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Abstract
For some teleosts, a role has been established for melanin-concentrating hormone (MCH) background adaptation and stress response. In teleost fishes, prepro-MCH (ppMCH) mRNA is expressed in the hypothalamus, predominantly in neurons of the nucleus lateralis tuberis (NLT) and in scattered cells of the nucleus recessus lateralis (NRL). The response of mature tilapia to different environmental challenges was studied by assessing ppMCH mRNA levels in these two hypothalamic nuclei by quantitative dot blot analysis. Changes in background colour induced pronounced differences in ppMCH mRNA expression in the NLT, but not in the NRL. The NLT of tilapia adapted to a white background contained 2.5 to 3 times more ppMCH mRNA than the NLT of black-adapted fish. The NLT of fish kept on neutral background contained intermediate levels of ppMCH mRNA, which were significantly lower than the levels in white-adapted fish. Oral administration of dexamethasone lowered plasma cortisol concentrations, but had no effect on ppMCH mRNA levels in white- and black-adapted fish. In tilapia exposed to strongly acidified water (pH 3.5), plasma cortisol and ACTH concentrations were highly elevated, and plasma chloride concentrations considerably lower than in controls. These fish responded with a 70% rise in ppMCH mRNA levels in the NLT, which is most probably associated with a stress response evoked by inadequate osmoregulation. After exposure to a milder acidification (pH 4.0) or to seawater no significant changes in ppMCH mRNA levels occurred in either the NLT or the NRL, nor in plasma chloride, cortisol and ACTH levels. A specific increase of ppMCH mRNA levels in the NRL was observed in repeatedly disturbed tilapia. We conclude that MCH neurons in the NLT and NRL of this teleost differentially respond to background colour, acidification and disturbance stress, and that this response is not strictly associated with changes in plasma ions and activity of the pituitary-interrenal axis.

Melanin-concentrating hormone (MCH) is a vertebrate neuropeptide, which is predominantly synthesized in the hypothalamus. MCH was first discovered in teleost fish as a neurohypophysial hormone which controls the skin colour (1). More recently, the occurrence of MCH and its preprohormone, ppMCH, were demonstrated in several non-teleostean vertebrate species by immunocytochemistry and molecular cloning (2-5). Subsequently, a number of other functions have been attributed to MCH and to a second peptide derived from ppMCH, called neuropeptide EI (NEI) in mammals and MCH gene-related peptide (Mgrp) in teleosts (1). Recent studies on mammalian MCH, NEI and MCH gene expression showed responsiveness of MCH cells to conditions, such as stress (6), osmotic stimuli (7-10), and lactation (11-14).

In teleosts, in addition to the melanin-concentrating function, a role for MCH in the stress-response has been postulated (1). Plasma MCH concentrations increased in trout stressed by injection, and this increase was antagonized by the synthetic corticosteroid dexamethasone, indicating a negative feedback control of cortisol on MCH secretion (15). Lowering the water level and injection stress also influenced MCH biosynthesis in trout (16). MCH appeared to have modulatory actions on the activity of the hypothalamus-pituitary-interrenal (HPI) axis at several levels: MCH inhibits the release of corticotropin releasing factor (CRF) from the hypothalamus and ACTH release from the pituitary gland in vitro (1, 17, 18). MCH also inhibits the release of α-MSH from the pituitary of some teleosts (19, 20); in fish α-MSH seems to function in background adaptation as well as in the stress response (21-23).

Osmoregulation and stress responses are intimately interrelated in fish (24), yet a role for MCH in hydromineral regulation has not been reported in teleosts. Therefore, in the present study the response at the mRNA level of MCH neurons to osmoregulatory challenges was investigated in the cichlid teleost Oreochromis mossambicus, a tilapia with impressive adaptive capacities, in particular with respect to osmoregulation (25). In this fish (26), as in other teleosts (1), MCH is synthesized in two nuclei of the hypothalamus. An abundance of magnocellular MCH neurons...
Differential MCH gene expression in tilapia

have been localized ventrally in the nucleus lateralis tuberis (NLT). Most of these neurons project into the pituitary. Scattered MCH cell bodies can be found near the lateral ventricular recess in the nucleus recessus lateralis (NRL). Axonal projections of these neurons have as yet not been identified (1). We recently cloned a hypothalamic ppMCH cDNA of tilapia (27), and developed a method to measure ppMCH mRNA levels of the NLT and NRL in individual animals (26). This enabled us to study whether these MCH-synthesizing neurons differentially respond to environmental challenges. Since MCH appears to be primarily involved in long-term adaptation processes (1, 16), an inventory was made of MCH gene expression in these two hypothalamic nuclei after a prolonged period of exposure to different background colours, dexamethasone treatment, and exposure to disturbance. In addition, levels of ppMCH mRNA were quantified after long-term exposure to seawater and low pH, treatments associated with hydromineral imbalance and endocrine responses (25, 28), which have been extensively characterized before in our laboratory (25, 29). As parameters for stress and hydromineral seclimation, plasma cortisol, ACTH and chloride concentrations were determined.

Results

Effect of background colour and dexamethasone on tilapia ppMCH mRNA expression

Tilapia kept up to 1 month in white or black tanks showed a pale or black skin-colour, respectively. As shown in Fig. 1, in the NLT of tilapia adapted for 2 weeks to a white background the ppMCH mRNA level was 2.5 times higher than the NLT of black-adapted fish, whereas the NLT of fish adapted to a neutral background contained intermediate levels of ppMCH mRNA, which were significantly lower than the levels found in white-adapted fish. No differences were found in the NRL region. Similar results were obtained when tilapia were adapted to black and white backgrounds for one month (Fig. 2). Stress-free, oral dexamethasone administration to white- and black-adapted tilapia significantly decreased plasma cortisol concentrations in both groups, but did not change plasma ACTH levels (Table 1). The dexamethasone treatment had no effect on ppMCH mRNA levels in either the NLT or NRL of white- and black-adapted tilapia (Fig. 2).

Effect of osmotic challenges and disturbance on tilapia ppMCH mRNA expression

In the following experiments fish were kept on a neutral background and no change of skin colour was observed. In tilapia, exposed to acidified water of pH 3.5, plasma cortisol and ACTH levels were strongly elevated compared with controls from pH 7.8 freshwater, while plasma chloride concentrations decreased by 35% (Table 2). These fish most of the time stayed on the bottom of the aquarium and did not react quickly to food. Levels of ppMCH mRNA in the NLT of the pH 3.5-exposed animals increased by 70% above controls, while no statistically significant changes could be measured in the NRL (Fig. 3). In a subsequent experiment, tilapia exposed to water of pH 4.0 showed no signs of discomfort, and no significant changes were measured in either plasma cortisol, ACTH, chloride (data not shown), or in hypothalamic ppMCH mRNA concentrations (NLT, C: 9.86±0.79 pg/g body weight (BW); pH 4.0: 12.57±1.51 pg/g BW; NRL, C: 0.77±0.09 pg/g BW; pH 4.0: 0.82±0.10 pg/g BW). Tilapia exposed for 10 days to 70% seawater showed no statistically significant differences in hypothalamic ppMCH mRNA levels, nor in plasma cortisol, ACTH or chloride concentrations when compared with freshwater controls (Fig. 3, Table 2).

Tilapia disturbed 3 times per day at irregular intervals, showed a 45% increase in ppMCH mRNA levels in the NRL when compared with control levels (Fig. 4). No changes in ppMCH mRNA levels were found in the NLT in response to this treatment.

Discussion

The present study provides evidence that hypothalamic MCH-neurons in the NLT and NRL differentially respond to environmental challenges. NLT neurons respond to changes in background colouration and acidification of the water, while MCH-neurons of the NRL respond to repeated disturbance.

The levels of ppMCH mRNA expression in the two hypothalamic nuclei of controls varied between different experiments. We consider this to be due to a reasonable range of biological variation, most probably related to the batch of fish. Within 1 experiment tilapia from 1 batch of eggs were used, but in the set of experiments described different batches were used. Moreover, in a series of background adaptation experiments, it was our experience that although absolute ppMCH mRNA values sometimes differ a factor two to four between experiments (compare for example Figs 1 and 2) the difference between white- and black-adapted fish was always a factor three.

A prominent rise in ppMCH mRNA expression levels was found in the NLT but not in the NRL when tilapia were kept on a white background instead of a black or neutral background. We conclude from our refined approach, to study the two hypothalamic regions separately, that the rise of tilapia hypothalamic ppMCH mRNA levels that we have previously reported (26) resulted specifically from a rise in MCH neurons of the NLT. Consistent with the enhancement of hypothalamic ppMCH mRNA levels, Baker and Bird (16) reported that de novo MCH synthesis in whole hypothalam of trout on a white background is doubled when compared with that of black-adapted animals. The only report specifically dealing with changes in the NLT, but not referring to neurons of the NRL, concerns a morphological study in Chinese grass carp indicating that MCH neurons are more active in white-adapted fish: the cells had larger cytoplasmic and nuclear areas and more prominent nucleoli than those of black-adapted animals (30). The specific rise of ppMCH mRNA levels in neurons of the NLT, projecting mainly to the neurohypophysis (1), is in accordance with the reported increase of MCH secretion from the pituitary in white-adapted teleosts. In white-adapted trout, more MCH is present in the blood than in black-adapted fish (1). Also in eel and carp the rate of MCH secretion differs in response to changes in background colouration, as judged from the MCH content of the pituitary gland (1).

As the neurons of the NRL were apparently not responding to changes in background colouration, we searched for other functions. Since in trout MCH appears to be involved in the modulation of the hypothalamic-pituitary-interrenal (HPI) axis (1, 15, 16, 17, 18), we followed several approaches to manipulate the HPI axis activity. We reduced the output of the interrenal tissue by feeding dexamethasone to white- and black adapted tilapia.

Differential MCH gene expression in tilapia

Fig. 1. Effect of background colour on ppMCH mRNA expression in tilapia NLT and NRL. Total RNA extracted from the NLT and NRL of tilapia adapted for 2 weeks to either a white, neutral or black background, was dot blotted and hybridized with a tilapia-specific anti-sense MCH cRNA probe. A sense MCH cRNA standard curve was used to convert hybridization signals into picograms of ppMCH mRNA per gram body weight. W, white-adapted tilapia (n = 7); N, tilapia adapted to neutral background (glass aquaria; n = 5); B, black-adapted animals (n = 9). **P < 0.01, ***P < 0.001 compared with the NLT of W.

This treatment lowered plasma cortisol levels, which is in line with similar observations on brown trout at neutral background (31). Plasma ACTH levels were not altered, which corresponds with findings after in vivo cortisol administration to this tilapia (32). The dexamethasone treatment did not alter ppMCH mRNA levels of both hypothalamic MCH neuron groups of tilapia kept on black as well as white backgrounds, which corroborates findings in trout. In these fish injection of dexamethasone suppressed the stress-induced rise in plasma MCH levels as effectively as it suppressed the stress-associated rise in plasma cortisol, but it did not significantly influence background colour related plasma MCH levels (15). Although to our fish a stress-free dexamethasone treatment was given, they were stressed by the capture procedure. Tilapia responds extremely fast to capture with a surge in plasma cortisol levels, which is ACTH-independent, but subject to feedback by cortisol (32) and dexamethasone (this study). This sampling effect occurs within minutes, whereas in the study by Green and Baker (15) fish received daily injections, and the experiment was terminated one hour after the final injection. It is conceivable that in our case the sampling period was too short for the MCH neurons to react. It is also possible that only MCH release, but not MCH biosynthesis, is influenced by dexamethasone, since dexamethasone treatment had no significant effect on levels of newly synthesized trout ppMCH (16).

We then exposed the fish to strongly acidified water (pH 3.5), a treatment which is known to evoke a prominent response of the HPI axis (25). In response to acidified water elevated plasma levels of cortisol, acting as a mineralocorticoid, are known to counteract the disturbed ionoregulation (25). Indications that exposure to water of pH 3.5 was experienced as a severe stressor by our fish are the extremely high plasma cortisol and ACTH values and the apparent discomfort of these animals. This challenge significantly increased ppMCH mRNA levels of the NLT, but not of the NRL. This stimulation of MCH neurons of the NLT is most probably associated with a stress response evoked by inadequate osmoregulation in these fish, evidenced by the above mentioned findings. Exposure to pH 4.0, or to 70% seawater did not alter ppMCH mRNA levels, which may indicate that the response of MCH neurons to challenges affecting osmoregulation is limited to conditions to which the fish do not acclimate, and in which the hydromineral balance is notably disturbed. Acclimation of tilapia to pH 4.0 and to seawater was apparent from the unchanged plasma cortisol and chloride values, consistent with results described before (25, 28, 29).

As a third type of stressor, tilapia were exposed to repeated disturbance. This treatment, which has no effect on plasma chloride and cortisol levels (Pelgrom, Balm personal communication), induced a significant increase of ppMCH mRNA expression in the NRL, whereas ppMCH mRNA levels of the NLT appeared to be unaffected. Thus MCH neurons in the NRL specifically respond to this disturbance, while they are not responsive to background colouration or osmotic challenges. Altogether, th
above findings demonstrate that ppMCH mRNA expression is differentially regulated in the NLT and NRL of tilapia, which suggests that these two MCH-synthesizing nuclei are part of different and stressor-specific pathways.

Our results confirm for tilapia the complex relationship between MCH and stress response, described for trout by Baker and coworkers (1, 15, 16). We found that after exposure of tilapia to acidification, ppMCH mRNA levels in the NLT and plasma cortisol concentrations were positively related. However, in the experiments dealing with background adaptation or dexamethasone no such relationship was found. In addition, no relationship was found between plasma cortisol values and ppMCH mRNA levels in the NRL of tilapia. Therefore, we conclude that stimulation or inhibition of MCH synthetic activity is not strictly coupled to the interrenal stress response.

**Materials and Methods**

**Fish**

Tilapia were bred in the laboratory and fed twice daily, each time 1% of BW, commercial dried fish food (Tetramin). The fish were held at 26°C in continuously aerated and filtered freshwater under a 12 h light, 12 h dark cycle. Sexually mature tilapia, both males and females were used. After the experiments the animals were netted and directly before sacrifice blood was collected from the caudal vessels in EDTA/aprotinin (1.5 mg Na2EDTA, 300 KIU aprotinin (Serva) ml⁻¹) and centrifuged at 4°C. The plasma was stored at -20°C until assay. After sacrifice by spinal transection, the hypothalamus was dissected from the brain. The NLT and NRL were separated by a transverse incision from the ventral side.

Fig. 3. Effect of osmotic challenges on ppMCH mRNA expression in tilapia NLT and NRL. ppMCH mRNA expression levels per gram body weight are shown for pH 7.8 freshwater tilapia (C), pH 3.5 freshwater animals (Acid) and 70% seawater exposed fish (SW). n = 8. *P<0.05 vs the NLT of control fish.

Fig. 4. Effect disturbance on ppMCH mRNA expression in NLT and NRL of tilapia. ppMCH mRNA expression levels per gram body weight are shown for freshwater controls (C, n=4) and disturbance treated fish (D, n=6). *P<0.05 vs the NRL of controls.

Differential MCH gene expression in tilapia of the hypothalamus, just caudal of the pituitary to the dorsal side of the optic chiasm (26).

Background adaptation
Male tilapia (BW 17.7 ± 0.5 g) were adapted to white or black backgrounds by transferring them to plastic white or black tanks, respectively, all-glass aquaria on a grey ground in which the stocks are kept were designated as neutral background. The tanks contained 80 l of Nijmegen tap water. After two weeks the fish were sacrificed.

Dexamethasone treatment
Male tilapia (BW 20.0 ± 0.8 g) were adapted to black and white backgrounds as described above. Four groups (2 black; 2 white) of 7 fishes were kept for 31 days in the tanks. The last 4 days before sacrifice 1 black and 1 white group of tilapia were fed twice daily with Tetramin flakes (1% of body weight per meal) containing 0.15 mg/g dexamethasone. These flakes were prepared by spraying with dexamethasone dissolved in ethanol, which was allowed to evaporate overnight at room temperature. Control groups received the same quantity of Tetramin flakes sprayed with ethanol only. At day 31, 16 h after the last feeding, the fish were captured.

Exposure to acidified and saltwater
Three groups of male tilapia (BW 20.5 ± 0.5 g) were kept 7 days before the start of the experiments on a neutral background in 120 l glass aquaria containing artificial freshwater, consisting of demineralized water supplemented with 1.3 mM NaHCO₃, 0.5 mM CaCl₂, 0.06 mM KCl and 0.2 mM MgCl₂ at pH 7.8 (29). For exposure to acidified water the pH was lowered gradually to pH 3.5 over a period of 73 h by addition of H₂SO₄ via a flow-through system. For exposure to 700 mosm/kg seawater the osmolality was raised gradually from 5 mosm/kg to 700 mosm/kg over a period of 48 h by addition of seawater (artificial fresh water salted with Wissel seasalts; Wieland & Co, Krefeld, Germany) via a flow-through system. The water pH and osmolality were controlled without disturbing the fish by taking water samples from the effluent outside the climate chamber. The fish were kept at low pH or in seawater for 10 days. Control fish were kept in artificial freshwater. Plasma chloride concentrations were measured by flame photometry as parameter for osmotic adaptation.

Exposure to disturbance stresses
Six weeks before the start of the experiments female tilapia (BW 14.8 ± 1.2 g) were divided into 2 groups, and kept on a neutral background in 80 l glass aquaria with continuously filtered and refreshed artificial freshwater. One group was exposed to disturbance stresses during 6 days. This group was housed in a separate identical climate room. The fish of this group were daily disturbed by confining them in a small net, switching off the light, and switching off the oxygen supply, each for 10 min in random order, and at irregular intervals to prevent habituation. Feeding and disturbance ended one day before sacrifice.

RIA for cortisol
Plasma concentrations of cortisol were determined in a RIA for cortisol as described previously (32). Plasma samples were diluted 10 times with distilled water before assay. The cortisol antiserum was purchased from Sterantis Res. Ltd. (UK). Cross-reactivity with dexamethasone was determined to be 3%. The antiserum was diluted to yield one third of the iter recommended by the suppliers. ³H labelled cortisol was from Amersham. Free and bound cortisol were separated by precipitation of the immunocomplex with dextran-coated charcoal. The inter-assay variation was 7.2%, and the intra-assay variation was 6.2%.

RIA for ACTH
Plasma concentrations of ACTH were determined in a RIA for ACTH as described previously (32). The antiserum against ACTH, was kindly provided by Professor R. Dores, University of Denver, USA. The final dilution was 1:38,500. The hACTH₃₉ standard was from NIBSS (London, UK). Iodination of ACTH was from Amersham. Free and bound ACTH were separated by precipitation with PEG 6000 (Merck) after incubation with a second sheep anti rabbit IgG antibody. The inter-assay variation was 7.2%, and the intra-assay variation was 6.2%.

Measurements of ppMCH mRNA
Levels of ppMCH mRNA in the NLT and NLR were measured by dot blot analysis as described previously (26). In short, total RNA was isolated from separated hypothalamic NLT and NLR regions using the acid guanidinium-thiocyanate phenol chloroform procedure (33), yielding about 3 µg total RNA per NLT and about 8 µg per NLR, in accordance with the size of the tissue samples. RNA was resuspended in 5 x SsPi (1 x SsPi is 0.18 M NaCl, 0.01 M Na₂HPO₄, pH 7.4, 0.01 M EDTA, 7.4% formaldehyde solution, and 25 or 35% of the NLT RNA and 10% of the NLR RNA was blotted on nylon membranes using a dot blot apparatus (Bio Rad, Hercules, USA). Dot blots containing a sense tilapia pMC-I cRNA standard dilution series were hybridized with a ³²P labeled antisense cRNA probe derived from clone TMe58, containing a 270-bp tilapia ppMCH cDNA fragment, encoding tilapia Mgrp and MCH and part of the 3’ untranslated region (27). Washing was performed until 0.1 x SsPi, 0.1% SDS, 68°C. Hybridization signals were quantified by densitometric scanning of the autoradiograms. The values were converted by the sense pMCH cRNA standard curve to pg ppMCH mRNA/g BW. The detection limit is 1 pg of ppMCH mRNA/dot.

Statistical analysis
Data are presented as mean ± SEM. For statistical analysis the two-tailed Student’s t-test was used after log transformation of data. Significance was accepted at P < 0.05.

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