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Melanin concentrating hormone (MCH) is involved in the regulation of growth hormone in *Cichlasoma dimerus* (Cichlidae, Teleostei)

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ABSTRACT

Growth hormone (GH) is the main pituitary hormone involved in somatic growth. In fish, the neuroendocrine control of GH is multifactorial due to the interaction of multiple inhibitors and stimulators. Melanin-concentrating hormone (MCH) is a cyclic peptide involved in skin color regulation of fish. In addition, MCH has been related to the regulation of food intake in both mammals and fish. There is only one report presenting evidences on the GH release stimulation by MCH in mammals in experiments *in vitro*, but there are no data on non-mammals. In the present work, we report for the first time the sequence of MCH and GH cDNA in *Cichlasoma dimerus*, a freshwater South American cichlid fish. We detected contacts between MCH fibers and GH cells in the proximal *pars distalis* region of the pituitary gland by double label confocal immunofluorescence indicating a possible functional relationship. Besides, we found that MCH increased GH transcript levels and stimulated GH release in pituitary cultures. Additionally, *C. dimerus* exposed to a white background had a greater number of MCH neurons with a larger nuclear area and higher levels of MCH transcript than those fish exposed to a black background. Furthermore, fish reared for 3 months in a white background showed a greater body weight and total length compared to those from black background suggesting that MCH might be related to somatic growth in *C. dimerus*. Our results report for the first time, that MCH is involved in the regulation of the synthesis and release of GH *in vitro* in *C. dimerus*, and probably in the fish growth rate.

1. Introduction

In vertebrates, growth hormone (GH) is the main pituitary hormone involved in somatic growth and differentiation. GH initiates many of its growing actions by binding to GH receptors and stimulating the release of the hepatic insulin-like growth factor I (IGF-I) [6,16,42]. GH is a single chain polypeptide produced mainly by cells located in the anterior pituitary and belongs to the structurally and functionally related prolactin (PRL), somatolactin (SL) and placental lactogen family [15]. In fish, the neuroendocrine control of GH is multifactorial with inhibitors such as somatostatin and a variety of inhibitory neurotransmitters, and stimulators such as growth hormone-releasing hormone, pituitary adenylate cyclase-activating peptide, ghrelin, dopamine, gonadotropin-releasing hormone, cholecystokinin, gastrin-releasing peptide, neuropeptide Y, thyrotropin-releasing and corticotrophin-releasing hormones [9]. Recent studies have demonstrated the presence of IGF-I in pituitary cells such as GH cells, and it is suggested that this pituitary IGF-I might act as an auto/paracrine mediator of feedback mechanisms [17].

Melanin-concentrating hormone (MCH) is a cyclic 17 amino-acid peptide, originally isolated from the pituitary of chum salmon as a hormone involved in the skin color regulation [23]. MCH is a melanophore-aggregating hormone involved in long-term background adaptation inducing physiological and morphological responses of melanophores such as pigment granule movement within these cells, which produce enlightening of the skin color. The distribution of MCH-immunoreactive (-ir) cell bodies and fibers in the brain has been described in several fish species [2–5,28,33,36] including *Cichlasoma dimerus* [39]. Particularly, in fish, MCH is synthesized in hypothalamic neurons and those from the lateral part of the *nucleus lateralis tuberis* (NLT) project their fibers towards the neurohypophysis [4,13,28,39]. In addition to the pigmentary role, MCH has been described as a neurotransmitter or neuromodulator [36]. Recent studies carried out in goldfish have

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proposed that MCH would act in the brain regulating the food intake rate as an anorexigenic peptide, in contrast to the orexigenic role reported in mammals [29–31]. Nevertheless, in barfin flounder, it is suggested that MCH acts as an orexigenic hormone [49,50]. Barfin flounders reared in white-colored tanks grew faster than fish reared in black-colored tanks which correlated well to a greater number of MCH-ir cell bodies. Segal-Lieberman et al. [45] demonstrated in human and mouse that the MCH receptor type 1 is localized in GH pituitary cells and MCH stimulates GH release from dispersed human fetal pituitary cells, GH secreting adenomas and mouse pituitary cells. It is possible that the stimulatory effect of MCH on GH release from pituitary cells is present in fish as well, and it could be an alternative explanation for the difference found in the growth rate observed in barfin flounder exposed to different background colors.

C. dimerus is a cichlid fish that can be easily maintained and bred in the laboratory. In its natural habitat and under laboratory conditions, this species shows pronounced changes in body color under stress, mating, and environmental changing conditions. Previous studies have reported the localization of GH, SL and PRL cells in the pituitary, and the distribution of MCH-ir neurons during the ontogeny and in adults of *C. dimerus* [39,40]. As differences in weight and length are observed in this species in a short period of time under laboratory conditions in juvenile animals, *C. dimerus* is a good model to study the neuroendocrine control of GH/IGF-1 axis (personal observations).

In the present study, we tested the hypothesis that MCH regulates GH expression and release in *C. dimerus*. We first obtained and described the *C. dimerus* MCH and GH sequences. Then, in order to test if MCH is involved in the regulation of GH, we examined MCH fiber innervations mainly focused on GH pituitary cells. In intact pituitary cultures, we evaluated the effect of MCH on GH synthesis and release. Finally, we manipulated background color (white or black) in order to influence MCH levels and analyzed whether MCH and/or GH expression and somatic growth were affected.

2. Materials and methods

2.1. Fish

Adult *C. dimerus* were collected from "Esteros del Riachuelo", Corrientes, Argentina (27°12′50″S, 58°11′50″W), transferred to the laboratory and maintained in fresh water tanks (400 L). They were acclimated and kept under a stable condition of temperature (25 ± 2 °C) and a 14L:10D photoperiod. Fish were daily fed with commercial pellets (Tetra Pond Variety Blend) prior to be used for experiments.

Offspring from different *C. dimerus* couples were separated from their parents 7 days after hatching (at a free swimming stage) and maintained in fresh water tanks (10 L) at 25 ± 2 °C under a 14L:10D photoperiod until they were used in background color adaptation experiments. During this time, fish were daily fed with *Artemia* spp. nauplii (during the first 3 weeks) and then with commercial ground food (Tetra Cichlid© flakes). Principles of laboratory animal care (Guidelines on the care and the use of fish in research, teaching and testing, Canadian Council on Animal Care, 2005) were followed.

2.2. C. dimerus MCH and GH sequence

Five adult *C. dimerus* of both sexes (50–70 g) were anesthetized with benzocaine 0.1% and brain (for sequencing MCH cDNA) and pituitary glands (for sequencing GH cDNA) were collected after decapitation. Total RNA from both tissues was extracted by TRI Reagent (MRC, Inc.) following the manufacturer's instructions. RNA samples were quantified and its purity was confirmed by spectrophotometry. RNA samples (2 μg) were then treated with DNase I (Sigma) and then first strand cDNAs were synthesized by using an AMV enzyme (Promega). Conditions for the reverse transcription (RT) reaction were: 45 °C for 50 min and 70 °C for 10 min. Degenerate primers (Table 1) were designed according to conserved regions of MCH or GH cDNA sequences of perciform fish available in the GenBank database. PCR amplification for MCH was performed in a final volume of 25 μl by using a GoTaq Flexi DNA polimerase (Promega). Following initial 2 min of denaturation at 94 °C, the PCR cycle was repeated 40 times with denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and elongation at 72 °C for 30 s with a last extension step at 72 °C for 10 min. After electrophoresis on a 1% agarose gel, a specific band, which was assumed to be MCH, was extracted and purified from the agarose gel by using an AccuPrep gel purification kit (Bioneer). The purified PCR product was subsequently sequenced (Unidad de Genómica-Instituto de Biotecnología, CICVyA, INTA, Argentina) and confirmed to be partial MCH sequence using the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi/).

Table 1
Primers used for MCH and GH sequencing.

Name	Sequence (5′ → 3′)
degMCH f	GCTTTTRCCTCSCTGCTGAAC
degMCH r	ATACACTCGTCCCACCATGCA
degGH f	CTCCATTGCHGTCARCAGAG
degGH r	CCTTGTGCATGTCCTTCTTG
MCH f	AGAGGAGAGCCGACGAAAACAAC
MCH r	CCTCGGAGCTCTGGCTTTGGTTACGA
GRacer f	CGACTGGAGCACGAGGACACTGA
GRacer r	GCTGTCAACGATACGCTACGTAACG

Full-length MCH or GH was obtained by RNA ligase-mediated rapid amplification of 5′ and 3′ cDNA ends (RLM-RACE) by using the GeneRacer Kit (Invitrogen). The 1st strand cDNA was synthesized according to the GeneRacer Kit instructions using 2 μg total RNA and SuperScript III RT (Invitrogen). The specific *C. dimerus* MCH reverse and forward primers were designed based on the obtained partial sequence. For GH amplification, the degenerate primers were used to obtain the full length sequence. PCR amplification was performed by using the primers described above and the primers described for the 5′ and 3′ cDNA ends from the kit (Table 1). The PCR conditions were as follows: 3 min of initial denaturation at 94 °C, the PCR cycles were at 94 °C for 30 s, 72 °C for 30 s for 5 times, 94 °C for 30 s, 70 °C for 30 s for another 5 times and finally 94 °C for 30 s, annealing at 65 °C (MCH) or 64 °C (GH) for 30 s and 72 °C for 30 s for 35 times. The last extension step was at 72 °C for 10 min. The PCR products were purified and subsequently sequenced as described above. The full-length *C. dimerus* MCH and GH sequences were confirmed by using the BLASTN program.

2.3. Double-label immunofluorescence of MCH and GH

Brains from adult *C. dimerus* were fixed for 24 h in Bouin's fluid, embedded in paraplast (Fisherbrand, Fisher, Wash., USA) and coronally sectioned. Sections of 10 μm were deparaffinized in xylene, rehydrated through graded ethanol gradient to phosphate-buffered saline (PBS, pH 7.4), and incubated with PBS containing 5% non-fat dry milk at room temperature (RT). They were next incubated at 4 °C overnight (ON) with an anti-rat MCH antiserum raised in rabbit (kindly donated by Dr. B.I. Baker, University of Bath, UK; 1:500 dilution). Then, they were washed in PBS and incubated with biotinylated anti-rabbit-conjugated secondary antibody (1:800) at RT for 1 h. Afterwards, sections were incubated with