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Bacterial lipopolysaccharide (LPS) modulates corticotropin-releasing hormone (CRH) content and release in the brain of juvenile and adult tilapia (*Oreochromis mossambicus*; Teleostei)

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Summary

Although immune endocrine interactions in teleost fish have been shown to involve adrenocorticotropin hormone (ACTH) and cortisol, the involvement of corticotropin-releasing hormone (CRH) has not been demonstrated. The present study investigates whether treatment with bacterial endotoxin (lipopolysaccharide, LPS) modulates brain CRH contents or *in vitro* CRH release in tilapia (*Oreochromis mossambicus*). 10 days LPS (*Escherichia coli*) exposure of juvenile tilapia (4.5 weeks post hatch) via the ambient water increased brain CRH and α-MSH content, whereas cortisol contents were not increased. This indicates that the elevation of brain CRH levels were not secondary to activation of HPI-axis. Adult tilapia were treated for 6 days with LPS (intraperitoneally) and were sampled before and after 24 h of confinement. Overall LPS pre-treatment modified the reaction of tilapia to the additional stressor of 24 h confinement, as interactions between LPS treatment and confinement were observed at the level of the hypothalamus (diencephalic CRH content), the pituitary (CRH and α-MSH content) and in plasma glucose levels. *In vitro*, LPS pre-treatment abolished CRH release from telencephalic tissues induced by norepinephrine, one of the CRH secretagogues released during stress *in vivo*. This effect might be a mechanism of action through which LPS *in vivo* abolished the up-regulation of telencephalic CRH induced by confinement stress. Our results provide evidence that the role of CRH in immune–endocrine interactions is a phylogenetically old mechanism, and we here demonstrate that LPS molecules are able to locally modulate CRH release in the central nervous system.

Key words: immune system, immune-endocrine communication, endotoxin, corticotropin-releasing factor, CRF, α-MSH, cortisol, plasma CRF, HPI-axis, pituitary, stress, teleost, telencephalon, NE, norepinephrine, 5-HT, serotonin.

Introduction

Corticotropin-releasing hormone (CRH) is recognised as the hypothalamic key factor in the neuro–endocrine stress response in vertebrates, including teleost fish. CRH also acts as a neurotransmitter outside the hypothalamus (Cook, 2002; Pepels et al., 2002a,b; Pich et al., 1995). In certain structures, such as the amygdala in the telencephalon, the peptide regulates anxiety, autonomic functions and coping behaviours in response to stress (Cook, 2002; Gray, 1993). In addition, at least in mammals, CRH is involved in the communication between the immune and the neuro–endocrine systems (Berkenbosch et al., 1987). Bacterial infections, or treatment with the endotoxin lipopolysaccharide (LPS; bacterial cell wall components of Gram-negative bacteria), stimulate the expression of CRH in the amygdala of the telencephalon and in the paraventricular nucleus (PVN) of the hypothalamus (see review by Turnbull and Rivier, 1999). The latter stimulation ultimately leads to an activation of the pituitary–adrenal (PA) axis.

The question whether LPS treatment also affects the amount of CRH peptide present in the brain of teleost fish has not been clarified and is investigated in the present study. Immune–endocrine communication also occurs in fish, as LPS administration modifies the activity of the pituitary–interrenal (PI) axis (Balm et al., 1995; Haukenes and Barton 2004; Holland et al., 2002; Wedemeyer, 1969; White and Fletcher, 1985). At present, it is unknown whether the *in vivo* modulation of the PI axis is an indirect effect caused by modulation of hypothalamic CRH or a direct effect of LPS on pituitary or head kidney tissue. *In vitro* treatment of tilapia (*Oreochromis mossambicus* Peters 1852) with LPS from *Escherichia coli* (*E. coli*) resulted in modulation of the activity of the HPI-axis at the level of the cortisol producing tissue, which became markedly less responsive to adrenocorticotropin hormone (ACTH; Balm et al., 1997).

In tilapia as in other fish, hypophysiotropic CRH-ir neurons directly innervate the pituitary gland and, under certain

stressful conditions, stimulate the release of pro-opiomelanocortin (POMC)-derived peptides, such as ACTH and α-melanocyte-stimulating hormone (α-MSH), which in turn stimulate the synthesis and release of cortisol from the interrenal cells located in the head kidney (Balm et al., 1994; Wendelaar Bonga, 1997). Besides its corticotropic role and its role in body coloration, α-MSH may be involved in immune–endocrine interactions in fish as well as in mammals (see review by Balm, 1997; Lipton and Catania, 1997).

In the brain of tilapia the largest CRH-ir cell population is found in the lateral part of the ventral telencephalon Vl (Pepels et al., 2002a,b). These CRH-ir cells in the Vl region are not directly involved in regulation of the pituitary because these Vl cells massively innervate the anterior part of the lateral dorsal telencephalon (Dla; see Fig. 1, original data in Pepels et al., 2002a). This Vl-Dla projection contains the highest amount of CRH-ir measured within the tilapia brain (Pepels et al., 2002a). We recently reported that during the acute stress associated with capture, CRH is secreted into the blood, probably from these telencephalic centres (Pepels et al., 2004; Fig. 1).

Fish are markedly tolerant to high doses of LPS compared with mammals (up to a 1000-fold; Berczi et al., 1966) and effects of LPS or Gram-negative bacterial infections become deleterious in fish after chronic infection, but are less severe after acute infection. Previously, we showed that chronic treatment with LPS (Balm et al., 1995) or with murine Interleukin-1 (IL-1; Balm et al., 1993) altered the activity of the PI axis. For these reasons we treated tilapia chronically. The present study compared the effects of LPS on hypophysiotropic and non-hypophysiotropic CRH-ir neurons with particular emphasis on telencephalic CRH. Initially, juveniles were treated stress-free with *E. coli* LPS via immersion. Fish larvae immersed in LPS-containing water have been shown to take up LPS (Dalmo et al., 2000). We recently have shown that rapid sampling and processing of juveniles can be achieved leading to whole brain CRH, α-MSH and tissue cortisol levels characteristic for unstressed fish (Pepels et al., 2002c; Pepels and Balm, 2004). Next, adult fish were used to obtain specific information on which of the CRH-immunoreactive (ir) brain regions are modulated by LPS treatment. LPS treatment was combined with confinement stress. In fish, the acute-phase response and the susceptibility to bacterial infections are modulated by stress (Maule et al., 1989). LPS is the bacterial constituent that triggers the acute-phase response and, thus, we anticipated that actions of LPS could be modulated by stress. Finally, to investigate whether LPS may directly act at the level of the CNS, the telencephalon of tilapia was superfused *in vitro* with LPS. Our previous results indicate that the tilapia telencephalon can be used *in vitro* to study regulation of CRH release (Pepels et al., 2002b).

In mammals there is still debate on the most likely route via which LPS *in vivo* stimulates cytokine release, and how these cytokines in turn stimulate CRH synthesis in the brain (reviewed by Turnbull and Rivier, 1999). Lipopolysaccharide tested on hypothalamic explants of rats, inhibited the *in vitro* CRH release (Pozzoli et al., 1994), although others reported no effects of LPS. In contrast to the CNS of mammals the CNS of fish contains extremely high numbers of sessile macrophages (Dowding and Scholes, 1993). These can act as intermediates for LPS actions as fish macrophages produce cytokines such as interleukin-1β (IL-1β; Zou et al., 1999) and tumour necrosis factor-α (TNF-α; MacKenzie et al., 2003), when encountering LPS or bacteria. Interleukin-1β is the common messenger that mediates LPS signalling to CRH neurons in mammals (Berkenbosch et al., 1987; Mirtella et al., 1994; Turnbull and Rivier, 1999), and the fish Il-1β gene has recently been sequenced (Zou et al., 1999). Holland et al., 2002 showed that homologues IL-1β stimulates PI-axis activity in trout and serves as an intermediate for LPS effects on the PI-axis.

Materials and methods

The research was approved by the Institution's Animal Care and the National Animal Ethical Committee and was conform

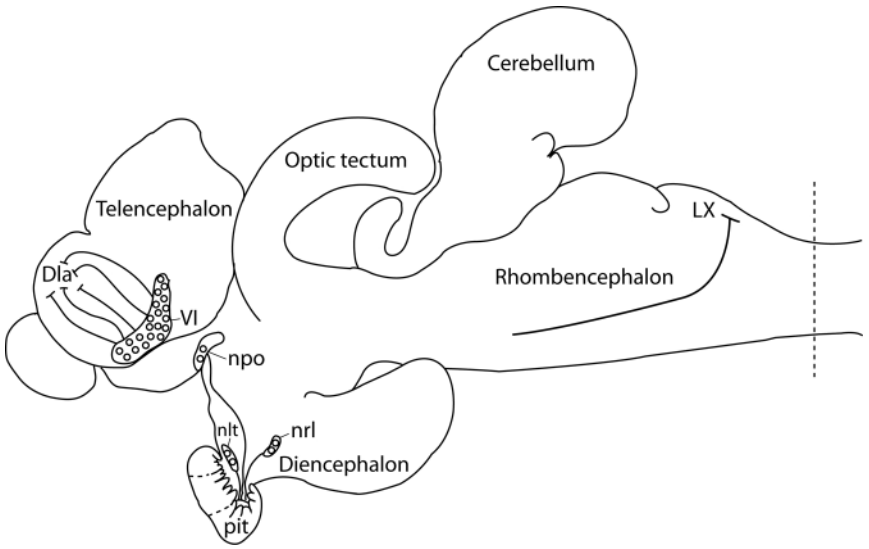

Fig. 1. Depicts the major CRH-ir cells groups and CRH-ir pathways in a sagittal section of the brain of tilapia (original data in Pepels et al., 2002a, 2004). Each circle represents approximately 25 CRH-ir cells. The hypophysiotropic CRH-ir neurons located in the nucleus preopticus (npo), nucleus recessus lateralis (nrl) and nucleus lateralis tuberis (nlt) project into the pituitary (pit). The largest CRH-ir cell group is found in the lateral part of the ventral telencephalon (Vl) and projects mainly into the anterior subdivision of the lateral part of the dorsal telencephalon (Dla). CRH-ir terminals have also been found locally in Vl. Many blood capillaries are found in close proximity of the CRH-ir cells in Vl and a collecting vein is located in the sulcus of Vl (Pepels et al., 2004). In the vagal lobe (LX) CRH-ir terminals are present.

to the guidelines of the UFAW (Universities Federation for Animal Welfare).

Exposure of juvenile tilapia to LPS

Tilapia (*Orechromis mossambicus* Peters 1852) breeding couples from our laboratory stock were kept in glass tanks of 500 l supplied with Nijmegen tapwater of 24°C under a 12 h photoperiod.

The developmental stage and corresponding age of the larvae was determined according to the classification criteria of Bauerle and Voss (1993). Tilapia life stages younger than 4 weeks post hatching (wph) are here described as larvae, and life stages older than 4 wph as juveniles. Larvae obtained from the mouth of breeding females were classified at stage 7 corresponding with 7 days post fertilisation (7 dpf) and 2 days post hatching (2 dph). Larvae were fed daily with Micro-min® (Tetramin GmbH, Melle, Germany) at a ration of 2.5% (w/w) of their total body mass. During the first week after hatching, larvae were maintained in a 120 l freshwater aquarium, after which period 64 larvae were equally divided among four transparent plastic tanks (height × depth × length = 25×20×30 cm) each containing 4 l of freshwater (Nijmegen tapwater). Water was continuously refreshed (4 l day^{-1} $tank^{-1}$) and aerated. After an adaptation period of 3.5 weeks in the 4 l tanks, flow-through was stopped and two tanks received either 8 ml of water (mixed by the air supply), or 8 ml of water containing LPS (*E. coli*, serotype 0111:B4, Sigma L-2630, lot no. 42K4120). Thereafter, the juveniles were observed for 10 min to see whether there was a behavioural reaction to the addition of water or LPS. At this moment the juveniles were approximately 4.5 wph. The LPS concentration in the fish water was 12.5 mg l^{-1}. Starting at exposure day 7, flow-through was resumed (1 l day^{-1} $tank^{-1}$) with water (control tanks) or with water containing LPS (12.5 mg l^{-1}). After 10 days of LPS exposure the two control and LPS tanks were sampled. Tanks were decanted above a fish net and larvae were immediately frozen by dipping the net into a mixture of dry ice and methanol. This sample procedure was completed within 30 s per tank. Larvae were removed from the dry ice methanol mixture, weighed individually and kept in Eppendorf cups at –20°C. Eight larvae from each group were processed further. After measuring body length, the head region and body were separated according to the procedure described previously (Pepels et al., 2002c). Briefly, the fish was put on its lateral side on an ice-cold plate under a binocular and the head was dissected with a scalpel. The plane of dissection ran from the rostral tip of the dorsal fin to the most caudal part of the operculum. Heads, including CNS, pituitary and head kidneys, were homogenised individually (Pepels et al., 2002b,c) in a mixture of methanol and 0.01 N HCl (3:1 volume) containing ascorbic acid (6 mmol l^{-1}) and aprotinin (Bayer: 250.000 KIU l^{-1}). CRH and cortisol levels were determined by radioimmunoassays (RIA) validated for tilapia larvae (Pepels et al., 2002b,c). α-MSH was determined by RIA (Balm et al., 1993) in which serial dilutions of juvenile head homogenates displaced radiolabelled α-MSH from the antibody in a parallel fashion with dilutions of the α-MSH standard (not shown).

The CRH values measured in head homogenates represent the sum of central nervous system (CNS), pituitary and head kidney CRH. Levels of head kidney CRH in comparison to the CRH content of the CNS are less than 1% in tilapia and thus negligible (Pepels and Balm, 2004). The α-MSH levels measured mainly represent pituitary MSH, as immunohistochemical α-MSH staining of the brain in young life stages did not reveal α-MSH-ir cells (Pepels and Balm, 2004).

Exposure of adult tilapia to LPS

Thirty-two tilapia (*Oreochromis mossambicus*) of mixed sex from our laboratory stock were equally distributed between two 120 l aquaria of neutral background. Water was continuously aerated, filtered (Eheim Pumps, GmbH, Deizisan, Germany) and refreshed (10 l h^{-1}). Water temperature was 24°C, the photoperiod was 12 h, and fish were fed twice daily with Tetramin tropical fish food (Tetramin GmbH) at a daily rate of 1.5% (w/w) of their body mass.

Fish were left undisturbed in their aquaria for 4 weeks. To obtain samples from undisturbed fish, on four alternate days one fish from each aquarium was quickly netted and sampled for blood and brain tissue (pre-injection sample). On each of these 4 days, aquaria were sampled in random order and sampling of two fish was completed within 2 min. Blood was collected in tubes containing EDTA (1.5 mg ml^{-1}) and aprotinin (3000 KIU ml^{-1}; Trasylol, Bayer AG, Leverkusen, Germany). Collected blood was centrifuged, and plasma was separated and stored at –20°C until analyses. Following spinal dissection, fish were weighed and sexed, fork length was measured, whole brains were removed including pituitary, and the tissue samples were frozen at –20°C until analyses. Starting 2 days after taking the final pre-injection sample, the remaining 12 fish per aquarium were injected intraperitoneally either with saline, or with 3 mg kg^{-1} body mass *E. coli* LPS (Sigma, serotype 0111:B4, lot no. 110K4060). Fish were injected on three alternate days (day 1, 3, 5) and were exposed to LPS for 6 days. 1 day following the final injection, six fish were sequentially sampled from each aquarium (pre-confinement). As the sampling protocol influences plasma cortisol (Balm et al., 1994) and plasma CRH (Pepels et al., 2004), strict care was taken to synchronise sampling of the two groups. Fish were sequentially sampled in alternating order (every other; 1st LPS fish, 1st saline fish, 2nd LPS fish, 2nd saline fish and so on until fish 6) at 2 min intervals. Sampling of the two groups was completed within 24 min. The remaining six fish per aquarium were confined in a net (approximately 1 l volume) in their home aquarium, and were sampled 24 h later. The same sampling protocol was used and within 24 min all fish were sampled.

Pituitary and brain samples were treated as described by Pepels et al. (2002b) with slight alterations of the dissection into various brain parts (see left upper panel in Fig. 2). Briefly, brains were dissected under a binocular on an ice-cold petridish into: telencephalon, diencephalons, rhombencephalon and

pituitary. The tectum–midbrain and spinal cord parts were discarded (grey parts in the Fig. 2). Brains from pre-injected fish were not further dissected. CRH levels in the tissue extracts were measured by RIA (Pepels et al., 2002b), and expressed as pg CRH tissue^{-1}. Plasma cortisol was assayed by RIA (Balm et al., 1994), plasma glucose was measured using a commercial kit (Boehringer Mannheim, Germany), and chloride was quantified by flame photometry. Plasma CRH, α-MSH (in pituitary and plasma) was assayed by RIA (Balm et al., 1993; Pepels et al., 2002b).

In vitro *basal and stimulated CRH release during LPS incubation*

Superfusion of telencephalic tissues was performed as described previously (Pepels et al., 2002b). Telencephalic tissues were derived from unstressed tilapia (*N*=60, bodymass 31±2 g), and cut into four pieces. In the first *in vitro* experiment four superfusion chambers, each containing telencephalic tissue from three tilapia, were superfused with artificial cerebrospinal fluid (acf-medium) containing 200 μmol l^{-1} ascorbic acid (Pepels et al., 2002b). After reaching a steady CRH release, tissues were during the time period 210–240 min pulsed with 5×10^{-6} mol l^{-1} NE (norepinephrine–hydrogentartaat; Centrafarm Company, RVG 50833 UR, Etten Leur, Netherlands), and during 270–300 min were pulsed with 5×10^{-6} mol l^{-1} serotonin (5-hydroxytryptamine-hydrochloride, 5-HT; Sigma H9523). Superfusion fractions were collected and stored at –20°C. CRH was analysed by RIA (Pepels et al., 2002b).

In the second *in vitro* experiment 16 superfusion chambers, each containing telencephalic tissue from three fish, were superfused for 225 min with acf-medium. Thereafter, eight control chambers were perfused with acf-medium and the remaining eight chambers were perfused with acf-medium containing 50 μg LPS ml^{-1}. The *Escherichia coli* LPS used (serotype 0111:B4) had been chromatographically purified by gelfiltration (Sigma L3012). This purified LPS was used because standard LPS preparations contain glutamate and adenosine contaminants (Hardy and White, 2001), which have been shown to stimulate the release of NE by cortex tissue in rats (Hardy and White, 2001). After 210 min of LPS treatment, tissues were stimulated for 30 min with 5×10^{-6} mol l^{-1} norepinephrine. Finally, to test whether the tissues were still viable and in a physiologically reactive state, all tissues received acf-medium containing a high potassium concentration (56 mmol l^{-1} KCl) between 500 and 515 min. Superfusion fractions were collected and stored at –20°C until use for CRH analysis by RIA. In the CRH RIA no interference of LPS was found when analysing acf-medium containing 50 or 500 μg LPS ml^{-1}.

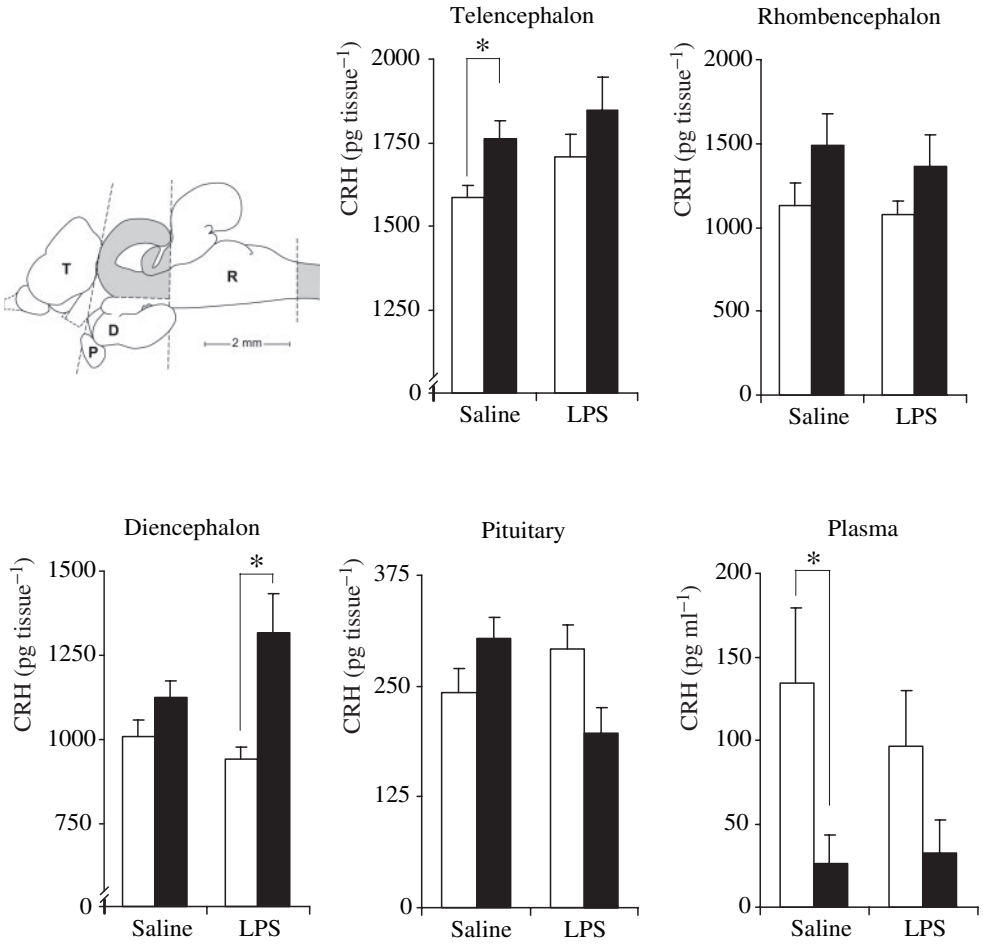

Fig. 2. Effects of injection with saline or *E. coli* LPS on brain and pituitary tissue CRH levels in tilapia (*N*=6 in all cases). Open bars indicate pre-confinement values and solid bars indicate values of fish sampled following 24 h confinement. The asterisks (*) indicate confinement effects within a saline- or LPS-treatment group (see *Presentation of data and statistics* for details). Upper left panel: sagittal overview of the brain of tilapia showing the dissected parts (telencephalon, diencephalon, rhombencephalon and pituitary). The grey areas indicate brain parts that were discarded: tectum and midbrain, and the rostral part of the spinal cord.

Table 1. *Effects of exposure on tilapia juveniles**

	Body mass (mg)	CRH (pg)	α-MSH (pg)	Cortisol (pg)
Control (*N*=16)	32.6±1.5	202±9	275±9	36.7±3.4
LPS immersed (*N*=16)	33.2±1.8	233±10	322±10	40.2±2.5
Control *vs* LPS	NS	$P<0.03$	$P<0.001$	NS

*10 days LPS (*E. coli*, 12.5 mg l^{-1} tank water). Hormone levels represent pg per fish (see text). NS, not significantly different.

Presentation of data and statistics

Values presented are means ± S.E.M. (*N*–1). In the experiment using juveniles, the data were subjected to analysis of variance (two-way independent ANOVA; SPSS statistical package 11.5 version for windows; SPSS Inc., Chicago, USA) with tank and LPS treatment as independent variable factors. Since the variables CRH and α-MSH content were not influenced by the tank factor ($P>0.533$ and $P>0.880$, respectively; two-way independent ANOVA, SPSS), we pooled the values from duplicate tanks to yield *N*=16. Student's *t*-test (two-sided) was used as *post-hoc* test, and $P<0.05$ was accepted as indicative of significant differences.

In the experiment using adult fish the condition (K) factor was calculated from 100 w l^{-3} (w = body wet mass; l = fork length). As observed previously (Balm et al., 1994) capture sequences were observed in plasma cortisol. Plateau plasma cortisol levels were calculated by taking the average of cortisol levels of fish 4, 5 and 6 from each group (Balm et al., 1994). Hormone or plasma parameters were subjected to analysis of variance (two-way independent ANOVA, SPSS) with LPS treatment and confinement stress as independent variable factors. Student's *t*-test (two-sided) was used as *post-hoc* test, and $P<0.05$ was accepted as indicative of significant differences. In the figures and tables, levels of significance are indicated as follows $P<0.05$*, $P<0.01$** and $P<0.001$***.

Maximally stimulated *in vitro* CRH release was calculated after determining the peak value of stimulation for each superfusion chamber. Differences between maximally stimulated and prepulse values were tested with the paired Student's *t*-test (two-sided).

Results

LPS treatment of juveniles

No behavioural reaction to the LPS addition was observed in the juvenile fish (~4.5 wph). During the 10 days of LPS exposure no difference in feeding response, nor in body colour was observed between groups. There was no mortality among the control or LPS-exposed juveniles. Body mass or length at the end of the experiment did not differ between the groups (Table 1). An effect of LPS on homogenate CRH and α-MSH contents was revealed by the ANOVA ($P<0.02$ and $P<0.01$, respectively). Heads of LPS exposed larvae contained significantly more CRH ($P<0.03$) and α-MSH ($P<0.001$), but not cortisol, when compared with controls (Table 1).

LPS treatment adult tilapia

Throughout the experiment, the feeding response as well as the body colouration remained unaltered, and none of the fish died as a result of the experimental treatments. Plasma cortisol levels of fish sampled pre-injection did not differ between the tanks (4.3±0.3 and 6.0±0.6 ng ml^{-1} in control and *E. coli* designated fish, respectively; *N*=4). Also whole brain CRH content (including tectum and pituitary) of fish sampled pre-injection did not differ between the 2 tanks (*N*=4/tank: 6119±419 and 6461±512 pg/fish in control and *E. coli* LPS designated groups, respectively).

In fish sampled 1 day following the final injection with saline or LPS no differences in body mass or K factor was observed between saline and LPS treatment groups (not shown, ANOVA: $P>0.226$ and $P>0.215$, respectively). The dissection of the brains was carried out according to the dotted lines in the diagram of Fig. 2 (left upper panel). Overall, confinement stress significantly affected diencephalic and telencephalic CRH, plasma CRH, plasma α-MSH, plasma cortisol and plasma chloride levels (ANOVA $P<0.02$, $P<0.01$, $P<0.01$, $P<0.001$, $P<0.02$ and $P<0.01$, respectively). In saline-treated fish confinement stress increased telencephalic CRH ($P<0.05$) content, plasma cortisol ($P<0.02$), plasma glucose ($P<0.01$) and decreased plasma CRH ($P<0.05$) and plasma α-MSH ($P<0.02$) levels (Fig. 2, Table 2). In LPS treated fish confinement increased plasma glucose ($P<0.001$), diencephalic CRH ($P<0.02$), and decreased plasma chloride levels ($P<0.05$; Fig. 2, Table 2).

Table 2. *Physiological parameters in tilapia treated with either saline or* E. coli *LPS*

	Saline		*Escherichia coli* LPS	
	Pre-confinement (*N*=6)	24 h confinement (*N*=6)	Pre-confinement (*N*=6)	24 h confinement (*N*=6)
Plasma α-MSH (pg ml^{-1})	130±41	37±13*	182±44	21±5**
Pituitary α-MSH (ng pit^{-1})	135±7	154±11	136±10	104±20
Plasma cortisol (ng ml^{-1})†	141±31	326±39*	222±20	331±28
Plasma glucose (mg 100 ml^{-1})	61±3	85±5**	49±5	106±10***
Plasma chloride (mmol l^{-1})	134±3	128±4	135±2	120±4*

†Plasma cortisol levels are plateau levels reached during sampling (*N*=3). The asterisks (*) indicate confinement effects within a saline or LPS treatment group (see *Presentation of data and statistics* for details).

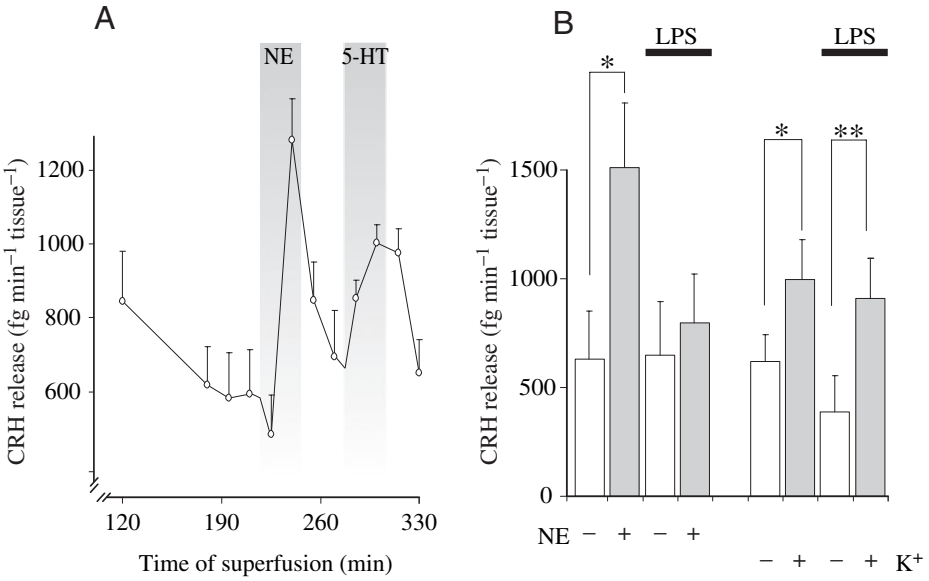

Fig. 3. (A) Basal and stimulated *in vitro* CRH release by telencephalic tissues. Tissues were stimulated for 30 min with 5×10^{-6} mol l^{-1} norepinephrine (NE) or 5×10^{-6} mol l^{-1} serotonin (5-HT). (B) Maximally stimulated *in vitro* CRH release by telencephalic tissue stimulated (grey bars) with 5×10^{-6} mol l^{-1} norepinephrine (NE) or 56 mmol l^{-1} K^+. Before stimulation, tissues were superfused with control medium or with medium containing LPS (50 μg LPS ml^{-1}).

LPS treatment alone did not affect the parameters studied, but interactions were observed between LPS treatment and confinement stress regarding diencephalic CRH, pituitary CRH, pituitary α-MSH and plasma glucose levels (ANOVA $P<0.05$, $P<0.02$, $P<0.05$, $P<0.02$, respectively).

Effects of in vitro *LPS treatment on CRH release from telencephalic tissue*

In the first 3 h of superfusion CRH release decreased after which period a steady state was reached with a basal release of approximately 550 fg min^{-1} $tissue^{-1}$ (Fig. 3A). Both neurotransmitters stimulated the CRH release (Fig. 3A). The maximally stimulated CRH release induced by NE was 1281±105 fg min^{-1} $tissue^{-1}$ ($P<0.001$ compared to prepulse values) and by serotonin was 1004±67 fg min^{-1} $tissue^{-1}$ ($P<0.02$). These rates corresponded to 223±66% and 187±36% of pre-pulse values, respectively.

Next, telencephalic tissues were superfused with or without LPS-containing medium. Superfusion with LPS did not alter the basal CRH release compared with controls (Fig. 3B). Prepulse release values were 629±223 and 647±248 fg min^{-1} $tissue^{-1}$ for control and LPS-treated tissues, respectively (Fig. 3B) However, LPS pre-treatment abolished the CRH response of the tissue to NE (Fig. 3B). Control tissues displayed a maximally stimulated CRH release of 1512±295 fg min^{-1} $tissue^{-1}$, when receiving NE, which corresponded to 240±47% of pre-pulse values. In reaction to the 56 mmol l^{-1} K^+ pulse, control tissues displayed a maximally stimulated CRH release of 997±186 fg min^{-1} $tissue^{-1}$ ($P<0.05$; Fig. 3B) and LPS superfused tissues displayed a maximal stimulated CRH release of 908±186 fg min^{-1} $tissue^{-1}$ ($P<0.01$; Fig. 3B). These rates corresponded to 142±26% and 219±46% of pre-pulse values, respectively.

Discussion

LPS effects in juvenile tilapia

The increase in whole brain CRH contents in juvenile tilapia following 10 days LPS exposure provides the first evidence for the involvement of CRH in immune–endocrine interactions in teleost fish. Previously, attempts had been made to stimulate immune defence mechanisms in young life stages of fish by LPS bath immersion (Dalmo et al., 2000), and by using LPS-coated feed (Guttvik et al., 2002). The rationale was that the increase in susceptibility of fish larvae and juveniles to bacterial infections, usually observed after handling or transport stress, would be less prominent following LPS pre-treatment.

Our results on the effectiveness of LPS treatment via the ambient water are consistent with previous findings in Atlantic halibut (*Hippoglossus hippoglossus*) larvae. Dalmo et al. (2000) reported that after 10 days of bath exposure to LPS (*Aeromonas salmonicida*) immunoreactive LPS was found in the gut lumen, intestinal epithelial cells, kidney ducts and epidermis, and fluorescein-labelled LPS was found in endothelial cells in veins adjacent to the intestinal tissue. This strongly indicates that LPS in yolk sac larvae is absorbed via the gut and the integument, and subsequently transferred via the circulation to the kidneys. We have not studied the uptake of LPS in our fish, but our juveniles had absorbed their yolk sac, and had already developed a skin with scales. As juvenile tilapia only feed in the short period during which the flake food floats on the water surface, the uptake of LPS precipitated on the feed may be neglected. Therefore, LPS must have been taken up predominantly by drinking. Indeed, using the freshwater drinking rate described for tilapia larvae by Lin et al. (2000), it can be calculated that during the 10 day experimental period our juvenile tilapia have ingested 22 mg LPS kg^{-1} body $mass^{-1}$ via the water. This dose is in the same order of magnitude as that administered to the adult tilapia in this study.

Our juvenile fish were 5.5 weeks (4.5 wph) of age at the start of the experiment and we propose that the response of their immune system to the LPS antigen triggered the neuro–endocrine effects observed. The first appearance of lymphocytes in thymus, head kidney, and in gut-associated lymphoid tissues of tilapia occurs already 1 wph (Dogget and Harris, 1987). In one-week-old carp (*Cyprinus carpio*), monocyte/macrophage-like cells are already present in thymus, head kidney, spleen, and importantly in blood and gut-epithelium (Romano et al., 1997). B cells appear in the carp head kidney in the second week post fertilisation (Romano et al., 1997). In fish the capacity to produce antibodies, which evidences the presence of mature immune cells and functional cytokine pathways, generally starts at an age between 3–8 weeks (see review Tatner, 1996).

The increases in CRH and α-MSH contents after LPS exposure were not related to differences in growth or to enhanced development, as mass and length of juveniles did not differ between LPS-treated and control groups. Growth stimulation by LPS has been reported in Atlantic salmon (*Salmo salar*) juveniles but only after long term (64 days) feeding of LPS coated feed (Guttvik et al., 2002).

Since cortisol levels did not differ between groups we conclude that LPS treatment effects were not secondary to HPI activation. The lack in cortisol response after LPS exposure can not be attributed to immaturity of the juvenile HPI-axis. In the first week post hatching the hypothalamus, telencephalon and pituitary gland of tilapia are CRH-immunoreactive (CRH-ir), POMC-derived peptides are expressed by the pituitary gland (Pepels and Balm, 2004), and the cortisol stress response is already present (Pepels and Balm, 2004). Results therefore suggest that LPS directly affected the CRH system after 10 days of treatment.

Similarly, the increase of α-MSH content after LPS exposure may represent a direct effect of LPS at the level of the pituitary. Previously we showed that *in vitro* LPS treatment alters the α-MSH release from pituitary melanotropes in tilapia (Balm et al., 1993, 1995). Since there was no HPI-axis activation or difference in body colour between experimental groups, the α-MSH increases after LPS exposure may subserve immune related actions. As early as 1938 an immunoregulatory role for melanotropins was reported as fishes' susceptibility to infectious diseases was influenced by tank colour (Sumner and Douderoff 1938). In mammals α-MSH acts as a potent immunomodulator, which inhibits fever and all types of inflammation (Lipton and Catania, 1997). In the periphery, α-MSH inhibits the production, release and actions of pro-inflammatory cytokines, such as IL-1, interferon (IFN) and TNF-α (Lipton and Catania, 1997). Interestingly, in fish the peptide has been reported to have predominantly stimulatory effects on immune responses *in vitro*, where it stimulates phagocytosis of leucocytes (see review Harris and Bird, 2000).

LPS effects on adult tilapia

To obtain more information concerning the specific CRH-ir brain regions influenced by LPS treatment further dissection of the brain was performed using adult specimens. The pre-injection results demonstrated that at the start of the experiment no differences in the HPI-axis activity existed between tanks as plasma cortisol and brain CRH levels were similar between groups. These CRH levels corresponded to previously measured CRH levels in adult unstressed tilapia (Pepels et al., 2002b). Overall, 6 days pre-treatment with *E. coli* LPS modified the reaction of tilapia to the additional stressor of 24 h confinement, as interactions between LPS treatment and confinement were observed at the level of the hypothalamus (diencephalic CRH), the pituitary (CRH and α-MSH contents) and in plasma glucose levels. Our results on the modulatory effect of LPS on the stress response appear to corroborate the results of Haukenes and Barton (2004), who found that crowding stress affected the HPI response to a single injection with LPS.

Confinement elevated telencephalic CRH levels in controls, but not in LPS-treated fish. Whether the small population of hypophysiotropic CRH-ir cells located in the nucleus preopticus (npo; CRH in these cells accounts for approximately 5% of total telencephalic CRH contents (Pepels et al., 2002a), or the large non-hypophysiotropic population of CRH-ir cells located in the lateral part of the ventral telencephalon (Vl) was affected by confinement cannot be ascertained from the present results. The stress-induced elevation of plasma cortisol levels observed in the controls only could point to activation of the npo cells in these animals. Similarly, Ando et al. (1999) observed an increase in npo CRH mRNA expression associated with confinement in rainbow trout (*Oncorhynchus mykiss*). Activation of the non-hypophysiotropic Vl CRH cells on the other hand would resemble the stress-induced activation observed in CRH cells located in the amygdala of rats (Pich et al., 1995) and sheep (Cook, 2002). We previously discussed that the lateral part of the ventral telencephalon (Vl) in tilapia might be comparable with the mammalian amygdaloid complex since in both situations these regions contain the largest extra hypothalamic CRH-ir cell population (Pepels et al., 2002a).

The most striking effect of the 6 days *E. coli* endotoxin pre-treatment was the modulation of the CRH response to confinement at the level of the diencephalon. The diencephalon of teleost fish including tilapia contains hypophysiotropic CRH-ir cells located in hypothalamic nuclei (lateral tuberal nucleus and recessus lateralis nucleus), which directly innervate the melanotrope and corticotrope cells in the pituitary gland (Pepels et al., 2002a). Notably, at the level of the pituitary combined effects of LPS and confinement were also found on CRH and on α-MSH. In fish the melanotropes are targets for the CRH-ir nerve endings in the pituitary.

Previously, we reported that CRH appears rapidly, within 5 min after the onset of capture, in plasma in tilapia (Pepels et al., 2004). This rise in plasma CRH was abolished when fish were confined for 48 h before capture. In the present study this inhibition in plasma CRH response was also observed in the control fish after 24 h confinement, but not in the LPS treated groups, which suggests that these compounds in some way

interfere with CRH release. During stress circulating CRH in tilapia may regulate circulating monocytes (Pepels et al., 2004), as in rats where CRH modulates IL-1 release by LPS-activated blood monocytes (Pereda et al., 1995).

In contrast with confined saline-treated fish, confined LPS-treated fish had difficulties maintaining their osmoregulation as plasma chloride levels were decreased in comparison to their unstressed LPS-treated counterparts. During stress, catecholamines increase the permeability of gills to water and ions, and cortisol counteracts this effect, partly by mobilisation of glucose (Wendelaar Bonga, 1997). In view of the combined effects of LPS and confinement found on plasma glucose levels, effects of LPS on energy reallocation may underlie the inability of the LPS treated fish to maintain ionic homeostasis.

Regulation of in vitro *CRH release*

We investigated whether *in vivo* effects of LPS could be caused by direct actions of LPS on brain tissue. Regarding mammals, there is still debate on the most likely route via which LPS *in vivo* stimulates cytokine release, and how these cytokines in turn stimulate CRH synthesis in the brain (reviewed by Turnbull and Rivier, 1999). One theory is that following IP administration, LPS is transported via the portal veins to the blood circulation (Lenczowski et al., 1997), and that LPS or LPS-induced blood-borne factors reach the brain to stimulate IL-1β production in brain regions where the blood–brain barrier is poorly developed (van Dam et al., 2000). Among the areas containing CRH-ir neurons in tilapia, the ventral telencephalon is most richly innervated by blood capillaries (Pepels et al., 2004). This vascular bed provides an opportunity for LPS to gain excess to the brain *in vivo*, and we therefore investigated CRH release from this tissue *in vitro* (Pepels et al., 2002b). We anticipated that stimulation of the CRH neurons by norepinephrine (NE) or serotonin (5-HT) would be required as monoamine utilisation in the CNS of mammals is altered by peripheral administration of LPS (Dunn, 1992). There is also substantial evidence for the critical and permissive role of medullary catacholaminergic innervation of the hypothalamus in the activation of the HPA axis in response to systemic LPS (Ericsson et al., 1994).

The stimulatory role of NE and 5-HT on *in vitro* CRH release is reported for the first time in lower vertebrates. Stress stimulates NE release in the fish brain. Hoglund et al. (2000) have demonstrated that turnover rates of 5-HT in the telencephalon, and turnover rates of NE in the brain stem and optic tectum, were positively correlated with plasma ACTH levels in socially chronic stressed (subordinate) Arctic charr (*Salvelinus alpinus*). Also in goldfish (*Carassius auratus*) there is support for a stimulatory role of 5-HT on CRH release, in relation to the regulation of feeding behaviour (de Pedro et al., 1998). The ventral telencephalon as well as the preoptic region of teleost fish are innervated by NE-ir or dopamine β-hydroxylase αir fibres (see review by Meek, 1994) and by 5-HT-ir fibres (Meek and Joosten, 1989). Similar to mammals, the NE-ir and DBH-ir cell bodies are located in the locus coeruleus and the brainstem and the 5-HT-ir cell bodies are mainly located in the raphe nuclei (Meek, 1994; Meek and Joosten, 1989). In mammals NE stimulates the *in vitro* CRH release from telencephalic tissues containing the amygdala (Raber et al., 1995). Previously, we demonstrated *in vitro* that the telencephalon synthesised and released CRH for at least 4.5 h of superfusion (Pepels et al., 2002b). The present stimulation of the *in vitro* CRH release by NE and 5-HT further confirms that *in vitro* CRH release is not caused by leakage but represents regulated release.

Between *in vitro* experiments the initial and basal CRH release rates in tilapia appeared remarkably comparable (see also Pepels et al., 2002b). Basal *in vitro* CRH release by the telencephalon was not affected following 3 h of LPS treatment/superfusion. Previously, we showed in tilapia that concentrations between 1–50 μg ml^{-1} of LPS *in vitro* applied in a similar superfusion set-up dose-dependently inhibited the basal release of α-MSH by the pituitary gland (Balm et al., 1993).

The major *in vitro* finding in our study was that LPS treatment inhibited the NE-induced CRH release. This effect was not due to non-specific effects, such as tissue damage by LPS, as the CRH neurons in the LPS-treated telencephalon reacted to the high concentration of potassium by increasing the CRH release in a similar way as the controls. Also, tilapia pituitary tissue superfused with equally high *E. coli* LPS concentrations remained viable (Balm et al., 1993). There are several possible mechanisms via which LPS may have affected the NE-induced CRH release, such as a LPS-induced nitric-oxide production, which inhibited CRH production in mammals (Kostoglou-Athanassiou et al., 1998). Alternatively, preincubation with LPS (or IL-1) could have desensitised adrenergic receptors because LPS stimulates the NE turnover in brain tissues of mammals (Ericsson et al., 1994; Francis et al., 2001). Neurons, microglia cells, sessile macrophages or endothelial cells may be among the cell types involved in these regulations (Kostoglou-Athanassiou et al., 1998; Turnbull and Rivier 1999). The observed *in vitro* inhibition of the CRH response to NE by LPS may explain why *in vivo* the confinement treatment in LPS pre-treated fish, in contrast to controls, was unable to increase telencephalic CRH.

In summary the present study is the first study in lower vertebrates to demonstrate changes in brain CRH levels after a challenge with the bacterial endotoxin LPS. Whereas the *in vivo* results were obtained following more-prolonged treatment, the *in vitro* results demonstrate that LPS in fish also can act within several hours. Our results together with those of Volkhoff and Peter (2004) establish that the role of CRH in immune–endocrine interactions is a phylogenetically old mechanism. A single *in vivo* LPS (ip or icv) treatment of goldfish elevated mRNA CRH levels in hypophysiotropic and non-hypophysiotropic brain regions (Volkhoff and Peter 2004). Since LPS treatment affects non-hypophysiotropic as well as hypophysiotropic CRH-ir cells, the role of the peptide in immune–endocrine interactions in fish is not limited to regulation of HPI-axis activity. Results indicate that, in particular, the non-hypophysiotropic CRH system located in

the ventral telencephalon of tilapia can be modulated directly by LPS.

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