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CRF-system activity, and indeed its mRNA levels are altered by restraint stress. Moreover, the unique structure and size of the CRFBP provide good tools in phylogenetic studies, that date the CRF-system to at least one billion years old. Pro-opiomelanocortin is produced and processed to ACTH and endorphin in the hypothalamic NPO and pituitary pars distalis ACTH-cells, to MSH and acetylated endorphins in the pituitary pars intermedia MSH-cells. ACTH is the prime corticotrope in acute stress conditions. In carp, MSH, considered a mild corticotrope in chronic stress responses in other fish, lacks corticotropic effects (in line with the absence of the melanocortin 5 receptor in headkidney); yet, an unknown corticotropic signal substance in the pars intermedia of carp awaits elucidation. Interesting observations were made on the CRF control of pituitary cells. CRF stimulates ACTH cells, but only when these cells experience a mild dopaminergic block. Endorphin, produced in the NPO and transported via axons to the pituitary gland in vivo, reverses the stimulatory CRF action on MSH-cells to a differential inhibition of N-acetyl beta-endorphin release in vitro (MSH release is not affected). We speculate that the consistently observed elevation of plasma MSH during chronic stress may exert central actions related to feeding and leptin regulated processes. A BOLD-fMRI study revealed the functional anatomy of the stress response at work in a paradigm where carp were exposed to a sudden water temperature drop. In carp (and other fish) the endocrine stress axis is already operational in very early life stages, viz. around hatching and comprises hypothalamic, pituitary and interrenal signaling to adjust the physiology of the hatchling to its dynamically changing environment. Understanding of stress during early life stages is critical as the consequent rises in cortisol may have long lasting effects on survival and fish quality.

*** Cover Letter**

Covering letter, 8/11/05

Dear Bob,
Herewith my revised manuscript for the Boston cymposium. I have now included three figures and all suggestions for revision were taken!

Cheers, Gert

Response to reviewers comments on ms GCE-05-166

Overall the reeferees were very enthousiastic about this paper and the criticism mainly concerned editorial aspects. Reviewer 1 gave an abundance of suggestions for revision that were all taken. Scientific matters are addressed below separately. Reviewer 2 would like to see illustration in the revised manuscript. I have included now three key figures that make the paper much more attractive. These are new figures in all instances, although similar figures (2 and 3) have been published in our work before. However, there is no copyright item here, as these are original figures not published before.

Reviewer 1

Abstract: the reviewer provided many valuable editorial suggestions that were all taken in the revision of the abstract. Indeed the abtract of the original ms was written in a great hurry and had an appaling number of mistakes; I apologise for that.

Introduction: all editorial suggestions were taken in the revision

The referee asks u to elaborate on the role of paracrine ACTH in the NPO. We must speculate here a little, but the immunohistochemistry is rather convincing. We see no ACTH in nerve tracts descending the NPO, but always find ACTH-positive cells in the NPO. We have written that the ACTH is either meant for local paracrine actions, or a by-product of endorphin production.

On the topic of opioid actions two references (Rene et al & Kapas et al) were included to substantiate statements on opioid receptor actions.

The text was condensed as suggested.

On the item of the CRFR's the text was adjusted as suggested and a reference included (Bale et al) to substantiate the reworded section.

On the topic of MCRs. We have assumed that the reader is informed about MC1R being the classical MSH receptor involved in color adaptation, MC2R the one for ACTH and MC5R a potential MSH receptor found in many instances in peripheral tissues. MC3 R and MC4R are generally considered receptors of the CNS. As we did not want to go into detail here too much we have left the text as short as posisble. In the meantime we have the 3 and 4 for carp as well and do not see them expressed in HK of carp.

MSH levels do rise in carp as in tilapia. This was an error to mention carp twice.

There is not a reference yet for our leptin work in carp; there is a paper under consideration with Endocrinology.

A paper on lipolytic action of MSH is included (Brennan et al., 2003)

The reference list was redone in GCEN style.

CRF and stress in fish

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Abstract

The endocrine stress response is pivotal in vertebrate physiology. The stress hormone cortisol - the end product of the endocrine stress axis - (re-)directs energy flows for optimal performance under conditions where homeostasis may be or become at risk. Key players in the continuous adaptation process are corticotropin releasing factor (CRF) from the hypothalamic nucleus preopticus (NPO), pituitary adrenocorticotropic hormone (ACTH) and cortisol produced by the interrenal cells in the headkidney (adrenal equivalent of fish). CRF is a member of a large family of related peptides that signals through CRF-receptor subtypes specific for central and peripheral actions of the peptide. CRF is "chaperoned" by an unique and phylogenetically very well-conserved binding protein (CRFBP); the functions of the CRFBP can only be speculated on so far, but its mRNA and protein abundance are important indicators of the central CRF-system activity, and indeed its mRNA levels are altered by restraint stress. Moreover, the unique structure and size of the CRFBP provide good tools in phylogenetic studies, that date the CRF-system to at least one billion years old. Pro-opiomelanocortin is produced and processed to ACTH and endorphin in the hypothalamic NPO and pituitary pars distalis ACTH-cells, to MSH and acetylated endorphins in the pituitary pars intermedia MSH-cells. ACTH is the prime corticotrope in acute stress conditions. In carp, MSH, considered a mild corticotrope in chronic stress responses in other fish, lacks corticotropic effects (in line with the absence of the melanocortin 5 receptor in headkidney); yet, an unknown corticotropic signal susbtance in the pars intermedia of carp awaits elucidation. Interesting observations were made on the CRF control of pituitary cells. CRF stimulates ACTH cells, but only when these cells experience a mild dopaminergic block. Endorphin, produced in the NPO and transported

via axons to the pituitary gland *in vivo*, reverses the stimulatory CRF action on MSH-cells to a differential inhibition of N-acetyl beta-endorphin release *in vitro* (MSH release is not affected). We speculate that the consistently observed elevation of plasma MSH during chronic stress may exert central actions related to feeding and leptin regulated processes. A BOLD-fMRI study revealed the functional anatomy of the stress response at work in a paradigm where carp were exposed to a sudden water temperature drop. In carp (and other fish) the endocrine stress axis is already operational in very early life stages, viz. around hatching and comprises hypothalamic, pituitary and interrenal signaling to adjust the physiology of the hatchling to its dynamically changing environment. Understanding of stress during early life stages is critical as the consequent rises in cortisol may have long lasting effects on survival and fish quality.

Introduction

For a dynamic interaction with the environment, crucial for survival, vertebrates continuously adjust their physiology ("adaptational responses") to an ever-changing environment. To do so, vertebrates may depend on a seemingly endless repertoire of physiological, endocrinological and immunological responses that allow them, indeed, to cope with physical, chemical and biological disturbances. Fish, in intimate contact with their aqueous environment via the elaborate and delicate epithelium of the gills – where unwanted chemicals and antigens may easily penetrate – were the first vertebrates that developed stress responses, which include interactions between the endocrine stress axis (a key player in the adaptive response with gills as an important target for cortisol the end product of the stress axis) and the immune system (crucial to eliminate intruding antigens). Bidirectional communication between the endocrine and immune system then is a prerequisite (Engelsma *et al.*, 2002). Indeed, evidence is accruing from molecular biological studies that in fish, representing the earliest vertebrates, the majority of chemical signaling between these systems is already operational. In this topical review we address the endocrine stress response in carp (*Cyprinus carpio*). We will focus on the functional anatomy of the stress axis in adult fish, and peculiarities of the CRF- and POMC-systems in the stress axis. In adult carp, we were able to visualise the endocrine stress axis at work, *in vivo*, by blood oxygen level dependent functional magnetic resonance imaging (BOLD fMRI). The early development of the stress axis activity was investigated in carp around hatching. This mini-review reflects our contribution to the symposium "Novel functions of the corticotropin-releasing factor system" held at the 15th International Congress of Comparative Endocrinology in Boston, May 2005; it is certainly not an exhaustive review, rather a document to stimulate further reading and discussion.

Some functional anatomy of the endocrine stress axis in adult fish

The focus in this section is on the hypothalamic nucleus preopticus (NPO), pituitary ACTH- and MSH-cells and the headkidney, where cortisol is produced. The hypothalamic nuclei involved in stress responses receive multiple input from sensory systems that continuously monitor and evaluate the internal and external conditions of the fish. Eventually much of this information converges to the NPO where it is integrated to direct the pituitary ACTH- and MSH-cells in their control over the interrenal cells and central systems that need adjustment during stress.

The CRF-system.

CRF-producing cells in the NPO are believed to play a crucial role in the process of adaptation to stressors. Importantly, other peptides like arginine vasotocin (AVT), thyrotropin releasing hormone (TRH) and the POMC-derived ACTH and endorphins are only some other players in this field. The peptides may be produced in the same CRF-producing cells or in seperate cells and intercellular communication with these signals may occur via synaptic(or synaps-like) contacts or be specified via localised receptor expression on the neuron somata. With an antibody to carp $ACTH_{10-23}$, which recognises the processed ACTH molecule (not its precursor POMC or the MSH sequence), we demonstrated (Metz *et al.*, 2004) the presence of ACTH in NPO-cells in the vicinity of CRF-cells. This ACTH could function in paracrine mode to modulate CRF-cell activity within the NPO, since we see no ACTH in axon bundles projecting to the pituitary gland. Paracrine release of ACTH in the NPO could be demonstrated by immunohistochemistry at the ultrstructural level on tannic acid treated tissue (to capture exocytosis), but such studies have not yet been carried out. We do not yet know the afferent input to these ACTH producing cells. A more

speculative yet attractive hypothesis is that these ACTH-producing cells are primarily an endorphin source. Processing of POMC to ACTH yields endorphin and, indeed, we do see endorphin-positive axons approaching and entering the pituitary gland. We do not rule out that (part of) the endorphin is N-terminally acetylated during transport towards the pituitary gland (Van den Burg *et al*., 2001). Acetylation of endorphin results in loss of opioid activity as the acetylated isoforms lose significant affinity for opioid receptors (Barg et al, 1993; Rene et al., 1998) and this may be required to fine-regulate endorphin levels as these peptides are rather powerful biochemicals. A remarkable observation is that endorphin, when given as a tonus *in vitro* to carp pituitary MSH cells, alters the response to CRF: CRF inhibits under such conditions the release of acetylated endorphin but not of MSH. Apparently, a differential regulation of processing and release of these peptides, that reside in a 1:1 ratio in the precursor POMC, is part of the complex pituitary response to hypothalamic stress signals (Van den Burg *et al*., 2005a). Acetylated endorphin has been assigned a potentiating role in corticotropic actions of MSH in the Mozambique tilapia (Balm *et al.* 1995). Every trial to confirm this concept in carp failed so far and we could not demonstrate mRNA of melanocortin-5 receptor, a predicted vector for MSH-signaling in head kidney (Metz *et al*., 2005). Little if any information is available on specific (activating) receptors for acetylated endorphins. Maybe acetylation of endorphins aims at production of opioid receptor antagonists (Rene *et al*., 1998). In that case the search for opioid receptors on fish (tilapia) cortisol producing cells is indicated. The consequence of such a discovery would be that the potentiating effect of acetylated endorphin on MSH corticotropic actions relies on antagonist action on opioid receptors. Such a complicated scenario is realistic as it was demonstrated in the mammalian adrenal cortex that the zona fasciculata cells express opioid mu- and kappa-receptors that stimulate corticosteroid production, via ACTH-independent pathways (Kapas *et al*, 1995). The search for specific,

activating receptors for acetylated-endorphin awaits further research and experimentation; that such receptors may exist follows from the consideration that highly specific antisera to acetylated endorphins can be made. This suggests that the acetylated molecule is highly specifically recognised by the immune system and thus is part of a known ligand-receptor combination.

Axons of the NPO CRF-cells project directly to the pituitary gland. This is a different situation from that in higher vertebrates (as of amphibia) where the hypothalamic releasing hormones are freed into the portal circulation of an eminentia mediana. The important difference between fish and higher vertebrates then is that the hypothalamic releasing hormones depend on receptor expression profiles of targets in higher vertebrates, whereas localised release (via synapses or into the circulation via neurosecretion in the pars nervosa) is typical for fish. Specificity is thus realised in the CRF-sending neurons in fish and in the receiving cells in higher vertebrates, respectively.

The CRF signal is eventually determined by at least three factors (Figure 1): the CRF structure, the presence of CRF binding protein (CRFBP) and the receptor for CRF (at least two known, R1 is the "central" receptor). Carp are tetraploid fish and make two CRF molecules that differ in a proline (neutral) and alanine (amphiphatic) on position four of the mature 41 amino acid peptide (Huising *et al*, 2004). As this is not a conservative replacement, one wonders whether the peptides have evolved into serving different functions. For sure the alanine would give the molecule less rigidity that could easily translate into a different tertiary structure. Little is known on this topic but it has been shown that messenger RNA's for the two very similar copies of POMC in this fish (Arends *et al*., 1998a) are differentially expressed among different strains of common carp when challenged with varying water temperatures (Arends *et al*., 1998b). This indicates that even very small differences in peptide molecules potentially yield advantages in adaptation

physiology and amplify the (stress) signaling repertoire of the fish. The same could hold for the two CRFs in carp. The high sequence identity between fish and human mature CRF (93%) substantiates a strong evolutionary pressure on conservation of this signal substance over the roughly 450 million years since the rise of fishes in the Ordovicium. The small, yet significant, changes within duplicate genes as occur in the tetraploid carp confirm this notion from observations on a single species; the genome duplication in carp is however more recent and estimated to have occurred 16 million years ago (Larhammar and Risinger, 1994). Another exciting hypothesis to test is whether different CRFs are produced in peripheral organs of the fish (headkidney, immune system, caudal neurosecretory system, gills) with a concomittant target specificity. CRF is projected towards ACTH-cells and towards the pars intermedia MSH-cells. Interestingly, CRF may be a releasing hormone for TSH in fish (as in birds via CRFR2 rather than CRFR1; De Groef *et al*., 2003) and this warrants studies on thyroid involvement in stress regulation. Intriguingly, CRF signaling to the pituitary gland could exert combined and concerted control over metabolism and metamorphosis via cortisol and thyroid hormones during early life stages and metabolism alone in adult stages. This notion further warrants studies into interactions of transcription factors for thyroid hormone (T3) and cortisol (and others such as retinoic acid receptors known to heterodimerise for their actions), and what a wonderful models fish such as carp provide to study the ontogeny of these aspects of the CRF system! We are presently screening the carp pituitary gland for CRFR1 and CRFR2 mRNAs to see if the chicken situation was maybe an “invention” of fish.

The abundant CRF-immunoreactivity in carp (and other fish) pars intermedia (Huising *et al*., 2004) may indicate that NPO axons terminate on pars nervosa blood vessels and release CRF there for peripheral purposes. This scenario could explain the extraordinarily high levels of CRF seen in fish plasma (Pepels *et al*., 2002). Whether

plasma CRF in fish is "chaperoned" by a binding protein is doubted by these authors, but this seems counterintuitive considering the situation in pregnant women: the high levels of CRF at term are buffered by CRFBP to protect the body from the potent CRF signal. Interestingly, the urophysis of fish is another potential source of CRF that could determine the high plasma levels in fish (Lu et al., 2004); whether the urophysis also contains CRFBP awaits further experimentation. Carp, pufferfish, and in all likelihood all fish, produce CRFBP (Huising *et al*., 2004), which may modulate CRF bioactivity or serve as a reservoir. The impetus for this research was given by data obtained with an antiserum to human CRFBP (donated by Dr W. Vale, Salk Institute, La Jolla ,CA), that allowed us to demonstrate a 35.8 KDa CRFBP in carp hypothalamus and pituitary extracts by western blot (Huising *et al.*, 2004). As will be discussed below, CRFBP is well-conserved during phylogeny and this explains the cross-reactivity at the basis of these observations. In carp CRFBP is produced by a separate group of small cells at the periphery of the NPO. The axons of these cells join those of the CRF-producing neurons on their way towards the pituitary gland. This leaves the CRF- and CRFBP-producing cells as two targets for regulation in fish (in higher vertebrates CRF and CRFBP are often produced by the same cell). Restraint stress elevates mRNA levels of both CRF and CRFBP in carp hypothalamus and results in release of both proteins from nerve terminals in the pituitary gland as shown by a comparison following immunohistochemistry of control and experimental tissue and thus we have to consider both proteins in our appreciation of the CRF signaling system. When parallels would exist with the IGF-system, we should at least extend our views on CRFBP functions to direct, CRF-independent effects of the binding protein on CRF targets (Bauchat *et al*., 2001). The rather well-studied IGF-system may fulfil a role model in this search. The IGF- and CRF-systems with their binding proteins share a long evolutionary history: both have been traced back in fishes (Bauchat *et al*., 2001; Huising *et al*., 2004).

Very recently CRFBP was demonstrated in the honeybee (Huising and Flik, 2005) and this would date the CRF system to at least one billion years old.

A third player in the CRF-system is of course the receptor moiety on the target cell for CRF. To date two categories of CRF-receptors (with splice variants; Eckart *et al*., 2002) are known, CRFR1 and CRFR2. Both are widely expressed in the central nervous system and peripherally. CRFR1 is *the* important receptor in the stress axis. CRFR2 is involved in a whole host of stress-related physiological and behavioral responses, from alterations in vascular tone and blood pressure to effects on feeding and anxiety-related behaviors; obviously CRFR2-mediated regulation of learning and anxiety control involves brain nuclei and areas outside the endocrine stress axis (Bale *et al*., 2002a&b). The involvement of the CRFR1 in the stress response of carp was demonstrated by a predicted down-regulation of the messenger RNA for this protein following a 24 h restraint (Huising *et al*., 2004). Fish (carp) possess an orthologue of the mammalian CRFR2 and its particular involvement in fish physiology and disease/stress awaits further experimentation. With the demonstration of the "complete CRF system" in fish, we hope to convince students in biology that we should look, where possible, for original functions of the components of this system in the early vertebrates (fish) and should realise ourselves that an anthropocentric approach in this particular field of research (extrapolation of data from human studies) may not always be justified, considering the high degree of specialisation of the human being and carp alike, and may leave surprises hidden. The versatility of the CRF-system may be at the basis of the success of fishes that, with an estimated 35.000 extant species, are the most successful vertebrates in this world, and that have explored and occupied essentially every thinkable niche. We only start to understand bits of the highly complex brain/hypothalamus regulation of pituitary output, which involves many more signals than those of the CRF-family (CRF, Urocortins, Urotensins), thyrotropin releasing hormone (TRH), arginine-

vasotocin (AVT) and proopiomelanocortin- (POMC-) derived peptides from the NPO which were addressed here; not to speak of similarly complex pituitary cocktails of pleiotropic signals meant to control the peripheral targets of the body for a properly concerted stress response. This complexity must be at the basis of the success of fishes and evolution of later vertebrates.

The POMC-system.

ACTH (figure 2). The expression of POMC in the NPO was addressed above "in the margin" . Our knowledge of ACTH actions in hypothalamic cells is rather limited, not in the least because of the scarcity of sensitive assays for ACTH in fishes (good antibodies are rare due to the extreme conservation of the ACTH molecule in most vertebrates). We succeeded in developing an ACTH-immunoassay for carp ACTH (Metz *et al*., 2004) that we used mainly to better understand the carp pituitary ACTH-cell. Carp ACTH-cells appear to be under negative dopaminergic control *in vivo*: ACTH-release is unleashed in ectopic pars distalis tissue. This notion was key to further study these cells *in vitro*. First attempts to measure CRF-regulated output from the pituitary pars distalis (which can easily be separated from the rest of the highly organised pituitary gland in fishes) directly in a perifusion setup enigmatically failed. The release rate of ACTH increased over time and CRF over a wide range of concentrations was without stimulatory effect. We then reasoned that the inhibitory dopamine tonus that these cells normally experience in the intact fish, with its consequences for second messenger make-up of the cells, could be a requirement for CRF action *in vitro*. Indeed, ACTH-cells do show CRF-dependency for ACTH-release only under mild dopamine block. It seems that the powerful ACTH signal is normally inhibited by dopamine for a balanced stress-axis output. Thus, proper ACTH-cell functioning *in vitro* is apparently only guaranteed when the cells experience a certain

dopamine tonus. Further, it was the peculiar and generally recognised power of the ACTH signal, e.g. reflected by picomolar surges during stress, that underlies the notion that an inhibitory tonus by dopamine is an integral part of stress-axis activity to allow for balanced and strictly controlled stress axis output and stress adaptation. More studies are needed. "Do both signals use cAMP?", "are other second messengers involved?", "is there cross-talk or bidirectional communication between their second messengers?", these are just some questions that have to be answered to understand the mechanisms underlying this observation.

Interestingly, hypothalamic dopamine production decreases in mice experiencing chronic stress (Konstandi *et al*., 2000). If the same holds for fish, a lower dopamine tonus due to stress could desensitise the ACTH cell for CRF, impeding acute stress signals. In chronically stressed (not in controls) Mozambique tilapia (Lamers *et al*., 1997) the pituitary MSH-cells express a stimulatory, high affinity dopamine D1 receptor, compatible with this notion of decreased dopamine levels and an increased MSH production during chronic stress. Apparently such situations of decreased dopamine activity do occur in fish.

MSH and N-acetylated endorphin (Figure 2). As mentioned above, the pituitary gland secretes many post-translationally modified POMC-derived peptides. MSHs and acetylated endorphins are two categories of products of the MSH-cell often considered in stress regulation. In a series of studies on tilapia in the nineties of the last century, MSH was proposed as a mild corticotrope (in concert with endorphin; Balm *et al*., 1995). The complex picture around the rather pleiotropic MSH-signal was narrowed down to its di-acetylated form as the specific corticotrope next to the non- and mono-acetylated isoforms (Lamers *et al*., 1992) and thyrotropin releasing hormone (not CRF) the prime hypothalamic regulator of its differential release (Lamers *et al*., 1994). The picture that emerged was that

of potent pars distalis ACTH in acute stress appearing in short surges to evoke sharp transient rises in cortisol and mild pars intermedia MSH chronically elevated to reset baseline cortisol to somewhat higher levels. A wonderful picture, but maybe a tilapia picture only. We could not confirm a similar concept in carp. Whatever combination of synthetic MSH- and endorphin-isoforms tested, no corticotropic action for MSH could be demonstrated in carp (Metz *et al*., 2005). Accordingly, no other than the MC2R, the "ACTH-receptor", could be demonstrated in carp headkidney tissue and this receptor was downregulated following stress in line with an anticipated negative feedback control. We anticipated that an effect of MSH would be mediated by an MC5R as this receptor is also expressed in mammalian adrenal cortex (Maia et al., 2002; Metz et al 2005; MC1R is the melanophore MSH-receptor, MC3R and MC4R are predominantly expressed in brain tissue). The intruiging observation remains that an aqueous extract of pars intermedia (conservatively dissected and thus mainly containing products of MSH- and somatolactin-cells *plus* CRF and CRFBP in the pars nervosa tissue therein) contains a corticotropic principle at least equipotent to ACTH (Metz *et al*., 2005). We are investigating the possibilities now.

Plasma MSH-levels do rise in chronically stressed carp (Metz *et al*, 2005) as in tilapia (Lamers *et al*., 1994), but maybe with another purpose? We consider the possibility of pituitary MSH feedback on central mechanisms to regulate food intake during stress (Cerda-Reverter *et al*., 2003). We have cloned carp leptin and assuming a rather pregnant role for leptin in control over neuronal activity involved in feeding behaviour, we now can evaluate anorexigenic (MSH, CART, Stat3, Socs3, CRF) and orexigenic (NPY, AgRF) signals to these neurons. Another function of MSH that requires more attention is its peripheral lipolytic activity in mammals (Brennan et al., 2003; Forbes *et al*., 2001) as well as in fish (Yada *et al*, 2002). Stress conditions may require an additional energy source

beyond glycogen/glucose. The consistent rises in plasma MSH seen during chronic stress in fish would be in line with this hypothesis.

The headkidney. The interrenal tissue of fish represents a peculiar anatomical situation with the cells producing cortisol and those that produce the catecholamines adrenalin and noradrenalin being intermingled and arranged along the cardinal veins in the headkidney of the fish; the endocrine cells lie embedded in haematopoietic tissue. Such organisation suggests that the endproducts of the stress axes (catecholamines and cortisol) have direct paracrine access to the cells of the immune system. *Vice versa*, chemical signals of the immune system could exert direct paracrine actions on the steroid and chromaffin cells. An example of such a concerted signal response was demonstrated by immersion vaccination of carp: cortisol surges are combined with upregulation of acute phase genes of the crucial innate immune components (interleukin-1β, *i*NOS, α2-macroglobulin, serum amyloid protein-A and tumor necrosis factor α), all meant to fight the imminent threat of pathogen invasion (Huising *et al*., 2003). Just with this concept in mind, this headkidney tissue deserves far more attention than it receives right now. We wonder about all the possible feedbacks that the combined headkidney signals may exert at every level of the stress axis.

The endocrine stress axis in action viewed by BOLD fMRI

No doubt exists that what we call the endocrine stress axis, *i.e.* the NPO-pituitary gland-interrenal cell-axis, is involved in the regulation of stress responses and this notion is based on extensive analyses of the physiology, biochemistry and molecular biology that show more or less predicted responses in stress paradigms. Yet, to the best of our knowledge there is no report on the visualisation of stress axis activity *in vivo*, and most certainly not in fish. In a wonderful collaboration with Dr. A. Van der Linden and colleagues we

developed a paradigm to study the stress axis activity in a high resolution MRI setup (Figure 3; Van den Burg *et al*., 2005b).

For this purpose, of course, we had to find out whether the (mild) anaesthesia required to immobilise the fish for imaging did not interfere with the initiation of the endocrine stress response. We thus set out to test several anaesthetics and succeeded to immobilise carp by irrigation of the gills with water containing low maintenance concentrations of MS222, while keeping the stress response intact. The stress response was monitored *i.a*. by changes in plasma cortisol levels. The actual stressor chosen was a sudden temperature drop (25 ºC to 15 ºC within 5 min), realised by switching between two water tanks from where the gills were irrigated. The fish was equipped with an antenna on top of its head and images were collected in the horizontal plane. This required the construction of a dedicated brain map for carp, which was made under supervision of our colleague Dr. Meek, renowned comparative brain anatomist (Meek and Nieuwenhuys, 1998). The "horizontal approach" was a requirement to avoid anticipated disturbances of the water irrigating the gills below the brain (the plane of gill adherence to the palatum is sufficiently distant from pituitary gland and brain basis). Just one other aspect addressed was the potential effect of noise that goes with MRI-analysis on the responses of the fish. To this end a sound CD was recorded of the actual setup in Antwerp and played with a gettho blaster at realistic decibels in the Nijmegen laboratory, where a fake MRI setup (but with the temperature switch) was constructed to sample carp for cortisol under conditions with and without noise. Fish certainly can perceive noises (have inner ears as higher vertebrates), and responded with a slight elevation of cortisol levels to handling and noise. However, the lightly anaesthetised carp responded with a very significant cortisol reponse within 5 minutes to the temperature drop.

Two major analyses were then carried out. The first concerned the distribution of blood by the use of injected iron particles as contrast during the temperature drop. A wealth of data was obtained, but the most remarkable observation was a massive drainage of blood from the brain to peripheral sinuses. We have interpreted these data as follows. Considering that the carp is a poikilotherm, the fish may benefit from a temporary insulation of the brain with the still warm blood. It should be noted that the brain is very close to the branchial circulation that can be considered as a heat exchanger system: any change in water temperature will change blood temperature quickly and this change is then reaching the brain first before the cooled blood reaches the body of the fish. In a poikilotherm, the biochemistry and thus the functioning of the brain is strictly dependent on the temperature of the ambient medium. The blood redistribution response to drastically changing ambient water temperature occurs as of 2 minutes following the onset of the temperature drop, which is 1.5 minutes after escape and stress responses are initiated (see below). In contrast to the blood volume reduction in the brain, the pars distalis of the pituitary gland received more blood, which not only accomodates increased activity of the ACTH-producing cells, but also facilitates the transport of ACTH to the interrenal tissue to stimulate production and release of cortisol.

To find out which areas of the brain were in volved in the initation of this endocrine response, a blood oxygen level dependent functional magnetic resonance imaging (BOLD-fMRI) study was done. The principle of this technique is that hemoglobulin with and without oxygen bound behaves magnetically differently and thus the oxygenation degree of the hemoglobulin in fact determines the constrast properties of the blood. Activated cells consume more oxygen, so that the local concentration of deoxygenated haemoglobuline increases and the signal intensity in the BOLD-fMRI images changes. It appeared that some areas were inactivated during or following the temperature drop, whereas others were

clearly activated (which may sound counterintuitive in a poikilotherm that is cooled down). Remarkable observations include activation of the NPO area (where the CRF-neurons reside), the tuberal hypothalamus (halfway NPO and pituitary gland) and the pituitary pars distalis (the pars intermedia is inactivated in this acute stress paradigm). More importantly, the activation followed a time-sequential pattern in line with activation of the endocrine stress axis: first the NPO (within 30 s), then the tuberal hypothalamus, followed by the pituitary pars distalis and then the interrenal cortisol release (as of 4-5 min). We concluded from these studies (Van den Burg *et al*., 2005b) that we succeeded to visualise the endocrine stress response in action! Presently we are working on other of the plethora of data then obtained that concern areas of the brain where sensory input from the buccal cavity is processed and relayed to the cerebellum for sensory processing and sensorimotor integration. The rationale behind this thought is that the fish would normally try to escape or avoid such cooler water.

Stress in very young fish, the onset of the endocrine stress axis

Understanding of the neuroendocrine regulation of the stress response of an organism is key to understand its general physiology and well being, as the signals involved in the stress response are powerful and influential. Cortisol (or corticosterone in some vertebrates) the key glucocorticosteroid stress hormone that is released following a cascade of signals from the brain down to the adrenal (or interrenal) cells, is an all-determining factor in steering the energy flows in the organism for optimal performance to cope with stressors; functions such as growth, reproduction and immune regulation are at risk when stressors persist and evoke too strong and long-lasting elevations of cortisol. Importantly, stress events (through actions of cortisol) at a given point in time may have effects on later development and life. For that very reason it is crucial to know when and how very young fish give their first

stress responses (Flik and Wendelaar Bonga, 2001) and how we can avoid such responses or how they become exaggerated in laboratory and aquaculture settings. In our work on carp larvae (Flik *et al*., 2002) we have observed that embryonic stages, still protected by the egg membrane, are able to produce cortisol when the embryo is disturbed by manipulation of the egg with forceps. We cannot exclude in this case that some direct pressure action of the squeezing procedure startled the cells that produce cortisol (although we very carefully squeezed to elicit three tail beats only), yet we favor a regulated (via CRF and ACTH) basis as also ACTH is produced very early during development of carp. Transient rises in ACTH in the prehatching stage indicate that the synthetic machinery is operational and up-regulated at such early stages. For cortisol this holds as well as cortisol is never stored but always produced according to needs. Admittedly, the yolk also harbors maternal sources of ACTH and cortisol, but then in the developing egg only a decline in levels could result from consumption, an increase can only be explained by embryonic synthesis. Interestingly, Pepels and colleagues (2004) working on Mozambique tilapia larvae quantified CRF immunoreactivity in the heads of larvae as young as five days post hatching, and a more sensitive immunohistochemistry extrapolated these results to even younger stages (2 days post hatch). An attractive hypothesis to test is that the fish is equipped with an endocrine stress axis some time before but certainly upon hatching. The consequences of such observations are obvious. We have to handle fertilised eggs and fish larvae with care. Experimentally we have shown that carp exposed to copper ions in the water respond differentially to levels of 0.3 and 0.8 micromolar. Both levels induce elevations in cortisol after hatching (the egg membrane is impermeable to copper ions), but the milder rise at the lower level of copper is enhancing growth and not affecting mortality, while the higher level results in a twenty percent higher cortisol content of the larvae, yet this results in retarded growth and significant increases in mortality. Apparently, the lower concentration

of copper evoked a cortisol response within the bandwidth of the carp, the higher concentration of copper presents an allostatic overload (McEwen and Wingfield, 2003). Understanding and establishing this bandwidth of the stress response will be a powerful tool in predicting the success of aquaculture practices.

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Figure 1. The vertebrate CRF system. Five members of the vertebrate CRF family have been identified, of which sauvagine has only been identified in a single frog species (*Phylomedusa sauvagei*). Note that urotensin-I is the fish ortholog of urocortin 1, found in tetrapods. All CRF family members signal via CRF receptors that belong to the superfamily of seven-helix G-protein-coupled receptors. Note that CRFR3 has to date only been identified in a single catfish species (*Ameiurus nebulosus*). The bioavailability of some CRF family members is modulated by CRF-BP. Recently, splice variants of CRFR1 and CRFR2 genes encoding truncate and soluble CRFRs have been described in human and rodents, which represents a novel way to modulate CRF-signaling.

Figure 2 . Serial, transversal sections of the pituitary gland of common carp immunostained with antisera raised against the C-terminus of carp beta-endorphin (A) and ACTH (B). Obviously, beta-endorphin-positive fibers project in the direction of the PI. These fibers most likely originate from the NPO, which is positive for both beta-endorphin and ACTH (NPO not visible in these sections). ACTH, however, is not immunoreactive in the pituitary-entering nerve fiber. Thus although derived from the same precursor, this suggests differential trafficking of the peptides beta-endorphin and ACTH. As shown, both POMC-derived peptides are also produced in the corticotrope cells of the PD. In the PI, ACTH is immediately further cleaved to alpha-MSH and CLIP, and thus leaves no ACTH immunoreactivity there. Note that the beta-endorphin immunoreactivity shown represents the non-acetylated full-length beta-endorphin, which we established immunohistochemically with the use of a N-acetyl beta-endorphin-specific antiserum (not shown in this figure). For clarity, nomenclature is shown only in panel A. Abbreviations: V, ventricle; NF, nerve fiber; PD, pars distalis; PI, pars intermedia.

Figure 3. Blood Oxygen Level Dependent functional Magnetic Resonance Imaging (BOLD-fMRI) in carp brain preoptic area (poa) and anterior midbrain tegmentum (mt), the tuberal hypothalamus (th) and in the pituitary gland following a 10°C temperature drop in the water irrigating the gills.

(A) BOLD-fMRI shows decreased (blue, mt) and increased (red, poa) BOLD-contrast, showing differential effects of the temperature drop on cellular activity; the area where the NPO CRH-cells reside (poa) is activated.

(B) (B) BOLD-fMRI of the tuberal hypothalamus; the area where a multitude of neural pathways between NPO and pituitary runs is activated.

(C) BOLD-fMRI of the pituitary gland; opposite responses in the pars distalis (pd; red/pink) and pars intermedia (pi; blue). Oxygen consumption increases in the pd (consistent with activation of ACTH cells) and decreases in the pi following an acute temperature drop.

(D) Cartoon depicting the time-sequential BOLD-fMRI events preceding the release of cortisol following an acute temperature drop (abbreviations as above). The observations are in line with the predicted sequence of events during activation of the endocrine HPI-axis.

Figure

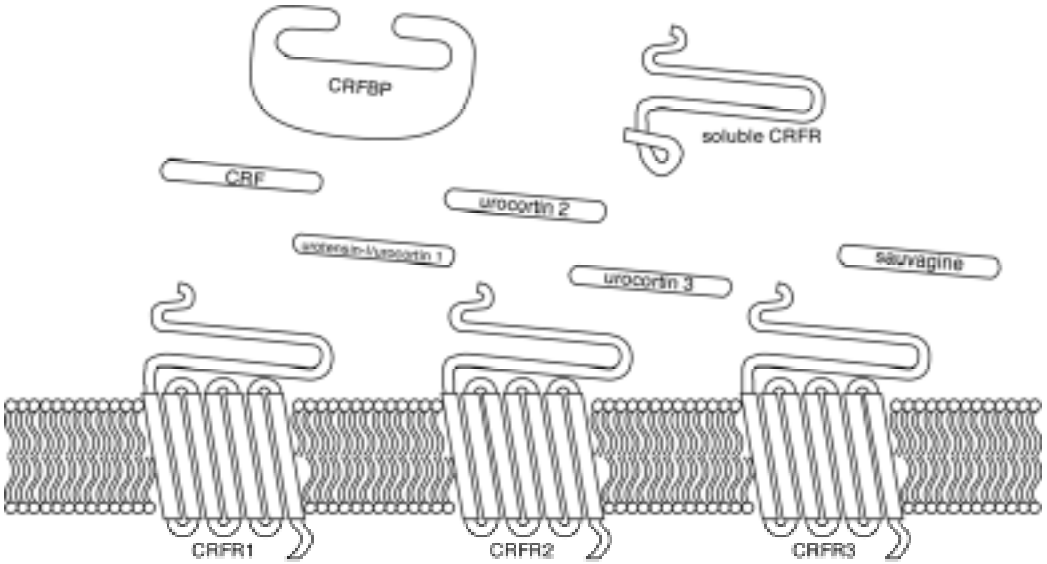
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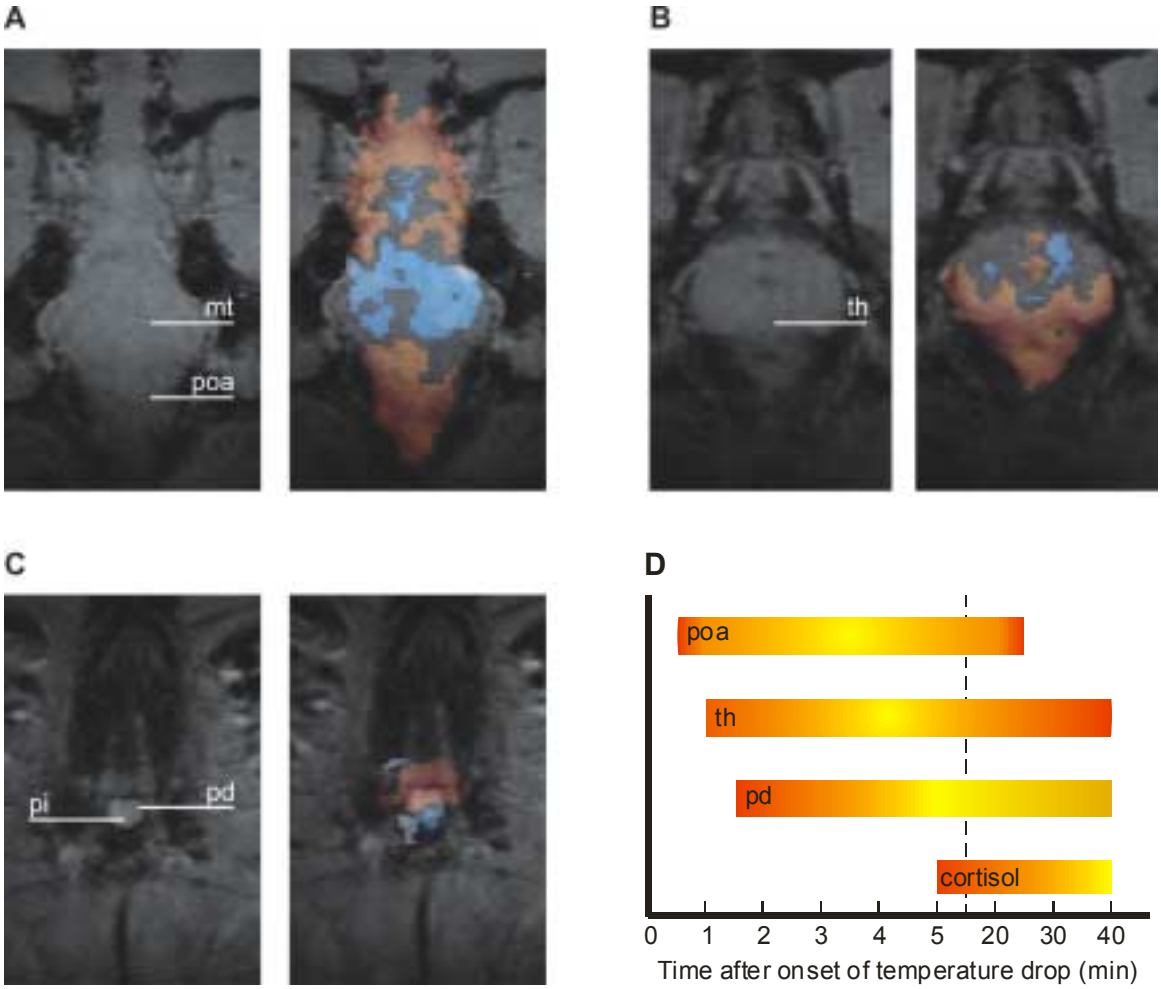
Figure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Time after onset of temperature drop (min)

Figure

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