Identification, Cellular Localization and in vitro Release of a Novel Teleost Melanin-Concentrating Hormone Gene-Related Peptide

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Abstract

The melanin-concentrating hormone (MCH) precursor encodes MCH and a second peptide named neuropeptide EI (NEI) in mammals, neuropeptide EV (NEV) in salmonids and MCH gene-related peptide (Mgrp) in other fish. The primary structure of the putative Mgrp of the cichlid fish tilapia (Oreochromis mossambicus) appears to be very different from mammalian NEI and salmonid NEV. To investigate the processing and release of tilapia Mgrp (tMgrp), in the present study an antiserum was raised against synthetic tMgrp. By immunocytochemistry, tMgrp immunoreactivity was colocated with MCH immunoreactivity in the tilapia hypothalamus and pituitary. In addition, a tMgrp enzyme-linked immunosorbent assay in combination with reversed phase HPLC was used to demonstrate the presence of processed tMgrp in tilapia hypothalamus and pituitary. The release of tMgrp from neuro-intermediate lobes (NILs) of tilapia pituitaries was demonstrated after in vitro incubation of chopped NILs. Depolarizing concentrations of potassium significantly stimulated tMgrp release. Six weeks of adaptation of tilapia to white or black backgrounds had no effect on in vitro tMgrp release or on the tMgrp content of NIL and hypothalamus. Tilapia Mgrp, unlike MCH, had no effect on tilapia scale melanophores, nor did it modulate the melanin-concentrating effect of MCH. We conclude that tMgrp is processed from the MCH preprohormone, that it is released in vitro, and that the peptide has no direct role in the melanin concentration of fish scale melanophores. Therefore a neuroendocrine or neuromodulatory function is proposed for tMgrp.

Introduction

Melanin-concentrating hormone (MCH) is a neuropeptide predominantly synthesized in the hypothalamus. In teleosts most hypothalamic axons project to the pituitary, where the peptide is stored and released [1]. In these fish, MCH was first discovered as a neurohypophysial hormone involved in the regulation of background adaptation. More recently functions in the regulation of stress response and ion balance have been attributed to this cyclic peptide in teleosts [1-3]. In mammals MCH-containing fibers are broadly distributed throughout the cen-
Fig. 1. Schematic representation of MCH preprohormone, and comparison of mammalian NEI, salmon NEV and tMgrp. A MCH preprohormone of tilapia. MCH, Mgrp and the signal peptide are noted. Black bars indicate two or three basic residues; the interrupted bar indicates the site where in mammals and salmonids NEI and NEV are processed. S-S = Disulfide bond between two cysteins. B Comparison of human NEV variant [13], human [11], rat [10], mouse [12], salmon-1 and salmon-2 NEV [6], and tMgrp [3]. Amino acids identical to mammalian NEI are black boxed; conservative amino acid substitutions are grey boxed.

central nervous system [4], and functions in osmoregulation, stress response and lactation have been attributed to mammalian MCH [1, 5].

Sequence analysis of MCH preprohormones of teleosts, such as salmonids [6-9] and tilapia [3], and mammals [10-13] revealed the presence of putative neuropeptides in the prohormone in addition to MCH. MCH is located at the carboxy-terminus of the prohormone and is highly conserved during evolution. The peptide directly preceding MCH in the prohormone is named neuropeptide glutamic acid-isoleucine amide (NEI) in mammals [10-12], neuropeptide glutamic acid-valine (NEV) in salmonids [8, 9] and MCH gene-related peptide (Mgrp) in tilapia [3]. In the mammalian MCH prohormone another putative peptide named neuropeptide glycine-glutamic acid (NGE) or neuropeptide proline-glutamic acid (NPE) precedes NEI. NEI and NGE/NPE are highly conserved between man, rat and mouse (85-100% identity), while the conservation between mammalian and salmonid NEI and NEV is considerably lower (30% identity and 65% similarity). However, the primary structure of the putative Mgrp of the teleost tilapia is clearly different in length and amino acid sequence if compared with the structures of the other known peptides (fig. 1).

Recently evidence has been provided that mammalian NEI is processed from the MCH prohormone and that the peptide is released in vitro [14]. Release of both rat NEI and MCH was stimulated by the secretagogues cAMP and cGMP, whereas only MCH release was increased by dexamethasone [14]. Biological actions of NEI are partly similar to and partly different from that of mammalian MCH [5]. Both peptides may contribute to lactation, since they stimulate oxytocin release from rat pituitary glands in vitro. Only NEI inhibits vasopressin release from rat pituitaries, indicating an involvement in osmoregulation [15].

The questions arise whether tilapia Mgrp (tMgrp) is processed from the MCH prohormone, whether this Mgrp is released and whether it has a function as a neuropeptide in fish. Therefore, in the present study an antiserum was raised against synthetic tMgrp, and subsequently tMgrp immunoreactivity was localized in the brain and compared with the location of MCH immunoreactivity. Furthermore, the presence of processed peptide in tilapia hypothalamus and pituitary, and the in vitro release of tMgrp from pituitary neurointermediate lobes (NILs) were investigated by an immunosorbent assay and HPLC analysis. To study whether Mgrp, like MCH, has a function in background adaptation, the Mgrp release from NILs was compared in white- and black-adapted tilapia, and the in vitro effect of Mgrp on scale melanophores was determined.

Materials and Methods

Fish

Tilapia of both sexes were bred in the laboratory and fed twice daily commercial dried fish food (Tetramin). The fish were held at 26°C in continuously aerated and filtered fresh water under a 12-hour light, 12-hour dark cycle. For all experiments sexually mature males or females were used. Body weight ranged from 70 to 200 g. After the experiments the animals were sacrificed by spinal transection. The brain and pituitary were removed.

Background Adaptation

Male tilapia were adapted to white or black backgrounds as described previously [16]. After 6 weeks the fish were netted and sacrificed.
Synthetic Tilapia Mgrp

Tilapia Mgrp was synthesized at the Department of Organic Chemistry of our university by solid phase peptide synthesis [17] using the Fmoc strategy for cleavage [18]. Synthetic tMgrp was coupled to bovine serum albumin (tMgrp-BSA) and to casein (tMgrp-Cas) by carbodiimide [19]. The conjugate was diluted in 0.1 M phosphate buffer (pH 7.0) and stored in aliquots at -20°C.

Tilapia Mgrp Antiserum

Antiserum to tMgrp was raised by Eurogentec (Seraing, Belgium). The antigenic conjugate, tMgrp-BSA (0.1 mg/0.2 ml), was emulsified in an equal volume of Freund's complete adjuvant and used for immunization of a rabbit by intradermic multisite injection. The rabbit was boosted on days 14, 28, 56, and 84, and bled 10 days after each injection from boost 2. Sera were collected and stored at -20°C.

Immunocytochemistry

Brains obtained from tilapia kept at a neutral background were fixed overnight in Bouin's fluid, dehydrated, and embedded in paraffin. Five-micrometer-thick sections were mounted on poly-L-lysine-coated microscope slides. Alternating sections were used for either MCH or tMgrp immunocytochemistry. MCH immunocytochemistry was performed as described before [16]. Immunocytochemistry with tMgrp antiserum was performed following the same procedure with an antiserum dilution of 1:1,000. For specificity control the MCH and tMgrp antisera were preadsorbed with 1 μM synthetic MCH (Peninsula) or 1 μM tMgrp prior to incubation on the slides. Sections were examined with a bright field microscopy. Nomenclature of brain areas was based on the work of Batten et al. [20].

In vitro Static Incubation and Tissue Extraction

Static incubation of tilapia NIL fragments was performed following a procedure modified from the one described for goldfish [21]. NIL fragments were obtained by cutting the freshly prepared NILs four times in different directions. NIL fragments were rinsed in 400 μl IM under the same conditions as the tissue. No significant differences were found between samples before and after incubation. The tissue recovered from the incubation well or freshly dissected tissue was homogenized in 300 μl 0.01 N HCl and the supernatant obtained after centrifugation was stored in aliquots at -20°C.

Reversed Phase-HPLC Analysis

To determine the processing of tMgrp, extracts of tilapia NIL and hypothalamus were submitted to HPLC analysis on a Spherisorb 10 ODS column (Bischof). The primary solvent was buffer B (0.5 M formic acid, 0.14 M pyridine, pH 3.0) and elution was accomplished with a gradient of 1-propanol at a flow rate of 2 ml/min [22]. Fractions of 1–4 ml were collected. The fractions were dried in a speed-vac concentrator (Savant), diluted into dilution buffer for tMgrp ELISA. The fractions measured were derived from 0.7 NIL-equivalent and 2.5 hypothalami. Synthetic tMgrp was used as a standard.

ELISA for tMgrp

A competitive ELISA was developed for tMgrp quantitation. This technique is based on competition for the tMgrp antibodies between free tMgrp in standard or samples and tMgrp immobilized on microtiter plates. Wells of microtiter plates (Nunc-Immuno Plate Maxisorp™, Nunc) were coated with 100 ng tMgrp-Cas in 200 μl of coating buffer (0.05 M sodium bicarbonate buffer, pH 9.6), except for the blanks which received only coating buffer. Coating lasted for 1 h at 37°C, followed by overnight storage at 4°C. Between incubation steps, coated plates were rinsed 3 times with 300 μl washing buffer (0.05 M sodium phosphate buffered saline, PBS pH 4.4, with 0.05% Tween-20; Bio-Rad). The tMgrp antiserum was pre-incubated in dilution buffer (washing buffer containing 2% porc serum) with 4 mg/ml BSA for 2 h. The antigen was diluted in dilution buffer, tissue extracts were lyophilized and diluted in dilution buffer. To IM samples 2% porc serum was added. For competition equal volumes of antigen and diluted antiserum (1:5,000) were incubated in 1.5 ml Eppendorf tubes for 1 h at 20°C. Tubes containing diluted antiserum only were incubated under the same conditions. After washing, the wells of coated plates were blocked with 200 μl blocking buffer (PBS containing 6% porc serum) for 2 h at 37°C. The plates were washed and filled with 100 μl/well of the antigen/antiserum mixture (each sample in duplicate), except for the blanks and Bfs, which received 100 μl of diluted antiserum. Plates were incubated for 1 h at 37°C. The wells were washed and incubated with 100 μl of goat anti-rabbit peroxidase conjugate (Nordic) in dilution buffer (1:5,000) for 1 h at 37°C. For quantification of the immunoconjugate bound to the wells, an enzymatic reaction was performed with o-phenylene-diamine (OPD; Sigma) as a substrate. After washing, 190 μl of substrate (0.05% OPD in citrate/phosphate buffer; 0.2 M Na2HPO4, 0.1 M citric acid, pH 5.0, with 0.025% hydrogen peroxide) was added per well. The reaction was allowed to proceed for 5–10 min and was stopped by adding 50 μl of 4 M H2SO4. Absorbance was measured at dual wavelengths (490 and 655 nm) in a microplate reader (Titertek). In the ELISA the detectable range was 8 pg to 8 ng per tube. Interassay variation was 14.3 ± 7.1% (n = 4). No cross-reactivity (0.02%) was found with MCH (Bachem), α-melanocyte-stimulating hormone (α-MSH; mono-acetylated α-MSH from Bachem), casein or cortisol (both from Sigma). Cross-reactivity with ovine corticotropin-releasing hormone was 0.1%. To test the parallelism of dilution curves the computer program PHARM/PCS-version 4.1 [23] was used.

Tilapia Scale Bioassay

Melanin-concentrating activity was estimated by an in vitro scale bioassay slightly modified from one described before by Kawaeze et al. [24]. Scales were taken from male tilapia and incubated in IM with 0.3 mg/ml BSA (IMB) for 5 min at 20°C followed by another 5-min incubation at 20°C with IMB containing 100 nM α-MSH, which is a physiological antagonist of MCH, known to act through its own specific α-MSH receptor. Four of five α-MSH-pretreated scales, in which the melanin granules were fully dispersed, were incubated in test solutions containing α-MSH, Mgrp and/or MCH dissolved in IMB. After 30 min the scales were examined under a dissection microscope, and the number of melanophores with fully aggregated granules, and the total number of melanophores were determined for...
Fig. 2. Immunocytochemical colocalization of Mgrp and MCH in tilapia hypothalamus and pituitary. A and B, C and D, and E and F are alternating sections. A, C, E Stained with MCH-antiserum. B, D, F Stained with Mgrp-antiserum. A, B NLT. Magnocellular neurons contain both MCH and Mgrp. Scale bars 50 µm. C, D Small cells immunoreactive for MCH and Mgrp (arrows) occur near the lateral ventricle (V) in the NRL. Scale bars 50 µm. E, F Pituitary. Mgrp and MCH immunostaining is visible in the neurohypophysis, predominantly in axons and also in a cell body (arrow). Scale bars 100 µm.

each scale. For each scale melanin-aggregating activity was expressed as the percentage of melanophores with fully aggregated granules after 30 min.

Data Analysis
Data are presented as the mean ± or SEM. For statistical analysis the paired or unpaired Student’s t test or one-way ANOVA were used where appropriate. p < 0.05 was accepted as indicating significant differences.

Results

Cellular Colocalization of Mgrp and MCH

Immunoreactivity
Immunocytochemical analysis revealed tMgrp-immunoreactive perikarya in the hypothalamus and pituitary (fig. 2). Three groups of cells could be distinguished. Many large cell bodies, 12–20 µm in diameter, were located ventrally in the hypothalamus in the nucleus lateralis tuberis (NLT; fig. 2B). Scattered small cell bodies (4–6 µm in diameter) were found near the lateral ventricle in the nucleus recessus lateralis (NRL; fig. 2D). Few Mgrp-immunoreactive cell bodies (about 12 µm in diameter) were observed in the neurohypophysis (fig. 2F). The majority of staining in the neurohypophysis was found in axonal nerves derived from Mgrp-containing hypothalamic neurons (fig. 2F). Most of the tMgrp-positive fibers penetrated the intermediate lobe of the pituitary, while some penetrated the anterior lobe. Mgrp-immunoreactive fibers were also observed in the brain, predominantly in the hypothalamus and in the ventral telencephalon. When tMgrp antiserum preadsorbed with synthetic tMgrp was used, the staining in the brain and pituitary was greatly reduced, whereas preadsorption of tMgrp with MCH antiserum, and of MCH with tMgrp antiserum had no effect on staining with the respective antisera (not shown). Immunoreactivity of tMgrp in hypothalamic NLT and neurohypophysial cells was colocated with MCH immunoreactivity (fig. 2A, B, E, F). Because of the small size of the Mgrp- and MCH-immunoreactive perikarya in the NRL, it was not possible to stain the same cells in alter-
nate sections (fig. 2C, D). The tMgrp and MCH staining of neurohypophysial axonal nerves was in the same region (fig. 2E, F).

Identification of tMgrp

The identity of tMgrp immunoreactivity was confirmed by two approaches. Firstly, the dilution curves of tMgrp immunoreactivity of tilapia NIL extracts and media paralleled that of synthetic tMgrp in the tMgrp ELISA (fig. 3). In both cases the slope of the log transformed curves was not significantly different from the tMgrp standard curve. Secondly, qualitative analyses of NIL and hypothalamus extracts by reversed phase HPLC revealed a single peak immunoreactive for tMgrp as measured by ELISA. The retention time (20.3 min) of this peak was identical to that of synthetic tMgrp (fig. 4). No signal was found in the tMgrp ELISA when trout or carp NIL extracts were used.

Release and Tissue Content of tMgrp

The tMgrp ELISA was used to measure the tMgrp content of medium in which chopped NILs had been incubated in vitro. Tilapia Mgrp was detectable in the medium of all NIL samples, indicating that in vitro tMgrp is released. Significant increases in the release of tMgrp were measured after incubation of NILs in the presence of 60 mM K+, whereas in controls the tMgrp release was not significantly changed (fig. 5A). After incubation, a considerable amount of tMgrp was still present in the NIL tissue (169.3 ± 34.2 ng/NIL, n = 5). No significant differences
Fig. 5. In vitro release of tMgrp in response to background color and depolarizing concentrations of potassium from NIL fragments. □ = IM with standard potassium (2 mM); ■ = medium with high potassium (60 mM). Linked bars represent subsequent incubations of 90 min each. A Effect of high potassium on tMgrp release from NILs of tilapia kept on a neutral background. Three NILs were incubated for two subsequent periods in standard K⁺ (left); another three were exposed to high K⁺ during the second incubation period (right). B Effect of background color on Mgrp release. WA = NIL of white-adapted tilapia (n = 5); BA = NIL of black-adapted tilapia (n = 5). * p < 0.05; ** p < 0.01 compared with low potassium incubation of same NIL by paired Student's t test after log transformation of data where appropriate; † p < 0.01 compared with the second incubation or with standard K⁺ by unpaired Student's t test.

Table 1. Mgrp content of NIL and hypothalamus of tilapia adapted white and black backgrounds for 6 weeks (n = 5)

<table>
<thead>
<tr>
<th>Body weight, g</th>
<th>Mgrp content, ng/gland</th>
<th>NIL</th>
<th>hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
<td>74.8 ± 7.9</td>
<td>237.0 ± 84.1</td>
<td>14.2 ± 3.4</td>
</tr>
<tr>
<td>Black</td>
<td>99.4 ± 5.5</td>
<td>204.2 ± 40.0</td>
<td>17.4 ± 3.2</td>
</tr>
</tbody>
</table>

were found in the release from NILs (fig. 5B), or in tissue contents of NIL and hypothalamus (table 1) between white- or black-adapted fish. The NIL/hypothalamus ratios of tMgrp tissue content were not significantly different between white- (18.2 ± 5.1) and black-adapted fish (13.7 ± 3.8).

Effect of tMgrp on Tilapia Scales
Melanin-concentrating activities of tMgrp, MCH and combinations of both peptides are compared in figure 6. Concentrations of 10 nM to 10 μM synthetic tMgrp had no melanin-concentrating effect in contrast to 1–100 nM MCH. When combinations of tMgrp and MCH were added, the effect was similar to the effect of only MCH addition. These findings indicate that tMgrp has no melanin-concentrating activity, and does not influence the effect of MCH.

Discussion
The present results provide evidence that tMgrp, a cleavage product of the MCH preprohormone, is present in the NIL and hypothalamus of this fish, and that tMgrp is released in vitro. Furthermore, tMgrp appeared to play no direct role in the melanin concentration in fish scale melanophores.

The first indication that tMgrp is synthesized in tilapia brain and pituitary was the colocation of tMgrp immunoreactivity with MCH immunoreactivity. Tilapia Mgrp immunoreactivity was detected in all regions previously reported to contain MCH and ppMCH mRNA [16]. The location of Mgrp in the tilapia hypothalamus greatly corresponds with the recently reported cellular localization of trout MCH and ppMCH mRNA [9]. In both fish species magnocellular neurons in the NLT are stained, while near the lateral ventricle a group of smaller cells occurs. However, the tilapia NRL neurons appeared to be located at the caudal site of the lateral ventricle, whereas the trout NRL neurons are situated near the dorsal surface. Secondly, Mgrp immunoreactivity in tilapia NIL and hypothalamus was, by the criteria of HPLC elution profiles and ELISA studies, indistinguishable from synthetic tMgrp; the sequence of the synthetic peptide was based on the sequence predicted from the tilapia MCH preprohormone [3]. This finding indicates that a tMgrp peptide of 22 amino acids, which differs considerably in length and amino acid sequence from other MCH precursor-associated peptides (fig. 1), is actually processed from the tilapia MCH preprohormone. This is the first time that processing of the MCH preprohormone to a teleost Mgrp has been demonstrated. For trout it has been demonstrated, by measuring MCH immunoreactivity, that the processing of the MCH preprohormone occurs via intermediates, one most probably being MCH coupled to a putative Mrp [25]. Since monobasic residues sometimes serve as cleavage sites [26], the 22-amino acid tMgrp might be further processed into two smaller forms if the lysine residue at position 11 is used as a processing site. However, it is doubtful whether this additional processing actually takes place since, according to the rules for monobasic cleavages, Lys11 is in an unfavorable position for cleavage [27].
Moreover, our antiserum probably would have recognized at least one of the two peptides resulting from such a processing. In that case the HPLC profile would have displayed more than one peak. It is not clear yet whether the tMgrp antiserum recognizes the MCH preprohormone and/or intermediates of the processing. The HPLC profiles of NIL and hypothalamus tissue extracts contained only one clear peak, indicating that the vast majority of ir-tMgrp in these tissues is tMgrp. However, it cannot be excluded that in studies on de novo tMgrp synthesis, the prohormone or intermediates will be recognized by the antiserum. The tMgrp antiserum appeared to be specific for tMgrp, since no ir-Mgrp was detected in the tMgrp ELISA in trout or carp NIL extracts.

The tMgrp contents of NIL and hypothalamus of tilapia were in the same range as values found for MCH in trout [28, 29] and 5–10 times higher than in carp [30] and eel [28]. Also the ratio between the tMgrp content of NIL and hypothalamus was comparable with values found for MCH in teleosts [28, 30]. No differences in Mgrp tissue content were found in tilapia adapted for 6 weeks to white or black backgrounds. Minor differences in MCH tissue content of teleosts adapted for several weeks to white and black backgrounds have been reported before for trout and eel [28]. A lack of difference in tissue content in itself does not mean that there are no differences in tMgrp synthesis and release between white- and black-adapted tilapia, since tissue content is the overall results of synthesis, release and breakdown. Therefore, Mgrp biosynthesis was indirectly determined by measuring ppMCH mRNA levels in the experimental animals, which yielded a 3 times increase in white-adapted tilapia (data not shown) as has been reported before for 2–4 weeks of background adaptation of this species [16, 31].

To study the release of tMgrp, an in vitro incubation system was developed. Evidence was provided that the presence of tMgrp in the IM of chopped NILs was the result of regulated tMgrp release, since Mgrp secretion was enhanced in response to depolarizing concentrations of potassium. This stimulatory effect of potassium and regulatory effects of other secretagogues have been reported before for the release of gonadotropin-releasing hormone (GnRH) from goldfish pituitaries in a similar experimental setup [21, 32]. The authors demonstrated differences in in vitro GnRH release between juvenile and adult fish, which appeared to be related to the GnRH content of the tissues of both groups of fish [32]. In tilapia we found no differences in basal or in potassium-stimulated tMgrp release between white- and black-adapted fish. Thus, in analogy with the GnRH results, the in vitro release of tMgrp may be related to the tMgrp content of the NIL, which appeared to be similar in white- and black-adapted tilapia. Probably, the regulation of tMgrp release from pituitary nerve terminals is under continuous control of hypothalamic Mgrp perikarya or presynaptic neurons. In the in vitro incubation system used, the tMgrp fibers in the NIL fragments are separated from their perikarya, and may explain why there is no difference in release between the tissues from white- and black-adapted fish.

In contrast to MCH, the amount of tMgrp released into the circulation may be of minor importance during background adaptation, since the peptide appeared not to influence pigment dispersion of tilapia scale melanophores. The effect of MCH on these melanophores was comparable with previously reported data [24]. Tilapia Mgrp did not influence the melanin-concentrating effect of MCH either. This indicates that tilapia Mgrp has no function similar to MCH in background adaptation,
which is in line with findings in mammals, where NEI and MCH had different effects on vasopressin secretion. However, NEI and MCH appeared to have a similar role in mammalian lactation by inhibiting oxytocin release in vitro [15]. A function for tMgrp can be sought in the control of the response to stressors such as disturbance, handling, or acid water, since these challenges are known to influence MCH biosynthesis [2] and/or ppMCH mRNA expression [31]. Possibly, tMgrp has a neuro-endocrine or neuromodulatory role in the response to these stressors.

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