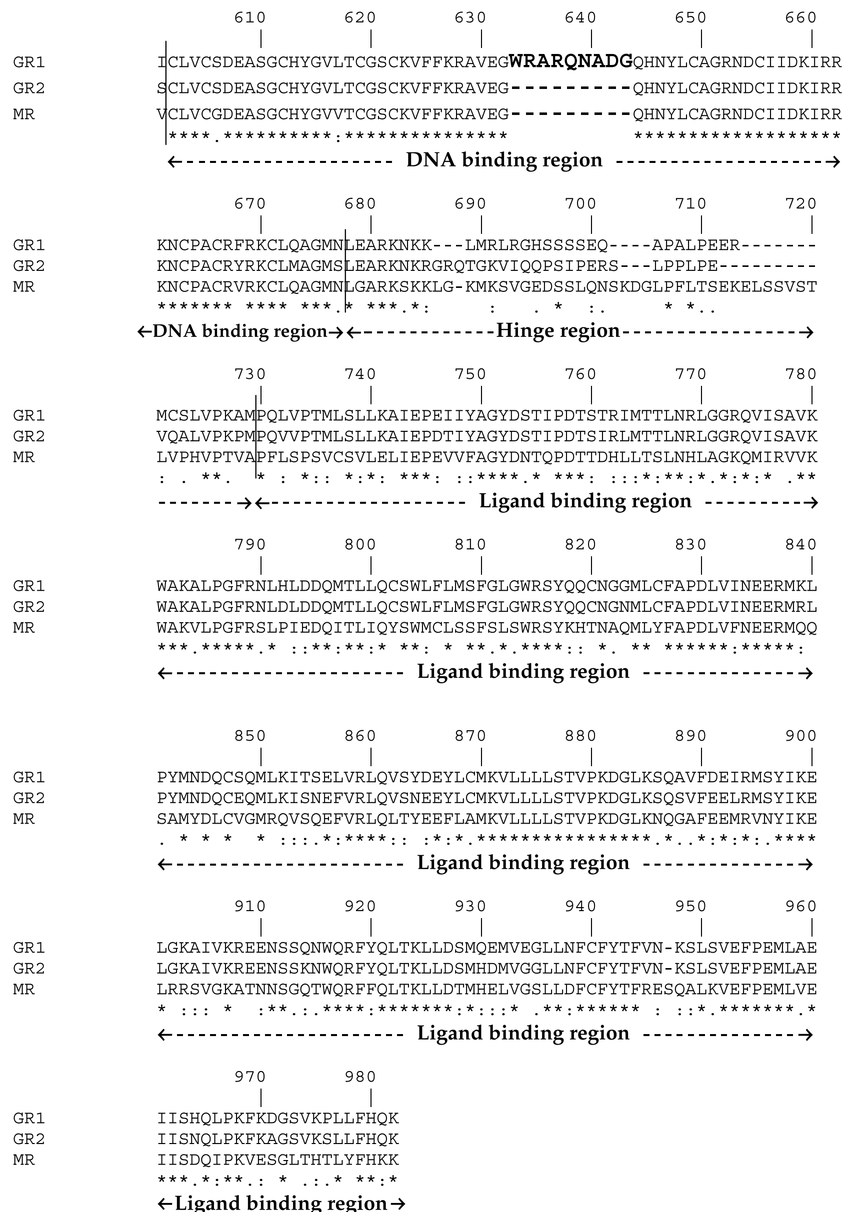

Figure 1 Alignment of corticosteroid receptor sequences of carp. Locations of probes are indicated; GR1 is underlined, GR2 is shown in bold and MR is shown in grey. Different regions of the receptors are indicated below the alignment; the transactivation region is located N-terminally from the other regions and has not been indicated. The nine amino acid insert present in the DNA-binding region of GR1 is shown in a larger, bold font. Asterisks indicate conserved amino acids, double points indicate replacement by amino acids with high similarity and single points indicate replacement with amino acids of low similarity.

tube and stored at −20 °C. Cortisol was measured by RIA (Arends *et al.* 1998), with 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-anilia-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. The samples were incubated overnight with 100 µl iodinated cortisol ∼1700 c.p.m./tube (^{125}I-labeled cortisol, Amersham) and 100 µl goat anti-rabbit γ-globulin (in RIA buffer). Bound and free cortisol in the assay 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). Tubes were centrifuged at 4 °C (20 min at 2000 ***g***), the supernatant aspirated and counted in a gamma counter (1272 clinigamma, LKB, Turku, Finland). Plasma glucose and Na^+ levels were measured

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with a Stat Profile pHOx Plus L Analyser (Nova Biochemical, Waltham, USA).

RNA isolation

RNA was isolated from tissues after extraction in Trizol reagent (Invitrogen), as suggested by the manufacturer. Total RNA was precipitated in isopropanol, washed with 75% ethanol and dissolved in nuclease-free water. RNA of separated pituitary glands (pars distalis and pars intermedia separate) was isolated as described by the RNeasy Mini Kit (Qiagen) strictly according to the manufacturer's instructions. RNA concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1·5% agarose gel before proceeding with cDNA synthesis.

DNAse treatment and first strand cDNA synthesis

For each sample, a '− RT' (non-reverse transcriptase) control was included. One microlitre 10× Dnase-I reaction buffer and 1 μl Dnase-I (Invitrogen, 18068-015) were added to 1 μg total RNA and incubated for 15 min at room temperature in a total volume of 10 μl. DNase I was inactivated with 1 μl 25 mM EDTA at 65 °C for 10 min. To each sample, 300 ng random hexamers (Invitrogen, 48190-011), 1 μl 10 mM dNTP mix, 4 μl 5× first strand buffer, 2 μl 0·1 M dithiothreitol and 40 Units RNAse Out (Invitrogen 10777-019) were added and the mix was incubated for 10 min at room temperature and for an additional 2 min at 37 °C. To each sample (not to the '−RT' controls), 200 U Superscript-II RNase H^- reverese transcriptase (RT; Invitrogen, 18064-014) was added and the reaction mixtures were incubated for 50 min at 37 °C. All reaction mixtures were filled up with demineralised water to a final volume of 100 μl and stored at −20 °C until further use.

Real-time quantitative PCR

PRIMER EXPRESS (Applied Biosystems, Foster City, CA, USA) and *PRIMER3* software were used to design primers for use in real-time quantitative PCR (RQ-PCR) (Table 2). For RQ-PCR, 5 μl cDNA and forward and reverse primers (300 nM each) were added to 12·5 μl Quantitect Sybr Green PCR Master Mix (Qiagen) and filled up with demineralised water to a final volume of 25 μl. RQ-PCR (15 min 95 °C, 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C followed by 1 min at 60 °C) was carried out on a Rotorgene 2000 real-time cycler (Corbett Research, Sydney, Australia). Raw data were analysed by use of the comparative quantitation of the Rotor-gene Analysis Software V5.0. Basal gene expression in organs and tissues of adult carp was determined as a ratio of target gene versus reference gene and was calculated according to the following equation: $\text{Ratio} = (E_{\text{reference}})^{C_t\ \text{reference}}/(E_{\text{target}})^{C_t\ \text{target}}$, where E is the amplification efficiency and C_t is the number of PCR cycles needed for the signal to exceed a predetermined threshold value. Expression following 24 h of restraint or cold water transfer was determined relative to the expression of non-restraint control fish according to the following equation (Pfaffl 2001), $\text{Ratio} = (E_{\text{target}})^{C_t\ \text{target(control−sample)}}/(E_{\text{reference}})^{C_t\ \text{reference(control−sample)}}$. Dual internal reference genes (40S ribosomal protein and β-actin) were incorporated in all RQ-PCR experiments; results were similar following standardisation to either gene. '− RT' controls were included in all experiments and no amplification above background levels was observed. Non-template controls were included for each gene in each run and no amplification above background levels was observed. Specificity of the amplification was ensured by checking the melting temperature and the profile of each melting curve. The product of each template was checked at least once by sequencing.

Transactivation assay

Clones encoding full-length open reading frame of common carp GR1 and GR2 were excised from pGEM-Teasy vector by EcoR1 and BamH1 and ligated into pcDNA3 expression vector cut with the same enzymes. Orientation and quality of the insert was confirmed by sequencing. COS-7 cells (derived from

Table 2 Primers used for gene expression studies

Gene	Sequence	Amplicon length (bp)	Genbank acc. no.
GR1	FW: 5′-AGA-CTG-AGA-GGC-GGA-GCT-ACT-G-3′ RV: 5′-GGC-GGT-GTT-GGC-TCC-AT-3′	113	AJ879149
GR2	FW: 5′-GGA-GAA-CAA-CGG-TGG-GAC-TAA-AT-3′ RV: 5′-GGC-TGG-TCC-CGA-TTA-GGA-A-3′	110	AM183668
MR	FW: 5′-TTC-CCT-GCA-GAA-CTC-AAA-GGA-3′ RV: 5′-ACG-GAC-GGT-GAC-AGA-AAC-G-3′	117	AJ783704
PRL	FW: 5′-CAT-CAA-TGG-TGT-CGG-TCT-GA-3′ RV: 5′-TGA-AGA-GAG-GAA-GTG-TGG-CA-3′	130	X52881
β-actin	FW: 5′-GCT-ATG-TGG-CTC-TTG-ACT-TCG-A-3′ RV: 5′-CCG-TCA-GGC-AGC-TCA-TAG-CT-3′	89	M24113
40S	FW: 5′-CCG-TGG-GTG-ACA-TCG-TTA-CA-3′ RV: 5′-TCA-GGA-CAT-TGA-ACC-TCA-CTG-TCT-3′	69	AB012087

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African green monkey kidney) were cultured as described previously (Sturm *et al.* 2005). Cells were transiently transfected using calcium precipitation method (Sambrook & Russell 2001), with the cells growing in log phase at 30–50% confluence. Cells were co-transfected with the following plasmids: expression vector with the appropriate hormone receptor cDNA (1 μg/24-well plate), reporter plasmid pFC31Luc that contains the mouse mammary tumour virus promoter upstream of the luciferase gene (10 μg/24-well plate); pSVβ (Clontech), a second reporter plasmid under control of the SV40 promoter and serving as a control for the transfection efficiency (2 μg/24-well plate); and finally pBluescript (Clontech) (7 μg/24-well plate), an irrelevant plasmid to increase transfection. Sixteen hours after transfection, medium was renewed and cortisol added from 1000-fold concentrated stock solution in ethanol. After 36-h incubation, cells were harvested using reporter lysis buffer (Promega) following the manufacturer's instructions; luciferase and β-galactosidase activities were determined as described previously (Bury *et al.* 2003). In addition to solvent controls (receiving only ethanol carrier instead of hormone), cells were transfected with an empty expression vector as control for luciferase activity in the absence of hormone receptor DNA. Experiments were repeated thrice independently, with triplicate cell cultures per treatment. Luciferase activity was corrected for 'well-specific' transfection efficiency (determined by β-galactosidase activity) and then expressed as percentage luciferase activity observed in cells treated with 10^{-7} M cortisol.

Half maximum activation concentration of ligand (EC_{50}) in the transactivation assay were assessed by fitting the data to a single ligand binding model using the Sigma plot^{R} software. Only converging data were included in data sets presented. Ligands were tested in the range of 10 pM to 1 μM. Data were normalised to maximum (100%) response and corrected for blanks prior to kinetic analysis.

Bioinformatics

Sequences were retrieved from the Swissprot, EMBL and GenBank databases using SRS and/or BLAST (Altschul *et al.* 1997). Multiple sequence alignments were carried out using CLUSTALW (Chenna *et al.* 2003). Calculation of pairwise amino acid identities was carried out using the SIM ALIGNMENT tool (Huang & Miller 1991). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al.* 2004). Phylogenetic tree was constructed based on the neighbour-joining method using the Poisson correction for evolutionary distance (Nei & Kumar 2000). Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

Statistical analysis

Statistic analysis was performed with SPSS 12.0.1 software (SPSS Inc., Chicago, IL, USA). Following ANOVA, differences between treatments were assessed by Mann–Witney *U* test, and $P<0{\cdot}05$ was accepted as fiducial limit. For RQ-PCR data, tests were performed for both internal reference genes (β-actin and 40S) and statistical significance is reported only if both reference genes showed a significant effect, where *indicates $P<0{\cdot}05$ and **indicates $P<0{\cdot}01$.

Results

CR characterisation

Cloning and characterisation of the MR and GR genes Full-length sequences of one MR and two different GR genes were obtained by homology cloning using a common carp brain cDNA library. The first GR gene (GR1; acc. no. AJ 879149) comprises 2190 nucleotides and encodes a protein of 730 amino acids. The second gene (GR2; acc. no.

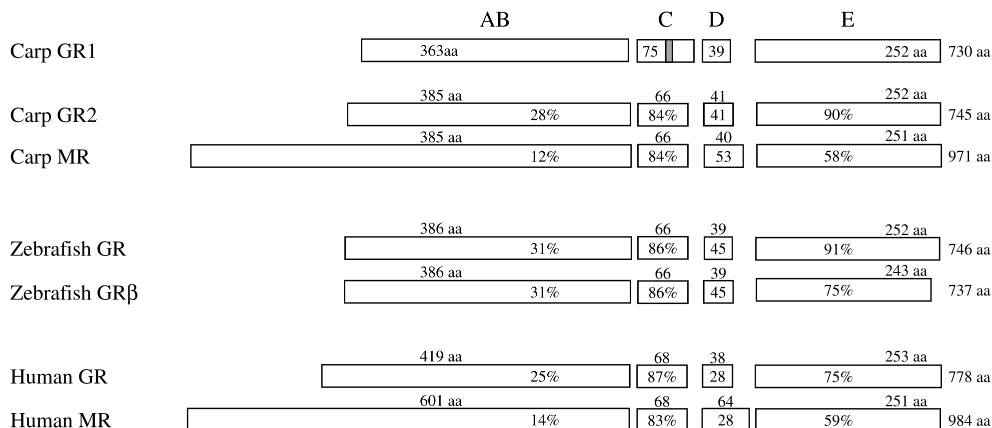

Figure 2 Similarities between the receptor domains (AB, transactivation region; C, DNA-binding region; D, hinge region; E, ligand binding region) of the common carp and other vertebrate corticosteroid receptors. Percentage of amino acid identity of the different domains is shown in the boxes. Amino acid length of particular domains is represented by the length of boxes and is also mentioned. Total length of each protein is shown at the right. Grey bar indicates nine amino acid insert as a result of alternative splicing.

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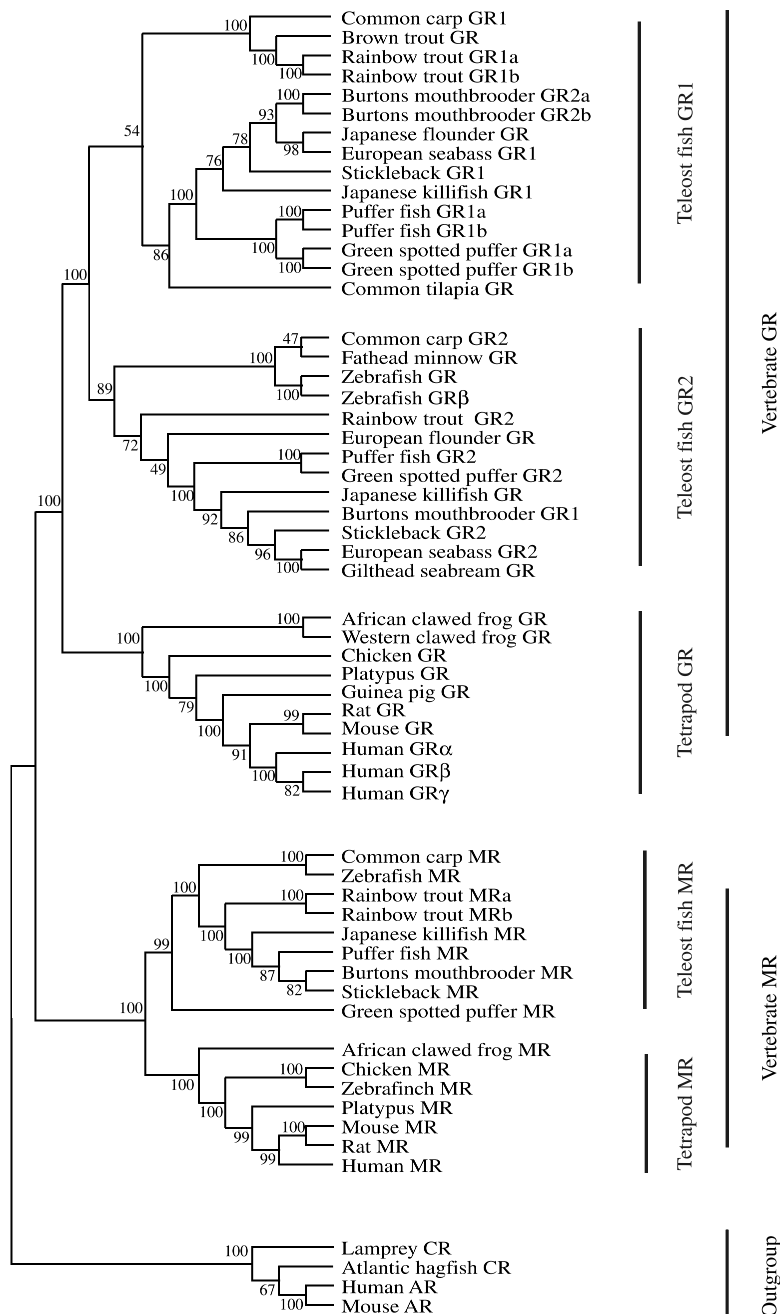
AM183668) contains an open reading frame of 2235 nucleotides and encodes a protein of 745 amino acids (Fig. 1). The predicted amino acid identity of these two GRs is 57%; both gene products show moderate sequence (45–60%) identity when compared with other teleostean fish and mammalian GR genes (Fig. 2). The MR gene (acc. No. AJ783704) has an open reading frame of 2913 nucleotides that codes for a 971 amino acids protein. The predicted MR amino acid sequence shows relatively high (65–90%) sequence identity to the other teleostean fish MR sequences and moderate (∼50%) sequence identity to the African clawed frog (*Xenopus laevis*) and mammalian MR sequences.

When the protein domains of the receptors (GRs and MR) are compared among different species, 85–100% sequence identity is found for the DNA-binding domain. For the ligand-binding domain (LDB), 50–60% sequence identity is



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found when GRs are compared with MRs between species, and 70–90% when LDBs of either GRs or MR of individual species are compared (Fig. 2). The N-terminal domains of GRs and MRs constitute the most variable region. Alignment of carp GR and MR genes yields low sequence conservation (28% for GR1 compared with GR2 and <15% for MR compared with either of the GRs; Fig. 1).

Phylogenetic analysis The neighbour-joining phylogenetic tree for corticosteroid receptor proteins (Fig. 3) resulted in a predicted cluster of GRs and MRs on separate branches together with mammalian orthologues; androgen and corticosteroid receptors from jawless fish represent an out-group. Within both the MR and GR branch, teleostean and tetrapod proteins form separate clades. The teleostean GR clade has a subdivision as a result of duplication of the GR gene.

Expression of mRNAs for corticosteroid receptors Expression of GR1, GR2 and MR genes in the brain (without hypothalamus and pituitary gland), ventral hypothalamus, pituitary gland of healthy and non-stressed carp was quantitated by real-time PCR shown in Fig. 4. The highest GR expression was found in the brain and hypothalamus. Expression of GR1 and GR2 genes was generally comparable; in the brain, however, GR1 expression was higher than GR2 expression. Receptor expression abundance in pituitary tissue was about half that was seen in the brain or hypothalamus. The MR gene also showed an about 50% lower expression level in pituitary tissue compared with the brain and hypothalamus (Fig. 4a).

To discriminate gene expression levels in the pars distalis (pro-opiomelanocortin, POMC cells producing ACTH) and pars intermedia (POMC cells producing melanocyte-stimulating hormone (MSH)), we dissected pituitary glands and confirmed tissue separation by assay of prolactin mRNA expression, a marker for the rostral pars distalis (RPD, insert Fig. 4b). GR1 and GR2 mRNA expression was significantly ($P<0{\cdot}01$) higher in pituitary pars distalis compared with pars intermedia. No such difference was observed for MR mRNA (Fig. 4b).

CR localisation The localisation of mRNA expression was further studied by *in situ* hybridisation. In the telencephalon, mRNA expression of all three receptors was detected, predominantly in the outer pallial layers. In other brain areas, GR1 and GR2 show the same distribution pattern, although relative expression levels were different, whereas MR mRNA showed a less wide distribution. In transverse sections of the hypothalamus, mRNA expression of all the three receptors was observed in the magnocellular part of the NPO (as assessed by comparison with paramedian sagittal slides (Huising *et al.* 2004)); expression was less pronounced in the parvocellular part (Fig. 5). In the pituitary pars intermedia, GR mRNA expression was low. In the pars distalis, strongest GR expression was found in the proximal pars distalis, in GH-producing cells. In the rostral pars distalis, ACTH cells express both corticosteroid receptors GR1 and GR2 and to a far higher degree than the prolactin cells (Fig. 6).

Transactivation activity GR2 was more sensitive to the different hormones tested than GR1. For both receptors, dexamethasone was the strongest agonist tested, followed by cortisol, deoxycortisol and corticosterone. Finally, aldosterone and DOC were very weak agonists. The physiologically important stress hormone cortisol was chosen as ligand to

Figure 3 Phylogenetic tree comparing the amino acid sequences of the vertebrate corticosteroid receptors. This tree was generated with MEGA version 3.1 software using the neighbour-joining method. Reliability of this tree was assessed by bootstrapping using 1000 bootstrap replications; values in percentage are indicated at branch nodes. Atlantic hagfish and sea lamprey corticosteroid receptors (CR) and human and rat androgen receptors (AR) were used as out-group. Common carp (*C. carpio*) GR1; AJ879149, GR2; AM183668, Rainbow trout (*Oncorhynchus mykiss*) GR1; P49843, GR2; AY4953720, Burton's mouthbrooder (*Haplochromis burtoni*) GR1; AF263738, GR2a; AF263739, GR2b; AF263740, Zebrafish (*Danio rerio*) GR2; EF436284, GR2β; EF436285 Japanese flounder (*Paralichthys olivaceus*) GR; (O73673), European sea bass (*Dicentrarchus labrax*) GR1; AY549305, GR2; AY619996, Brown trout GR (*Salmo trutta*); AY863149, Fathead minnow (*Pimephales promelas*) GR; AY533141, Puffer fish (Fugu) (*Takifugu rubripes*) GR1; GENSCAN00000003615 (scaffold 1264) & GENSCAN00000029451 (scaffold 4328), GR2; SINFRUG00000143550 (scaffold 59), Green spotted puffer (Tetraodon) (*Tetraodon nigroviridis*) GR1; GIDT00024792001 (Chr. 7), GR2; GSTENG00017027001 (Chr. 1) Stickleback (*Gasterosteus aculeatus*) GR1; ENSGACP00000027400, GR2; ENSGACP00000024074, Japanese Killifish (Medaka) (*Oryzias latipes*) GR1; ENSORLP00000001939, GR2; ENSORLP00000007570, Mozambique or common tilapia (*Oreochromis mossambicus*) GR; BAA23662, African clawed frog (*Xenopus laevis*) GR; P49844, Western clawed frog (*X. tropicalis*) GR; CR848477, Chicken (*Gallus gallus*) GR; ENSGALP00000011948 (Q8JHA4 partial), Platypus (*Ornithorhynchus anatinus*) GR; ENSOANP00000009152, Pig (*Sus scrofa*) GR; AY779185, Cow (*Bos taurus*) GR; AY238475, Guinea pig (*Cavia porcellus*) GR; P49115, Mouse (*Mus musculus*) GR; P06537, Rat (*Rattus norvegicus*) GR; NP_036708, Human (*Homo sapiens*) GRα; P04150, Human GRβ; NP_001018661, Human GRγ; NP_001019265, Carp (*C. carpio*) MR; AJ783704, Zebrafish (*D. rerio*) MR; ENSDARP00000053817, Rainbow trout (*O. mykiss*) MRa; Y495584, MRb; AY495585, Burton's mouthbrooder (*H. burtoni*) MR; Q8JJ89, Puffer fish (Fugu) (*T. rubripes*) MR; NEWSINFRUP00000129848, Green spotted puffer (Tetraodon) (*T. nigroviridis*) MR; GSTENT00032894001, Stickleback (*G. aculeatus*)*MR;*ENSGACP00000022713, Japanese killifish (Medaka, *O. latipes*) MR; ENSORLT00000009439, Chicken (*G. gallus*) MR;ENSGALP00000016283 (Q8QHI2, partial), *Platypus* (*O. anatinus*) MR; ENSOANT00000008378, African clawed frog (*X. laevis*) MR; BC081082, Mouse MR; XP_356093, Rainbow trout MR; AY495584, Human MR; M16801, Sea lamprey (*Petromyzon marinus*) CR; AY028457, Atlantic hagfish (*M. glutinosa*) CR; DQ382336 Mouse (*M. musculus*) AR; M37890 Human (*H. sapiens*) AR; P10275. NB: Burton's mouthbrooder nomenclature is different; GR1 has no splice variants (comparable with common carp and rainbow trout GR2) and GR2 has two splice variants (comparable with rainbow trout GR1); Burton's mouthbrooder GR2b has a nine amino acid insert.

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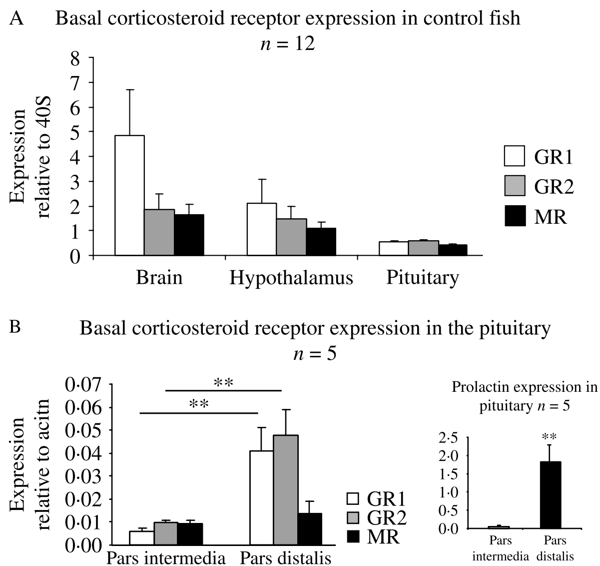

Figure 4 Basal corticosteroid receptor expression in stress axis of control fish (A). Comparison of quantitative real-time PCR data was based on samples of four untreated fish and controls from 24-h netting experiment ($n=4$) and controls of the cold water transfer experiment ($n=4$). Data are plotted as average of the mean of each of the three experiments relative to the housekeeping gene 40S; error bars indicate standard error of the means of these three experiments. NB: Gene expression data relative to housekeeping gene β-actin ($n=8$) showed the same pattern (not shown). Basal corticosteroid receptor expression in different sections of the pituitary gland ($n=5$) (B). Insert shows prolactin mRNA expression in different sections of pituitary.

compare sensitivity between the different receptors in a transactivation assay. Carp GR1 was less sensitive than the GR2, both to cortisol (EC_{50} 7·1±2·9 nM and 2·4±0·4 nM for GR1 and GR2 respectively) and dexamethasone (EC_{50} 2·4±3·8 nM and 0·7±1·4 nM for GR1 and GR2 respectively) (Table 3). The carp MR has intermediate sensitivity for cortisol (EC_{50} 4·1±2·0 nM) when compared with the GRs. The sensitivity of the MR to aldosterone and DOC was comparable and approximately tenfold higher than to cortisol.

Physiology

Corticosteroid receptor expression and stress To study the corticosteroid receptor expression after stress, we used two different stress paradigms. Restraint of carp for 24 h resulted in significantly elevated plasma cortisol and plasma glucose values as assessed upon completion of the treatment. Plasma sodium concentrations had significantly decreased in stressed animals, indicative of stress-related loss of integumental permeability to water and ions (Wendelaar Bonga 1997, Metz *et al.* 2003). Corticosteroid receptor expression in any of the tissues tested was not affected by this restraint. When fish were repeatedly subjected to temperature drops, they showed strongly elevated plasma cortisol levels and a down-regulation of mRNA expression of the GRs and MR in the brain; in hypothalamus and pituitary gland, no statistically significant changes were seen (Fig. 7).

Discussion

In vertebrates, corticosteroids are deeply involved in general metabolism, stress adaptation, reproduction, osmoregulation, growth and bone formation. The phenomenal pleiotropy of corticosteroids would suggest radiation of multiple isoforms and splice variants for their receptors during evolution. Indeed, research on mammals has focused on isoform and splice variant incidence of cortisol receptors (GR receptors, GRs) and how these variants translate for instance into regulation of the stress

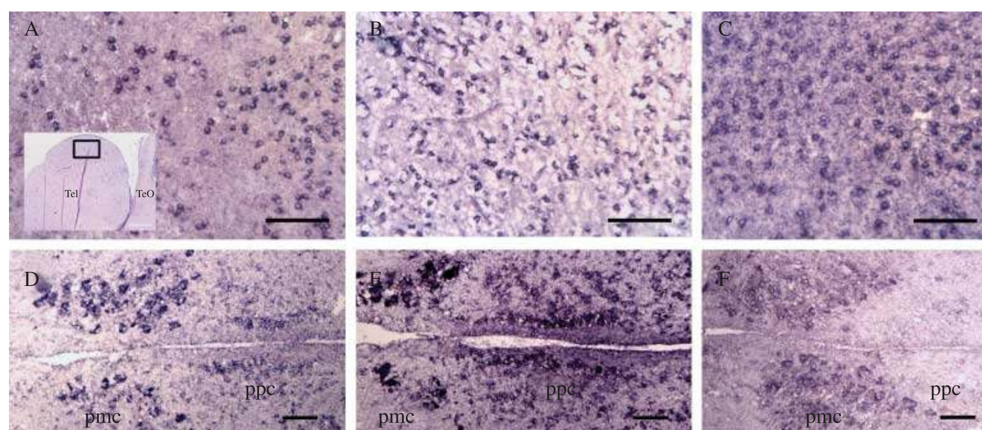

Figure 5 Localisation of GR1, GR2 and MR expression by ISH in cells of the telencephalon and the NPO. (A–C) telencephalon; (A) insert, overview of the telencephalon with haematoxylin and eosin staining. Anti-sense probe for GR1 (A), GR2 (B) and MR (C). (D–F) NPO, nucleus pre-opticus of hypothalamus; anti-sense probe for GR1 (D), GR2 (E) and MR (F). GR1 with: Tel, telencephalon; TeO, tectum opticum; pmc, nucleus pre-opticus magnocellularis; ppc, nucleus pre-opticus parvocellularis. Scale bars indicate 500 μm (A insert) or 100 μm (B–F).

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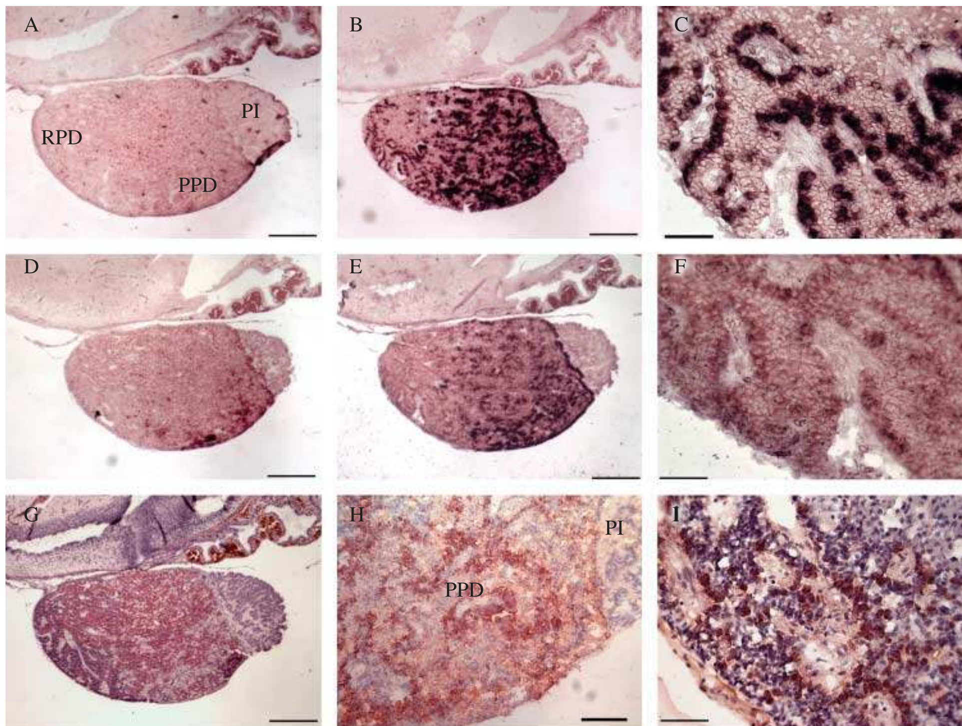

Figure 6 Localisation of GR1 and GR2 expression in the cells of pituitary. (A) Sense control for GR1, (B) anti-sense probe for GR1, and (C) detail of rostral pars distalis (RPD) with anti-sense probe for GR1. (D) Sense control for GR2, (E) anti-sense probe for GR2 and (F) detail of RPD with antisense probe for GR2. (G) Overview of pituitary stained with haematoxylin and eosin, (H) detail of proximal pars distalis (PPD) with antibody against GH, and (I) detail of RPD with antibody against ACTH. PI, pars intermedia. Scale bars indicate 500 μm (A, B, D, E and G), 50 μm (H), 100 μm (C, F and I).

axis. The extant teleostean fishes are representatives of the earliest true vertebrates and exhibit a complex receptor profile. With two genes encoding functionally different GRs, this system is even more complex than that observed in mammals, which warranted investigation into the role of these different receptors in stress axis regulation.

CR characterisation

Receptor evolution in fishes Different GR genes were demonstrated in distantly related teleostean species (Bury *et al.* 2003, Greenwood *et al.* 2003) and for that very reason not necessarily result from the tetraploidisation of common carp (Greenwood *et al.* 2003, Stolte *et al.* 2006). In the green-spotted puffer, two duplicates on different chromosomes are found that makes a single gene duplication less likely (Stolte *et al.* 2006). Moreover, all known teleostean GR1 proteins share a conserved insert of nine amino acids (WRARQNTDG, or Wrarqnadg in carp) in the DNA-binding domain which are not found in other vertebrates. We rate it highly unlikely that all teleosts independently duplicated a single gene and inserted every time again a nine amino acid sequence. Most convincing for this debate is that our phylogenetic analysis yields two distinct clades of GR genes in the teleostean lineage that argues against

Table 3 Transactivation capacity of corticosteroid receptors[a]

	Cortisol		Dexamethasone		Aldosterone		DOC	
	EC_{50} (nM)	S.E.M.	EC_{50} (nM)	S.D.	EC_{50} (nM)	S.D.	EC_{50} (nM)	S.D.
GR1	7·1	2·9	2·4	3·8				
GR2	2·4*	0·4	0·7	1·4				
MR	4·1	2·0			0·46	4·1	0·25	3·6

*GR2 is significantly more sensitive to cortisol than GR1 ($P<0·05$).
[a]EC_{50} values of GR1, GR2 and MR for different hormones. Cortisol for GRs; average of three separate experiments with standard error of the mean; cortisol for MR, dexamethasone, aldosterone and DOC; single experiments. DOC, 11-Deoxycorticosterone.

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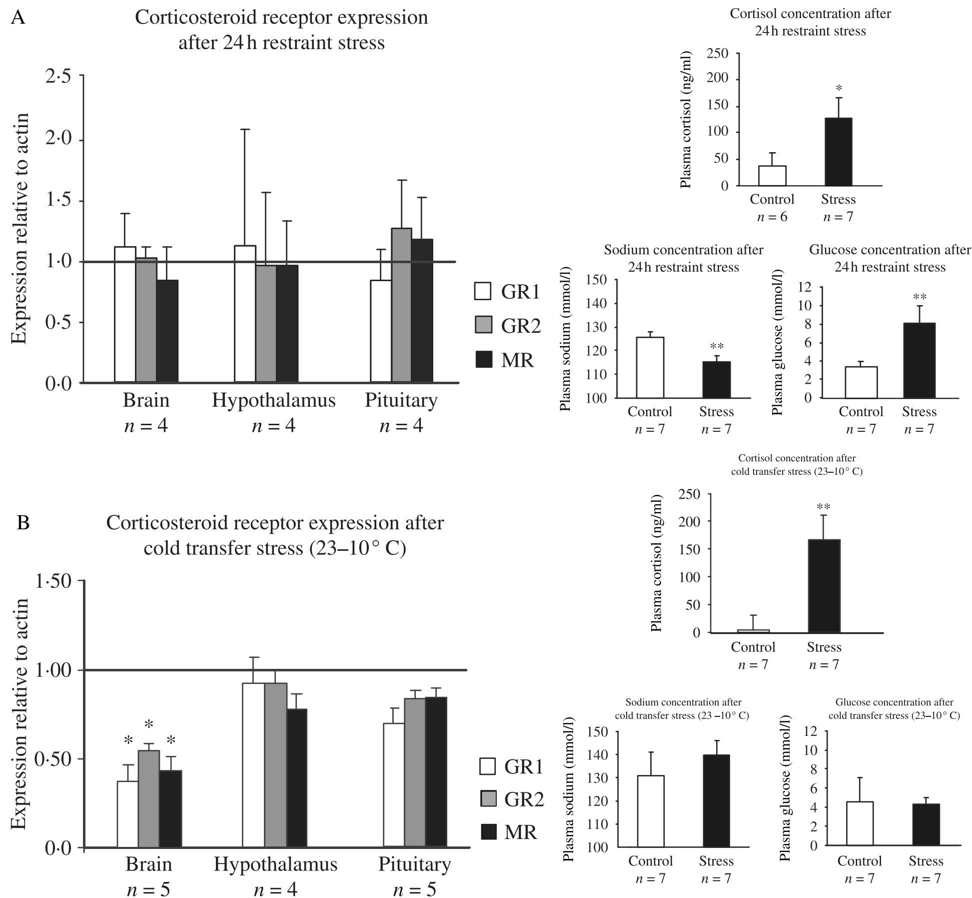

Figure 7 Corticosteroid receptor expression in stress exposed fish. Gene expression of corticosteroid receptors in stress axis organs after 24-h restraint stress (A) and cold transfer (23 °C to 10 °C) stress (B). Inserts of plasma cortisol, glucose and sodium levels are shown for control and stressed animals for the respective experiments. Gene expression is shown in comparison with unstressed control fish of the respective experiment and relative to β-actin.

duplication in the tetrapod lineage. The duplication in all likelihood results from an early genome duplication 300–450 million years ago, and only after the divergence of the tetrapods from the fish lineage (Volff 2005). If we proceed from a notion of a major genomic duplication in fishes, it follows that one MR has apparently been lost during evolution as we were not able to detect a second MR-coding gene in carp or in genomic databases for zebrafish (*Danio rerio*), puffers (*Fugu* species) or rice fish (*Oryzias latipes*). Three possibilities arise after gene/genome duplication: non-functionalisation, the fate of most duplicated genes (Brunet *et al.* 2006); neo-functionalisation, the acquirement of a new function; or sub-functionalisation, where each copy loses part of the ancestral function and both copies are required to maintain the full function (Force *et al.* 1999). The duplicated GR genes of fish escaped a fate as non-functional pseudogene: expression levels and differential sensitivities for cortisol are more, so in-line with neo- or sub-functionalisation (Bury *et al.* 2003, Greenwood *et al.* 2003). Interestingly, zebrafish has only one GR copy that clusters with other fish GR2 sequences. However, zebrafish has acquired a splicing β-isoform of the GR (Fig. 2) that resembles the dominant negative GRβ of humans in structure, expression level and function. This could reflect an alternative regulatory mechanism to compensate for the loss of a functional second GR gene (Schaaf *et al.* 2008).

Receptor functional definition by transactivation capacity In carp, transactivation capacity of cortisol (capacity of hormone to initiate or repress CR-mediated transcription of downstream genes) is about three-fold higher for GR2 (EC_{50} 2·4±0·4 nM) than for GR1 (EC_{50} 7·1±2·9), and this would facilitate differential regulation by basal and elevated cortisol levels. The carp MR sensitivity (EC_{50} 4·1±2·0 nM) is intermediate to that of the GRs, and this is in stark contrast to data for Burton's mouthbrooder and trout, with MRs more sensitive to cortisol than the GRs (Greenwood *et al.* 2003, Sturm *et al.* 2005). In fish, levels of up to 10 nM DOC were published (Campbell *et al.* 1980). The low EC_{50} of DOC (0·25 nM) for MR transactivation in carp certainly does not

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exclude a mineralocorticoid function for DOC in fishes. In carp plasma basal, total levels of cortisol are around 5 ng/ml (i.e. 13·8 nM); following stress, cortisol levels easily reach 150 ng/ml (>400 nM). As only 20% is available as unbound cortisol (Flik & Perry 1989), this corresponds to 2·8 and > 80 nM respectively, for which in carp apparently specific receptor subtypes are present: at rest both GR2 and MR may be occupied and activated, whereas GR1 is preferentially activated by stress levels of cortisol. In rodent brain, a similar system was demonstrated: an 80% MR and 10% GR corticosterone occupancy was established in non-stressed situations (Reul *et al.* 1987). Even though the majority of MRs is occupied at low cortisol levels, it still is a dynamically regulated receptor. MR activity could be increased by an agonist to further inhibit HPA axis activity (Buckley *et al.* 2007). We assume the same applies for common carp GR2 and MR. This means that although both receptors are partly or even largely occupied with cortisol, continuous modulation of HPI axis activity could be mediated by increasing cortisol levels after stressful events via GR1.

Receptor functional definition by localisation As we focus on the involvement of the different receptors in stress axis regulation, our areas of interest are the HPI axis organs. Hypothalamus and pituitary gland of unstressed carp showed comparable mRNA levels for both the GRs and MR, suggesting functional importance of all the three. Only in the brain (without hypothalamus and pituitary gland), a consistent two-fold higher mRNA expression for GR1 over GR2 was seen. A receptor-defined duality in GR function in the brain of fishes seems of wider occurrence as similar preferential expression was seen in the brain of rainbow trout and Burtons' mouthbrooder (Bury *et al.* 2003, Greenwood *et al.* 2003). The higher mRNA expression level could translate into higher protein levels but may also reflect higher turnover. We have no data on brain cortisol levels but two receptors with significant difference in receptor sensitivity would allow differential responses to basal and stress levels of the steroid. In carp and trout (Sturm *et al.* 2005), the brain shows strong MR mRNA expression; unexpectedly, typical osmoregulatory organs such as gills, kidney and intestine show far lower MR mRNA expression. This suggests that a widely accepted but only presumed hypothesis of MR involvement in osmoregulation may be wrong, and is a consequence of extrapolation of the situation for aldosterone and MR function in mammals. However, also in the mammalian brain, MRs play a key role in corticosteroid-regulated processes (De Kloet *et al.* 1998). The mammalian hippocampus exerts inhibitory control over the HPA axis activity: a dominant inhibition by MR activity is attenuated by GR activation (De Kloet *et al.* 1998). Our results support such a combined function for steroids mediated by GR and MR activities in the regulation of stress coping and learning, and this is apparently a very early function developed in our ancestral fishes.

Steroid receptors distribution in telencephalic regions may contribute to define hippocampal regions, especially involved in (learning) behaviour and sentience. Our demonstration of GR and MR mRNA in carp telencephalon expands and further substantiates the notion that GR and MR corticosteroid receptors have the basic functions in the regulation of learning and memory. In goldfish (Vargas *et al.* 2006) and cod (Nilsson *et al.* 2008) learning and spatial memory may involve the telencephalic lateral pallium, the homologue of the hippocampus of mammals (Meek & Nieuwenhuys 1998).

In the hypothalamus, mRNA of all the three receptor genes is expressed in parvo- and magnocellular neurons of the pre-optic nucleus. We confirmed (data not shown) by immunohistochemistry on adjacent sections that expression of GR- and MR-mRNA co-localises with CRH (Huising *et al.* 2004). So far, the presence of GR protein in these cells was shown for rainbow trout (Teitsma *et al.* 1998) and Mozambique tilapia (*Oreochromis mossambicus*) (Pepels *et al.* 2004). However, the antiserum used in these studies does not distinguish between GR gene species. To the best of our knowledge, no data on MR distribution in other fish brains are available. In mammals, the MR was described in the ventromedian and arcuate nuclei of the hypothalamus, and the GR in the arcuate nuclei, paraventricular and supra-optic nucleus of the hypothalamus (Reul & de Kloet 1985); the hypothalamic arcuate nucleus is an important target for metabolic and hormonal signals controlling food intake and feeding behaviour (Meister 2007). Clearly, future studies on GR and MR localisation in the fish analogues of these structures are warranted as we know that regulation of stress on feeding (arcuate nucleus in fish) are strongly intertwined, also in fish (Bernier *et al.* 2004).

In carp pituitary gland, the two GR genes co-localise in the same areas. Remarkably, little expression was seen in pars intermedia and would imply limited feedback by cortisol on MSH- and somatolactin-producing cells. This reminds of the situation reported for trout (Teitsma *et al.* 1998), but not for tilapia that has significant GR-immunoreactivity in the pars intermedia (Pepels *et al.* 2004), although mRNA expression could not be detected (Kitahashi *et al.* 2007). The strongest signal for GR mRNA was seen in the proximal pars distalis GH cells (in line with observations for GR1 on trout (Teitsma *et al.* 1998) and tilapia (Pepels *et al.* 2004, Kitahashi *et al.* 2007)). Stress/cortisol induced suppression of growth, for instance seen after handling or confinement of fishes is thus explained by down-regulation of GH cells (Auperin *et al.* 1997). In the rostral pars distalis finally, ACTH cells express significant levels of GR mRNA, as one could predict as a basis for feedback control in the stress axis. Interestingly, recent data in tilapia showed no mRNA expression of either of the duplicated GRs in ACTH-producing cells (Kitahashi *et al.* 2007), whereas immuno-histochemical studies with an antibody against GR showed positivity in ACTH cells in tilapia and rainbow trout (Teitsma *et al.* 1998, Pepels *et al.* 2004). Prolactin cells, however, known to become activated during chronic stress (Auperin *et al.* 1997), showed no detectable GR expression (Teitsma *et al.* 1998). This only seems at variance with recent reports on direct effects of cortisol on prolactin cells (Uchida *et al.* 2004): a G-protein-coupled membrane receptor for cortisol should be considered. Indeed, the reported effects of

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cortisol on prolactin cells reported are fast, faster than one would predict for GR/MR-mediated genomic effects.

MR mRNA was far more abundant in the pars distalis than in the pars intermedia. Based on the comparisons with GR staining, we predict the MR expression to be scarcely expressed in the α-MSH cells of the pars intermedia and strongly expressed in the GH cells of the pars distalis. Unfortunately, we were unable to demonstrate MR mRNA in ACTH cells specifically. Assuming (by analogy to the situation for GR mRNA) absence of MR mRNA in prolactin cells, our RQ-PCR data would favour the presence of MR in ACTH cells; this part of our study requires future attention.

Physiology

Restraint by netting for 24 h resulted in hyperglycaemia, hyponatraemia, typical signs of strong catecholaminergic activation and inherent loss of control over integumental permeability to water and ions (Wendelaar Bonga 1997). A persistent hypercortisolinaemia was observed, but the duration of the stress condition proved insufficient to induce feedback by cortisol. However, a more persistent stress of repeated cold transfer induced high cortisol levels and hyperglycaemia and mild hypernatraemia indicative of adoption to the stressor. Mild hypernatraemia is typical for enhanced prolactin activity and successful restoration of blood mineral levels to a new set point (Metz *et al.* 2003). This longer term adaptation process did involve measurable down-regulation of CRs in the brain (without hypothalamus and pituitary gland). This observation suggests a central initiation of down-regulation of cortisol release, rather than direct feedback via NPO or pituitary gland. In tilapia, a similar regulation system was suggested; cortisol feedback on CRH release is exerted via the forebrain medial part of dorsal telencephalon (Pepels *et al.* 2004). Our observation of GR and MR mRNA down-regulation only following the persistent stress is further consistent with experiments on Coho salmon (*Oncorhynchus kisutch*) that showed no effect of acute stress on GR expression, whereas chronically elevated cortisol levels in several other paradigms did (Maule & Schreck 1991).

The MR mRNA in carp brain was down-regulated to the same extent as the mRNA levels of both GRs. This seems in contrast with the mammalian model where GR stimulates HPA axis activity, whereas MR has an inhibiting effect (De Kloet *et al.* 1998). However, a similar result was found in hippocampus of rats exposed to increasing the corticosteroid levels (Hugin-Flores *et al.* 2004). And in mouse pups with high corticosteroid levels due to 24-h maternal deprivation, both GR and MR mRNA expression were significantly decreased (Schmidt *et al.* 2003). These discrepancies between different stressors are thus of wider occurrence and require further and species specific approaches.

In conclusion, we show that carp express separate GRs for regulation under basal and stressful conditions. We predict an ancestral role not only for the duplicated GRs but also for MR in stress physiology, based on the receptor localisation and expression profile after chronic stress and provide a receptor profile in the brain conform memory and learning functionalities in fishes as in higher vertebrates. A role for DOC in stress physiology in fishes has never been considered but seems a reasonable hypothesis for future research. More in-depth studies on specific roles of these receptors in learning and stress physiology would therefore elucidate early strategies in vertebrate evolution to control the basics of life.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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