Localization, expression and control of adrenocorticotropic hormone in the nucleus preopticus and pituitary gland of common carp (*Cyprinus carpio* L.)

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Abstract

Adrenocorticotropic hormone (ACTH) takes a central role in the hypothalamo-pituitary-interrenal axis (HPI axis), which is activated during stress. ACTH is produced by the corticotrope cells of the pituitary pars distalis (PD) and is under control of factors from the nucleus preopticus (NPO). The distribution of ACTH in the hypothalamo-pituitary system in common carp (*Cyprinus carpio* L.) was assessed by immunohistochemistry. ACTH and β-endorphin immunoreactivity was observed in the ACTH cells in the PD and in the NPO. Nerve fibers, originating from the NPO and projecting to the pituitary gland, contain β-endorphin, but not ACTH, and these fibers either control the pituitary pars intermedia (PI) through β-endorphin or release it to the blood. The release of pituitary ACTH (studied in a superfusion setup) must *in vivo* be under predominant inhibitory control of dopamine. Release of ACTH is stimulated by corticotropin-releasing hormone, but only when ACTH cells experience dopaminergic inhibition. The expression of the precursor pro-opiomelanocortin in (POMC) NPO, PD and PI was studied in an acute restraint stress paradigm by real-time quantitative polymerase chain reaction (RQ-PCR). POMC gene expression is upregulated in these three key tissues of the hypothalamo-pituitary complex, revealing a hitherto unforeseen complex role for POMC-derived peptides in the regulation of responses to stress.

Journal of Endocrinology (2004) **182,** 23–31

Introduction

Adrenocorticotropic hormone (ACTH) is a 39-amino-acid peptide hormone derived from the hormone precursor pro-opiomelanocortin (POMC). ACTH is predominantly produced in the corticotrope cells of the pituitary pars distalis (PD). In the melanotrope cells of the pituitary pars intermedia (PI), ACTH is immediately processed to α-melanocyte-stimulating hormone (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP). POMC further gives rise to β-MSH and β-endorphin (for review, see Castro & Morrison 1997).

Pituitary ACTH takes a central role in the vertebrate stress response. When a fish meets stressful conditions, its hypothalamo-pituitary-interrenal axis (HPI axis) is activated; this axis is the teleostean equivalent of the hypothalamo-pituitary-adrenal axis of mammals. Neurons in the nucleus preopticus (NPO) of the hypothalamus, the homolog of the paraventricular nucleus of mammals, are activated to release corticotropin-releasing hormone (CRH) in the vicinity of the corticotrope cells of the PD. Consequently, the corticotrope cells secrete ACTH, the principal stimulator of cortisol release from the interrenal cells. Cortisol is responsible for the redistribution of energy flows, required to cope with the stressor (Wendelaar Bonga 1997).

Production and release of ACTH from the pituitary gland is under multiple control of hypothalamic factors. In contrast to mammals, teleost fish do not have a median eminence. ACTH cells are directly innervated by neurons from the NPO (Jorgensen & Larsen 1967, Fellmann *et al.* 1984). CRH is considered to be the dominant stimulatory factor and key in ACTH release during acute stress responses (Rotllant *et al.* 2000, Van Enckevort *et al.* 2000). Arginine vasotocin (AVT), colocalized with CRH in the NPO (Yulis & Lederis 1987, Huising *et al.* 2004), is believed to synergize CRH-driven ACTH release in trout (Baker *et al.* 1996), but not in goldfish (Fryer *et al.* 1985*a*). Release of ACTH is under inhibitory control by agents such as dopamine

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(Olivereau *et al.* 1988) and melanin-concentrating hormone (MCH) (Baker *et al.* 1986).

POMC-derived peptides have been demonstrated in several other tissues, such as gonads, liver, headkidney (Varsamos *et al.* 2003), thymus (Ottaviani *et al.* 1997) and several areas in the brain (Vallarino *et al.* 1989, Olivereau & Olivereau 1990, 1991). The function of ACTH in the fish brain is poorly understood and not necessarily linked to stress axis activity. In mammals, centrally produced ACTH is involved in learning and memory processes, analgesia and thermoregulation (Tatro, 1990).

In the present study, we addressed the distribution of ACTH in the nucleus preopticus and pituitary gland by immunohistochemistry. We raised a highly specific antiserum against carp ACTH, directed against the α-MSH-CLIP transition of ACTH (^{10}Gly-^{23}Tyr). We developed a radioimmunoassay with a commercially available antibody against human ACTH[1–24] that enabled us to quantify ACTH in carp NPO and pituitary homogenates as well as ACTH released in culture medium. The control of ACTH release by CRH and dopamine was studied in a superfusion setup (e.g. Van den Burg *et al.* 2003). The expression of POMC in the NPO and pituitary PD and PI was assessed with real-time quantitative polymerase chain reaction (RQ-PCR) in an acute restraint stress paradigm.

Materials and Methods

Animals

Male isogenic carp, *Cyprinus carpio* L., strain E4xR3R8 (Bongers *et al.* 1998), were obtained from the Wageningen University fish culture facility (De Haar Vissen, The Netherlands). Fish weighed around 100 g and were kept in 150 l tanks at 22 °C, with recirculating 80% filtered tap water, under 16 h of light alternated with 8 h of darkness. Fish were fed commercial fish food (Trouvit, Trouw, Putten, The Netherlands) at a ration of 1·5% of the estimated body weight per day. Experimental protocols were according to Dutch legislation and approved by the ethical committee of the University of Nijmegen.

Sampling

Fish were anesthetized in 0·1% (v/v) 2-phenoxyethanol (Sigma). Within 2 min, blood was taken by puncture from the caudal vessels, using Na_2EDTA as anticoagulant. Blood was transferred to ice-cold tubes containing 1 trypsin-inhibiting unit (TIU) aprotinin (Sigma) to prevent proteolysis. After 5-min centrifugation (5000 ***g***, 4 °C), plasma was separated from blood cells and stored at − 20 °C until analysis.

Directly after blood collection, fish were placed on ice. The pituitary gland and brain were removed; the hypothalamus containing the NPO was separated from the brain. Tissues were immediately frozen in liquid nitrogen and stored at − 80 °C until RNA isolation.

For ACTH radioimmunoassay, pituitary glands were removed and homogenized on ice with a Potter-Heveljem device in 200 µl 0·01 M HCl. After centrifugation (15 000 ***g***, 10 min, 4 °C), to remove cellular debris, the supernatant was stored at − 20 °C until analysis.

Plasma analyses

Plasma cortisol was measured by radioimmunoassay (RIA), as previously described (Metz *et al.* 2003). Plasma pH, Na^+, glucose and lactate were determined by a Stat Profile pHOx Plus analyzer with automatic two-point calibration. Sodium and pH were determined with ion-selective electrodes. Glucose and lactate were determined with enzymatic electrodes (Nova Biomedical, Waltham, MA, USA).

Immunohistochemistry

Carp were anesthetized and transcardially perfused with 0·9% (w/v) NaCl containing 0·1% (v/v) 2-phenoxyethanol immediately followed by Bouin's fixative. Brains with pituitary glands attached were removed and postfixed in the same solution for 24 h. Tissue was dehydrated through a graded series of ethanols and embedded in paraffin. Sections of 7 µm were mounted on gelatinized glass slides and dried. After removal of paraffin, endogenous peroxidases were neutralized with 2% (v/v) H_2O_2, and nonspecific antigenic sites were blocked with 10% (v/v) normal goat serum. The slides were incubated overnight with the polyclonal antiserum against CRH, AVT (Huising *et al.* 2004), N-Ac β-endorphin (Takahashi *et al.* 1984), carp β-endorphin[20–29] (Van den Burg *et al.* 2001) or carp ACTH. The antiserum against ACTH was raised against a carp ACTH-specific peptide that was designed and synthesized (Eurogentec, Herstal, Belgium) based upon sequence comparison with α-MSH and CLIP (Arends *et al.* 1998). A Cys residue was N-terminally added to allow coupling to keyhole limpet hemocyanin (KLH). The sequence of the peptide thus designed was Cys-ACTH[10–23]: Cys-Gly-Lys-Pro-Val-Gly-Arg-Lys-Arg-Arg-Pro-Ile-Lys-Val-Tyr. Two rabbits were immunized at days 0, 14, 28 and 56. The rabbits were killed and bled at day 80. The antiserum did not show any cross-reaction with α-MSH or CLIP. This was established immunohistochemically, by ACTH- and α-MSH-stained serial sections of carp pituitary glands. The final dilution for immunohistochemistry was 1:5000. Goat antirabbit (Nordic Immunology, Tilburg, The Netherlands) was used as secondary antibody at a dilution of 1:150. Slides were subsequently incubated with 1:150 diluted rabbit peroxidase anti-peroxidase (M-PAP; Nordic). Staining was performed in 0·025% (w/v) 3,3′-diaminobenzidine (DAB) and 0·0005% (v/v) H_2O_2.

ACTH RIA

The ACTH concentration in pituitary and NPO homogenates and superfusates was measured by radioimmunoassay, using a commercially available antibody against human ACTH[1–24] (Biogenesis, Poole, UK). This antibody has full cross-reactivity with ACTH[1–39] and less than 0·02% cross-reactivity with α-MSH. The standard was human ACTH[1–39] (Sigma). The standard, labeled with ^{125}I (ICN, Costa Mesa, CA, USA) by the iodogen method (Salacinski *et al.* 1981) and purified through solid phase extraction (octadecyl Bakerbond column), was used as tracer. All constituents were in phosphate-EDTA RIA buffer: 0·05 M Na_2HPO_4, 0·01 M Na_2EDTA, 0·02% (w/v) NaN_3, 0·1% (v/v) Triton X-100, 0·25% (w/v) bovine serum albumin (Sigma) and 250 KIU/ml aprotinin (Trasylol; Bayer, Leverkusen, Germany).

Samples or standards of 25 µl were preincubated in duplicate with 100 µl 1:1000 diluted antibody for 24 h at 4 °C. A volume of 100 µl ^{125}I-labeled human ACTH[1–39] (approximately 5000 cpm) was added and incubated for another 24 h. A volume of 100 µl 1:20 diluted goat antirabbit IgG (Biotrend, Köln, Germany) in 0·005% (w/v) rabbit IgG (Sigma) was added and incubated for 30 min at room temperature. Immune complexes were collected after addition of 1 ml ice-cold 7·5% (w/v) polyethylene glycol 6000 and centrifugation (10 min, 2000 ***g***, 4 °C). Supernatants were decanted and pellets were counted in a gamma counter (1272 Clinigamma, LKB Wallac, Turku, Finland). The detection limit of the assay was 0·5 fmol. The interassay variation was 12 ± 3%, and the intra-assay variation was 4 ± 1% (n=6).

In vitro *superfusion*

To assess ACTH release *in vitro*, freshly collected pituitary glands were carefully dissected in PD and PI by use of a stereo microscope. PD tissue was placed on a cheesecloth filter in a superfusion chamber and superfused with a 0·015 M HEPES/Tris-buffered medium (pH=7·4) containing 128 mM NaCl, 2 mM KCl, 2 mM $CaCl_2.2H_2O$, 0·25% (w/v) glucose, 0·03% (w/v) bovine serum albumin and 0·1 mM ascorbic acid. Medium was saturated with carbogen (95% O_2 /5% CO_2) and pumped through the chambers at 30 µl/min by a multichannel peristaltic pump (Watson-Marlow, Falmouth, UK). After 120 min, medium was supplemented with 10^{-7} M ovine CRH (Sigma) or 10^{-5} M dopamine (Sigma) for 30 min. A 30-min 60 mM KCl pulse was applied at t=240 min to depolarize the tissue and release ACTH aspecifically. In a second series of experiments, the effect of a 30-min 10^{-7} M CRH pulse halfway through a 90-min 10^{-7} M dopamine application was tested. Ten- or 15-min fractions were collected, stored on ice and immediately analyzed for ACTH as described above. Basal, unstimulated release was calculated based on the three values preceding the pulse and designated 100%. Stimulation is expressed as percentage of basal release.

Stress experiment

Groups of eight carp were transferred from stock tanks to experimental tanks and acclimatized for at least 4 weeks. At the day of the experiment, eight fish at a time were confined in a net in their own aquarium. After 24 h, fish were sampled. Controls were left undisturbed.

POMC expression

Relative expression of POMC was assessed by quantitative RT-PCR. Fresh tissue, rapidly removed after anesthesia, was homogenized in TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA). Total RNA was extracted according to the manufacturer's instructions. To remove any traces of genomic DNA, 1 µg RNA was incubated with 1 unit DNase I (amplification grade; Gibco BRL) for 15 min at room temperature. A volume of 1 µl 25 mM EDTA was added and the sample incubated for 10 min at 65 °C to inactivate DNase and linearize RNA. Thereafter, 1 µg RNA was reverse-transcribed (RT) with 300 ng random primers (Gibco BRL), 0·5 mM dNTPs, 10 units RNase Inhibitor (Gibco BRL), 10 mM dithiothreitol and 200 units Superscript II RT (Gibco BRL) for 50 min at 37 °C. For quantitative PCR analysis, 5 µl 50 times diluted RT-mix was used as template in 25 µl amplification mixture, containing 12·5 µl SYBR Green Master Mix (Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands) and 3·75 µl of each primer (final concentration 300 nM). The primer sets used in the PCR were for POMC: *forward* 5′-TTG GCT CTG GCT GTT CTG TGT-3′, *reverse* 5′-TCA TCT GTC AGA TCA GAC CTG CAT A -3′; for β-actin: *forward* 5′-CAA CAG GGA AAA GAT GAC ACA GAT C-3′, *reverse* 5′-GGG ACA GCA CAG CCT GGA T-3′; and for 40S: *forward* 5′-CCG TGG GTG ACA TCG TTA CA-3′, *reverse* 5′-TCA GGA CAT TGA ACC TCA CTG TCT-3′. After an initial step at 95 °C for 10 min, a real-time quantitative PCR of 40 cycles was performed (GeneAmp 5700, Applied Biosystems), and each cycle consisted of 15- s denaturation at 95 °C and 1 min annealing and extension at 60 °C. Cycle threshold (C_T) values were determined and expression of POMC was calculated as a percentage of β-actin or 40S expression; both genes yielded similar results and were thus considered true housekeeping genes. All results presented here are expressed relative to β-actin expression.

Statistics

Superfusion data were analyzed by comparison of the average ACTH release at the three time points preceding

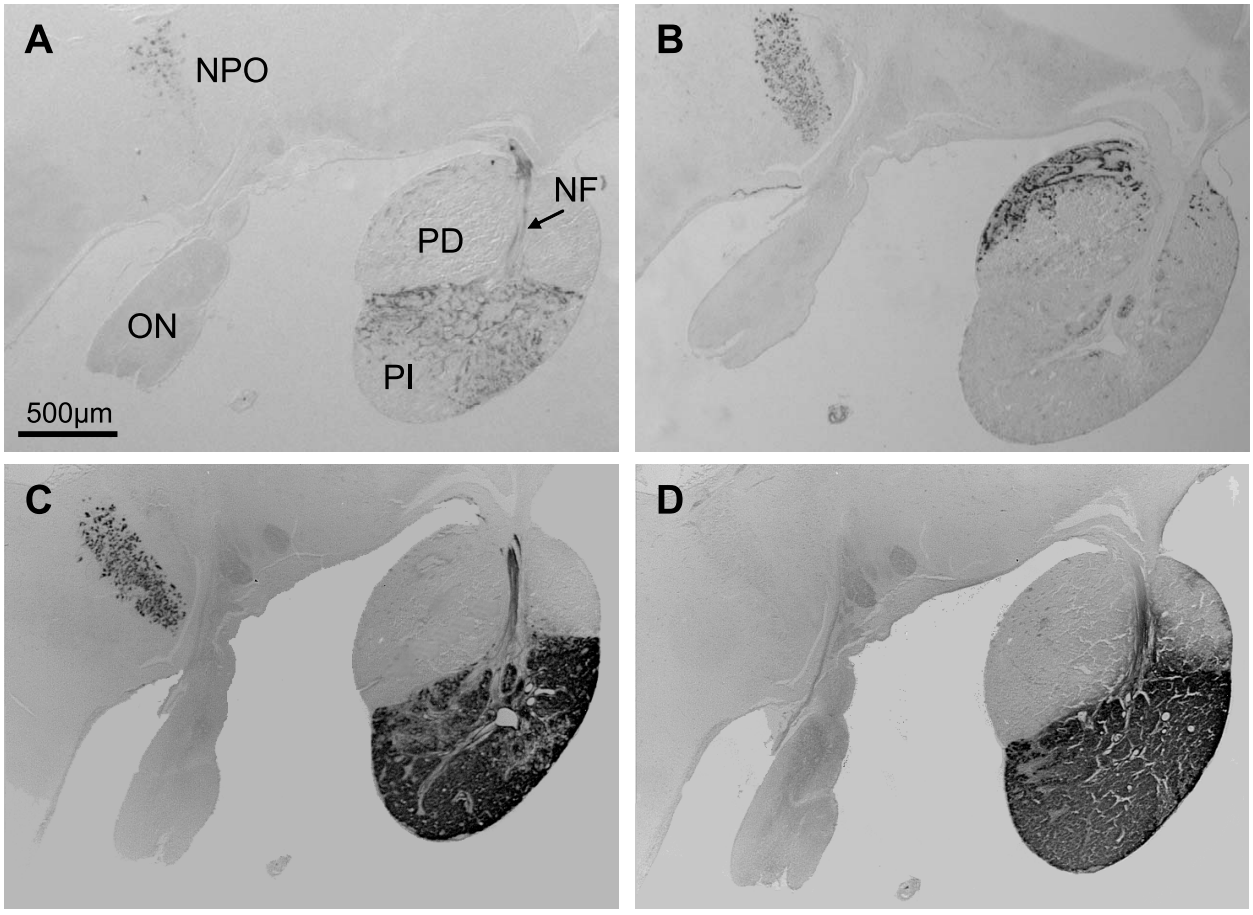

Figure 1 Immunohistochemistry of sagittal sections of the hypothalamus–pituitary complex of common carp. (A) Overview, stained with antiserum against CRH. (B) Stained with carp-specific antiserum against ACTH. (C) Stained with antiserum against the C-terminus of carp β-endorphin. The antibody recognizes N-Ac as well as nonacetylated β-endorphin. (D) Stained with antiserum specific for N-Ac β-endorphin. This section contains the NPO; under higher magnification, perikarya were clearly visible (not shown). Figure 1C and D have been previously published (Van den Burg *et al.* 2001). For clarity, nomenclature is shown only in panel A. Abbreviations: ON, optic nerve; NPO, nucleus preopticus; PD, pars distalis; PI, pars intermedia; NF, nerve fiber.

the pulse with the release during and after the pulse. Data were analyzed by ANOVA and Tukey post hoc testing.

Differences among groups in the expression of POMC were assessed by the nonparametric Mann–Whitney U-test.

All values are expressed as means ± standard deviation (S.D.). Significance was accepted at $P < 0{\cdot}05$.

Results

Figures 1, 2 and 3 show immunostained sagittal and transverse sections of the hypothalamus-pituitary system of carp. The nucleus preopticus of the hypothalamus shows prominent staining for CRH, ACTH and nonacetylated β-endorphin (Fig. 1A–C respectively). The magnocellular and parvocellular areas in the NPO both contained CRH, ACTH and nonacetylated β-endorphin-positive neurons. No N-Ac β-endorphin staining was observed in the NPO (Fig. 1D). The nerve fiber bundles of the pars nervosa, which project mainly onto the PI, were positive for CRH and nonacetylated β-endorphin, but not for ACTH and N-Ac β-endorphin.

Higher magnification of the rostral pituitary PD shows that the ACTH-containing corticotrope cells are organized in sheets around the nerve branches (Fig. 2A). ACTH-negative cells in the rostral PD appeared to be prolactin cells, as was established immunohistochemically (not shown). Higher magnification of the ACTH-containing cells in the NPO shows that immunoreactivity is present in the secretory granules of cells with a large nucleolus-containing nucleus (Fig. 2B).

Serial transverse sections of NPO stained with AVT (Fig. 3A) and ACTH (Fig. 3B) show that perikarya were positive either for ACTH or for AVT. No cells were observed that were positive for both these hormones.

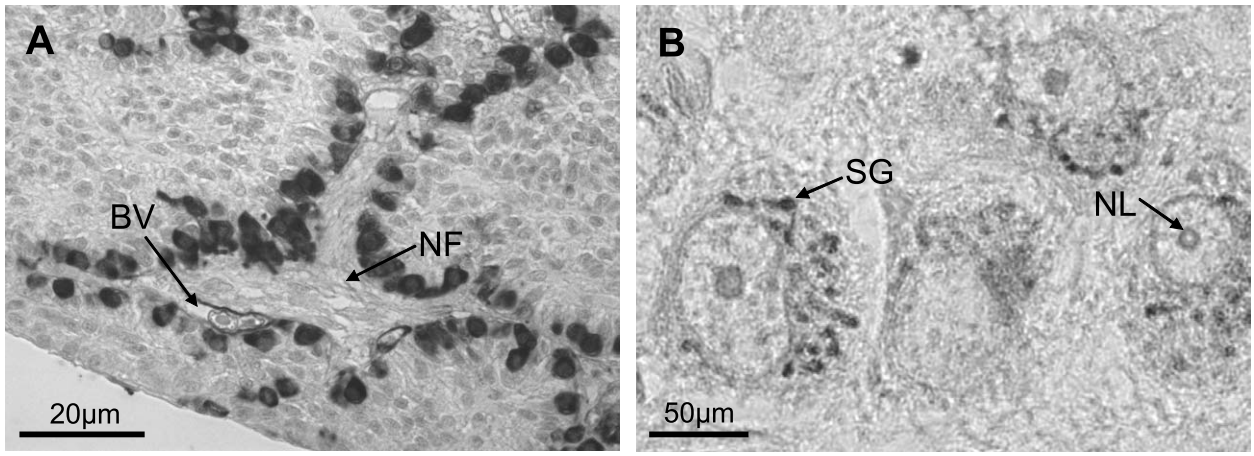

Figure 2 (A) Magnification of the rostral PD stained for ACTH. (B) Magnification of ACTH-positive perikarya in the NPO. Abbrevations: BV, blood vessel; NF, nerve fiber; SG, secretory granules; NL, nucleolus.

Figure 4 shows the binding curve for standard ACTH. Serial dilutions of PD and NPO homogenate and superfusate displaced radiolabeled ACTH from the antibody in parallel with dilutions of the standard.

As shown in Fig. 5A, unstimulated release of ACTH from the PD steadily increased during the 210-min duration of the experiment. The initial release, measured at t=75 min, averaged 5 ± 3 fmol/min/PD ($n=6$). A 30-min pulse of 10^{-7} M CRH did not affect ACTH release (Fig. 5B). Dopamine at 10^{-5} M inhibited ACTH release within 20 min (Fig. 5C). Under the influence of a 100-fold lower concentration of dopamine (10^{-7} M), ACTH release plateaued for at least 70 min (Fig. 5D). Stimulation with CRH, during such a low dopamine exposure, evoked a clear stimulation of ACTH release (Fig. 5E). Cells depolarized by means of a 60 mM KCl pulse consistently released ACTH to the superfusate (Fig. 5F).

Plasma cortisol values increased more than sevenfold in response to a 24-h restraint stress (Table 1). Plasma glucose levels rose to over 9 mmol/l, while lactate and sodium significantly decreased. Plasma pH remained unchanged. Expression of POMC in the nucleus preopticus, as well as in the PD and PI, increased significantly (Fig. 6).

Discussion

This study provides three novel key findings. (1) The POMC-derived peptides ACTH and β-endorphin are

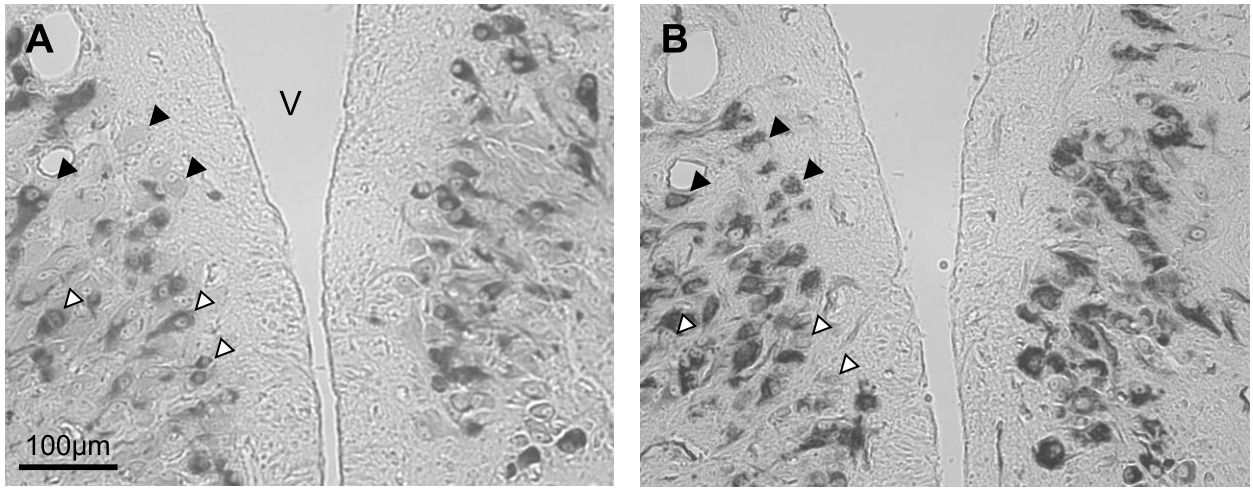

Figure 3 Serial, transverse sections of the NPO stained for AVT (A) and ACTH (B). Perikarya were positive for either AVT (white arrowheads) or ACTH (black arrowheads). Abbreviation: V, ventricle.

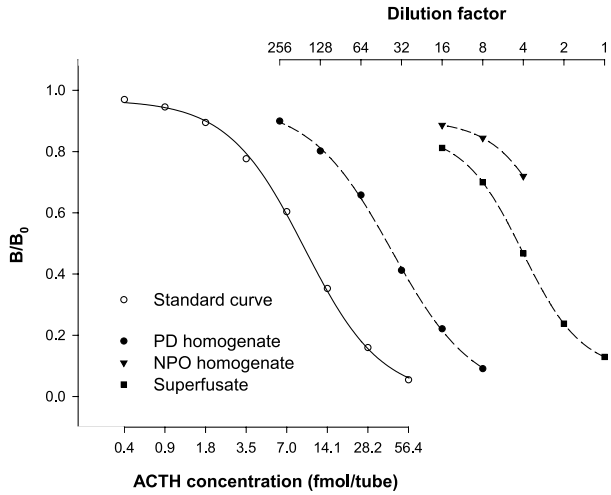

Figure 4 ACTH radioimmunoassay binding curves for standard ACTH and dilution curves of superfusate, PD and NPO homogenates.

present in the carp NPO. Nerve fibers projecting on cells of the pituitary gland are positive for β-endorphin, but not for ACTH. We postulate β-endorphin release via the pituitary gland, while ACTH serves another function or is produced as an unnecessary byproduct. (2) ACTH release from the corticotrope cells of the PD is under inhibitory control of dopamine and stimulated by CRH during a low dopamine tone. (3) POMC expression is upregulated during 24-h restraint stress in NPO, PD and PI. These three major conclusions will be discussed separately.

Immunohistochemistry

The ACTH antiserum did not cross-react with α-MSH and CLIP, the POMC-derived products from the PI. Since the ACTH-producing cells in the PD are closely located to nerve fibers and blood vessels, we postulate that their activity must be under tight control of factors from the hypothalamus and that ACTH can be quickly released into the circulation, as during acute stress. We found a considerable ACTH immunoreactivity in the NPO. ACTH-containing cell bodies were negative for AVT and vice versa. In carp, AVT and CRH are colocalized (Huising *et al.* 2004), and their axons project directly on the cells in the pituitary gland. We did not observe ACTH-positive fibers in the pituitary gland and therefore conclude that ACTH plays no direct role in the control of hormone release from the pituitary gland. On the contrary, considerable β-endorphin staining was observed in the nerve fibers entering the PI. Apparently, ACTH and β-endorphin, although derived from the same precursor, are differentially packaged and transported along the axons. This endorphin must be nonacetylated β-endorphin[1–29] and/or [1–33], as this antiserum was raised against carp β-endorphin[20–29], and we did not observe staining with the N-Ac β-endorphin-specific antiserum. Teleost fish lack a median eminence and portal vessels directing to the pituitary gland, but our observations point towards release of nonacetylated β-endorphin produced by the NPO into the circulation. Nonacetylated β-endorphin could also play a role in the release of ACTH and/or α-MSH from the pituitary gland, but as yet we could not demonstrate such an effect (J R Metz, personal observations). The function and targets of ACTH from the NPO remain unclear. POMC expression in the NPO, however, is upregulated during acute stress, and thus a central stress-related function may be anticipated. In a few other fish species examined, POMC-derived peptides, including ACTH, have been demonstrated in the NPO area (Vallarino *et al.* 1989). Possibly, ACTH serves a function other than in the pituitary gland, or it is produced as a byproduct of β-endorphin production.

Control of ACTH release

The release of ACTH from the PD is classically considered to be under positive control of CRH. Several studies have shown that CRH is a stimulator of ACTH release in fish (Fryer *et al.* 1984, Baker *et al.* 1996, Rotllant *et al.* 2000, Van Enckevort *et al.* 2000). However, when we repeated such studies in carp, we failed and anticipated that the failure was related to the use of heterologous CRH (human/rat and ovine) or oxidation of CRH during the superfusion experiment. Therefore, mass spectrometric analysis was performed on CRH in oxygenated medium. We detected only the intact peptide; oxidation or breakdown of CRH could not be established.

As the pituitary PD shows a slowly but consistently increasing basal, unstimulated release of ACTH *in vitro*, we concluded that ACTH release *in vivo* must be under predominant inhibitory control. This contrasts with the release of α-MSH and β-endorphin from the pituitary PI, where unstimulated release in time is stable or slowly decreases (Lamers *et al.* 1991, Van den Burg *et al.* 2003). Apparently, in a nonstressed situation, suppression of ACTH release is necessary to keep circulating levels of ACTH at a low level. Thus, *in vivo*, ACTH cells may receive both dopaminergic and CRH signals to control ACTH release in concert. Indeed, our experiments show that CRH is stimulatory only when the ACTH cell is under a dopaminergic inhibition. Dopaminergic neurons from the preoptic region innervate the pars distalis (Kah 1986) and the pars intermedia (Fryer *et al.* 1985*b*). In an ACTH cell, the inhibiting effect of dopamine is achieved via a D2-like receptor (Selbie *et al.* 1989, Civelli *et al.* 1993, Stefaneanu *et al.* 2001). CRH binds the CRH-receptor type 1 (CRH-R1) (Chen *et al.* 1993; Huising *et al.* 2004). Both these receptors are 7-transmembrane G-protein-coupled receptors. However, the D2-receptor inhibits, while CRH-R1 activates, adenylyl cyclase. We

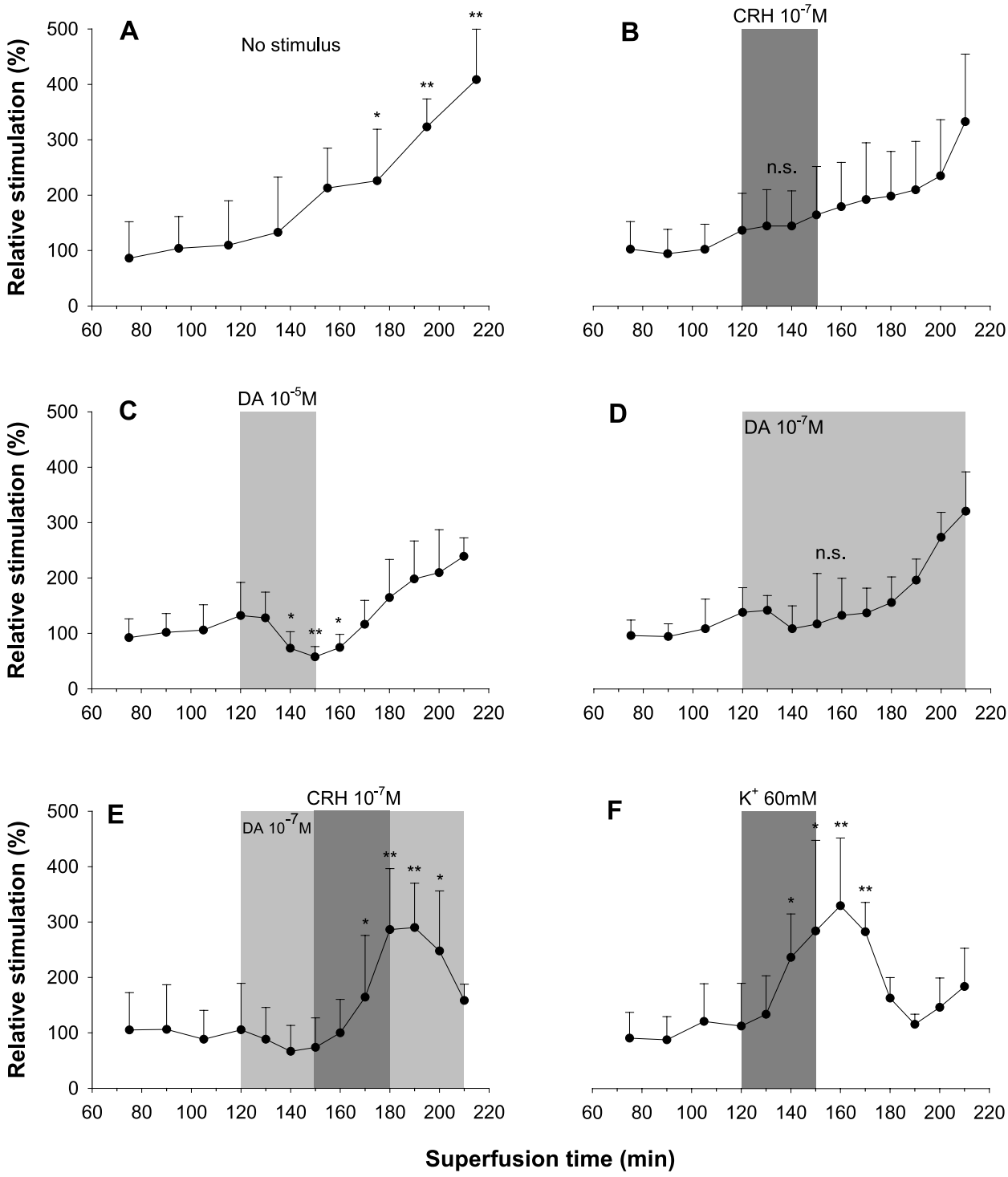

Figure 5 *In vitro* ACTH release from carp PDs in a superfusion setup. We assessed unstimulated release (A, $n=12$), and stimulation with 10^{-7} M CRH (B, $n=12$), 10^{-5} M dopamine (C, $n=8$), 10^{-7} M dopamine (D, $n=8$), 10^{-7} M CRH during a 10^{-7} M dopamine exposure (E, $n=12$) and 60 mM KCl (F, $n=8$). Stimulation is expressed as percentage of basal, unstimulated release. Asterisks indicate statistical differences with basal release. *$P<0{\cdot}05$; **$P<0{\cdot}01$.

Table 1 Plasma parameters of control ($n=8$) and 24-h restraint stressed fish ($n=8$). Significance among groups is indicated

	Control	24-h restraint	Significance
Cortisol (nmol/l)	176 ± 133	1319 ± 211	$P<0.001$
Glucose (mmol/l)	2·56 ± 0·46	9·22 ± 1·48	$P<0.001$
Lactate (mmol/l)	3·27 ± 0·36	2·58 ± 0·41	$P<0.05$
pH	7·61 ± 0·02	7·60 ± 0·03	n.s.
Na^+ (mmol/l)	115 ± 6·6	97·5 ± 6·8	$P<0.001$

conclude that activation of both receptors is necessary to initiate an intracellular pathway eventually leading to secretion of ACTH.

POMC expression during stress

The stress of a 24-h restraint caused an increase of plasma cortisol and glucose, and a decrease of plasma lactate and sodium. Enhanced plasma cortisol is considered a primary stress response marker (Wendelaar Bonga, 1997). The observed changes in glucose, lactate and sodium are typical for the secondary stress response (Wendelaar Bonga, 1997). This indicates that the restraint stress given disturbed homeostasis, and, consequently, physiological mechanisms to restore these disturbances must be activated. This indeed appeared to be true, as the persistent stimulation of the stress axis during the 24-h restraint period may have recruited the POMC-derived peptides from stores in NPO, PD and PI, and this may have necessitated upregulation of POMC gene expression. This effect is likely to be mediated by the glucocorticoid receptor, which is highly expressed in the CRH-producing neurons of the NPO (Teitsma *et al.* 1998).

The intriguing observation of POMC processing into ACTH and β-endorphin in NPO neurons and the differential localization of these peptides suggests a peripheral role for β-endorphin and a central (paracrine?) role for ACTH. Whatever their function, an involvement in stress regulation seems evident. Our future studies will extend our understanding of the regulation of the stress response of common carp.

Acknowledgements

This study was financially supported by the Dutch Research Council (NWO-ALW) under project number 805-46-033. The authors would like to thank Mr F A T Spanings for animal care and experimental setup.

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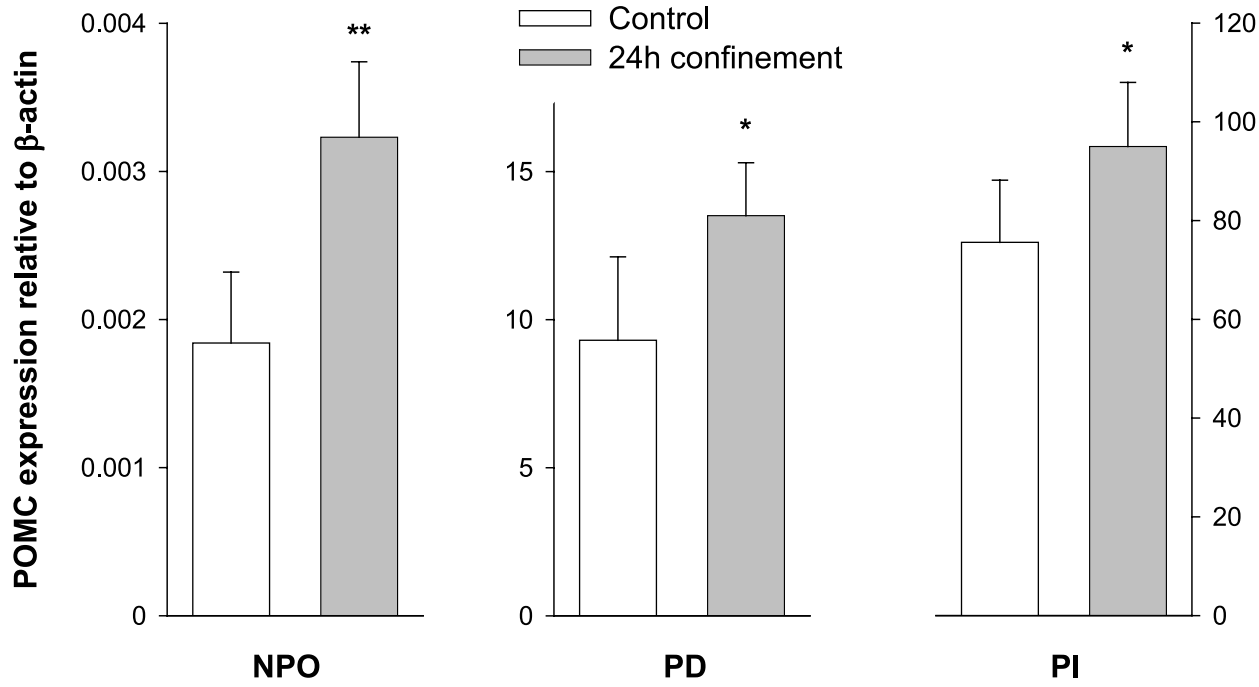

Figure 6 Relative expression levels of POMC in the NPO, PD and PI in controls and 24-h restraint-stressed carp. *$P<0.05$; **$P<0.01$.

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Received 20 January 2004
Accepted 31 March 2004
Made available online as an
Accepted Preprint 13 April 2004