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REGULAR ARTICLE

Effect of salmon melanin-concentrating hormone and mammalian gonadotrophin-releasing hormone on somatolactin release in pituitary culture of *Cichlasoma dimerus*

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Abstract We detected a close morphological association between melanin-concentrating hormone (MCH)-immunoreactive (ir) fibers and somatolactin (SL)-ir cells in the pars intermedia of the cichlid fish *Cichlasoma dimerus* by double-label immunofluorescence. Male pituitaries obtained from adult *C. dimerus* were incubated with 0.1–10 μM salmon MCH, and the amount of SL released into the culture medium was semi-quantified by Western blot. This assay showed an increase of SL release in a dose-dependent manner (linear regression: $P<0.05$). A close association of GnRH-ir fibers with SL-ir cells was also detected at the pars intermedia level. Male pituitaries were also incubated with 0.1–10 μM of mammalian GnRH, and SL release was semi-quantified by Western blot, showing an increase of released SL levels in a dose-dependent manner (linear regression: $P<0.05$). In contrast, SL release was unaffected from female pituitaries incubated with salmon MCH; however, an increasing tendency was observed when mammalian GnRH was used. Hypothalamic close association of MCH-ir perikarya and GnRH-ir fibers was found by double-label immunofluorescence indicating a possible relationship between them. These results suggest that SL, like other pituitary hormones, is under hypothalamic control and is involved in diverse physiological processes including background adaptation and reproduction. This study has also shown that the in vitro culture of a single *C. dimerus* pituitary is a feasible method for studying the control of SL release and other pituitary hormones.

Keywords Hypothalamus · Immunofluorescence · Western blot · (Teleostei, Perciformes, Cichlidae) *Cichlasoma dimerus*

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Introduction

Hypothalamic melanin-concentrating hormone (MCH) is a neuropeptide, with an apparent neuromodulatory role, that is present in all vertebrates. This hormone was originally isolated from the chum salmon, *Oncorhynchus keta* (Kawauchi et al. 1983) and was found to be a 17-amino-acid cyclic peptide. Characterization of mammalian MCH has demonstrated that this neuropeptide has been highly conserved throughout vertebrate evolution (Nahon 1994). In mammals, MCH has been shown to have central roles as a neurotransmitter or neuromodulator, regulating food intake, energy homeostasis, stress, reproduction, behavior, sensory perception, and neuroendocrine responses (Forray 2003; Griffond and Baker 2002; Kawauchi and Baker 2004; Pissios and Maratos-Flier

2003). In teleost fish, MCH has been described as a neurohypophysial hormone with a pale-pigmentary role, in addition to its aforementioned function in mammals as a neurotransmitter and neuromodulator (Baker and Bird 2002). MCH has also been proposed as a hypothalamic regulator of the release of pituitary hormones (Baker et al. 1985; Balm and Gröneveld 1998).

Gonadotrophin-releasing hormone (GnRH) is a decapeptide that is synthesized primarily by neurons within the central nervous system. Its main biological function is to induce the production and release of gonadotrophins. To date, several distinct forms of GnRH have been identified in vertebrates (Adams et al. 2002). In particular, several species of perciform teleosts express three different forms of GnRH in their brains: GnRH I, GnRH II, and GnRH III (White and Fernald 1998). GnRH I is always expressed in the preoptic area, from where GnRH fibers project to the pituitary, and acts mainly as an hypophysiotropic hormone; GnRH II is expressed in the midbrain tegmentun, whereas GnRH III is expressed in the ventromedial olfactory bulb (Weltzien et al. 2004).

Teleost fish have no median eminence or portal system; therefore, the nerve fibers originating from the hypothalamus are in close contact with pituitary cells. Immunocytochemical studies have demonstrated that neurohormonal fibers and terminals are specifically localized in the pituitary in association with endocrine cells. Because of this special anatomical arrangement, teleosts are a unique experimental model for determining putative brain peptides or monoamines involved in the regulation of endocrine cells of the pituitary (Peter et al. 1990).

Somatolactin (SL) is a pituitary hormone that is only present in actinopterygian fish and the sarcopterygian lungfish *Protopterus annectens* and that belongs to the growth hormone (GH) family (Kawauchi and Sower 2006). Its synthesis and secretion is restricted to the somatolactotroph cells of the pars intermedia. SL is involved in various physiological processes, such as the regulation of some aspects of reproduction and the response to stress (Planas et al. 1992; Rand-Weaver and Swanson 1993; Mousa and Mousa 2000; Vissio et al. 2002; Rand-Weaver et al. 1993; Johnson et al. 1997). Other studies have reported the relationship between SL, the acid-base balance (Kakizawa et al. 1995, 1997), and calcium regulation (Kaneko and Hirano 1993). In several pieces of work, SL has been associated with background adaptation (Kakizawa et al. 1995; Zhu and Thomas 1995, 1996, 1997, 1998; Zhu et al. 1999; Fukamachi et al. 2004; Cánepa et al. 2006). In the medaka, *Oryzias latipes*, the "color interfere" mutant (an abnormal proliferation and morphogenesis of chromatophores) occurs because of a truncated SL gene (Fukamachi et al. 2004). Moreover, SL is absent in mouse and human genomes, suggesting that these species have lost the gene and its background adaptation function during evolution (Fukamachi et al. 2004). These findings provide strong support for pigmentation control being one of the functions of SL.

Although several studies have focused on SL function, little information is available concerning the regulation of SL secretion. Kakizawa et al (1997) have shown that salmon GnRH, corticotrophin-releasing factor, and serotonin stimulate SL release in cultured pituitaries of rainbow trout, *Oncorhynchus mykiss*. In masu salmon, *Oncorhynchus masou*, salmonGnRH elevates SL mRNA expression in primary pituitary cell cultures (Onuma et al. 2005).

Cichlasoma dimerus (Perciform), a South American cichlid fish, represents an interesting experimental model because of its high survival and reproductive rates under laboratory conditions. This cichlid shows a marked color display during stress, mating, and environmental changes. Among other studies, the localization of SL-immunoreactive (ir) cells (Pandolfi et al. 2001a, b) and the expression of GnRH, MCH, and alpha-melanocyte-stimulating hormone (α-MSH) cells (Pandolfi et al. 2002, 2003, 2005) have been reported for *C. dimerus*.

In this species, MCH-ir and GnRH-ir fibers have been observed reaching the three regions of the adenohypophysis (ADH), specially the pars intermedia (Pandolfi et al. 2002, 2003) in which SL and αMSH cells are localized (Pandolfi et al. 2001a). In addition, SL cells, together with MCH and αMSH, have been shown to be involved in background color adaptation (Cánepa et al. 2006). In other teleost species, several studies have demonstrated that SL cells express GnRH receptors (Parhar et al. 2005; Stefano et al. 1999). On the basis of these previous results, we have hypothesized that MCH and GnRH fibers are morphologically and functionally related to SL cells. Therefore, we have analyzed, by double-label immunofluorescence, the relationship of MCH and GnRH fibers with SL cells. We have also analyzed the effect of salmon MCH and mammalian GnRH (which belongs to the type I GnRH group) on SL release by performing *in vitro* cultures of *C. dimerus* pituitaries to validate the possible regulating function of MCH and GnRH implied by their morphological association. This study provides evidence that MCH and GnRH have an effect on SL cells, by using these two independent techniques. Additionally, we have established that our *in vitro* system with individual pituitaries is a reliable method for studying the regulation of pituitary hormones and its correlation with morphological associations.

Materials and methods

Fish

The reproductive adults of *C. dimerus* (n=95; length: females 12.6±0.2 cm, males 12.4±0.3 cm; weight: females

45.6±1.2 g, males 48.1±0.9 g; gonadosomatic index: females 3.19±0.15, males 0.090±0.003) used in this study were captured in Esteros del Riachuelo, Corrientes, Argentina (27'25°S, 58'15°W), were maintained in well-aerated conventional tanks with external filtration at 27±1°C under a 12:12 h photoperiod, and were fed with commercial pellets prior to processing. As a dominant male or a reproductive female will probably show different hormone-release levels and cell immunoreactivity than non-reproductive fish, animals with an extremely high GSI were discarded.

Principles of laboratory animal care (NIH Guide for the Care and Use of Laboratory Animals, 1996) were followed.

Tissue processing for immunofluorescence

Ten fish were anesthetized with MS222 solution (Sigma; 150 mg/l) and killed by decapitation. Brains with pituitaries attached were removed and fixed in Bouin's solution at 4°C for 24 h. Samples were then dehydrated, embedded in Paraplast (Fisherbrand, Fisher, Wash., USA), coronally sectioned at 7 μm, and mounted on gelatin-coated slides.

Double-label immunofluorescence

Preparation of tissue sections was as previously described for *C. dimerus* (Cánepa et al. 2006). Briefly, sections were deparaffinized in xylene, rehydrated through graded ethanol to phosphate-buffered saline (PBS, pH 7.4), and incubated with PBS containing 5% non-fat dry milk at room temperature (RT). Next, they were incubated at 4°C overnight (ON) with rabbit *Sparus aurata* (sa) SL antiserum (1:1,000 dilution; Astola et al. 1996), washed in PBS, and incubated with anti-rabbit fluorescein-conjugated secondary antibody at RT for 40 min. Subsequently, the sections were incubated at 4°C ON with synthetic rabbit MCH antibody (kindly donated by Dr. B.I. Baker, University of Bath, UK; 1:1000 dilution), washed, incubated at RT for 40 min with an anti-rabbit rhodamine-conjugated secondary antibody, and analyzed by confocal laser microscopy (Olympus FV-30 attached to an Olympus Bx-61 microscope). This system with two rabbit primary antibodies was performed since SL does not co-localize with MCH. We obtained two micrographs, one showing SL cells (green) and the other showing MCH fibers (red). These images were digitally superimposed.

Double-label immunofluorescence with anti-GnRH (1:5,000 dilution; LRH13) monoclonal antibody and saSL antiserum was performed. SL cells were revealed as before. GnRH-ir fibers were developed with the catalized signal amplification system and rodamine-conjugated streptavidin (Dako, Carpinteria, Calif.) as recommended by the manufacturer, at RT. The double-label immunofluorescence was then analyzed by confocal laser microscopy.

In addition, double-label immunofluorescence with anti-GnRH (1:5,000; LRH13) monoclonal antibody and MCH antiserum was performed as before. GnRH was visualized by using an amplification system and rodamine-conjugated streptavidin and MCH with an anti-rabbit fluorescein-conjugated secondary antibody.

Specificity tests for the SL antiserum were as previously described in *C. dimerus* for immunohistochemistry and Western blot, and for the LRH13 monoclonal antibody and MCH antiserum for immunohistochemistry. Briefly, for the specificity controls, all antisera were preadsorbed with an excess of antigen, and then these solutions were used as primary antibodies. In addition, primary antibodies were omitted in both immunohistochemistry and Western blot. All specificity tests were negative (Pandolfi et al. 2001b, 2002, 2003; Cánepa et al. 2006).

SL release from *in vitro* cultures of pituitaries of *C. dimerus*

Pituitaries were removed from male (n=40) and female (n=40) *C. dimerus*. Sampling procedures were similar to those described previously by Astola et al. (2004). Five glands per treatment were obtained from *C. dimerus* of both sexes; these were individually incubated in 80% (v/v) Leibovitz L_{15} medium (Gibco), pH 7.4, containing 10% fetal bovine serum, 10 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml of streptomycin and maintained in a dark incubator at 27°C. Pituitaries were individually incubated for 1 day (24 h) in culture medium in order to establish basal conditions of SL release. Subsequently, the medium was removed and stored frozen at -20° for further analysis. Immediately following removal of the medium, fresh medium was randomly added containing either salmon MCH or mammalian GnRH (0.1 μM to 10 μM) or without salmon MCH/mammalian GnRH as an internal control. Pituitary culture assay was carried out on intact pituitaries only, and so 10 μM was used as the highest concentration to ensure that salmon MCH and mammalian GnRH reached SL cells localized in the inner portion of the pituitary. After 24 h of treatment (day 2), the medium was removed and stored frozen. Finally, pituitaries were fixed in Bouin's solution at 4°C for 24 h, dehydrated, and embedded in Paraplast (Fisherbrand) in order to check the morphology of SL cells and the integrity of the gland tissue.

As a control of heterologous hormones, the effect of salmon MCH on scale cultures was carried out by using saline solution (NaCl, 160 mM; KCl, 8.6 mM; $CaCl_2$, 3.0 mM; $MgCl_2$, 2.5 mM; $NaHCO_3$, 10 mM; Na_2HPO_4, 1.3 mM; D-glucose 5.0 mM). The effect of increasing concentrations of salmon MCH, from 0.1 to 100 nM, was analyzed in order to validate the use of an heterologous hormone. Photographs of the same part of a representative scale from the dorsal trunk were taken. The expected results

were obtained: whereas scale cultures without salmon MCH showed disperse pigment granules in their melanophores, salmon MCH caused the aggregation of granules in a dose-dependent manner (data not shown).

A pituitary culture assay with mammalian GnRH from 0.01 to 1 μM followed by Western blotting for β-luteinizing hormone (βLH) was performed in order to validate the use of mammalian GnRH (GnRH type I group). The stimulatory effect of GnRH on LH pituitary cells is well known, and so LH release was evaluated as a positive control. Results showed a clear effect on LH release as evidenced by Western blot (Fig. 1) with *Fundulus heteroclitus* LH antisera, whose specificity was previously well- characterized in *C. dimerus* (Pandolfi et al 2006)

Western blot analysis

The presence of SL in the pituitary culture media was examined by immunoblots with rabbit saSL antiserum as the primary antibody. Briefly, samples (15 μl) of each pituitary culture medium were separated on 15% sodium dodecylsulfate-polyacrylamide gels by electrophoresis (SDS-PAGE). Proteins and molecular markers (SeeBlue Plus2 PreStained Standard, Invitrogen) were then transferred onto a nitrocellulose membrane (Amersham Biosciences, UK) for 75 min at 75 V, as previously described by Cánepa et al (2006). The membranes were washed in TRIS-buffered saline with Tween (TBST) at pH 7.5 (100 mM TRIS–HCl, 0.9% NaCl, 0.1% Tween-20) and blocked with TBST containing 3% non-fat dry milk and 3% bovine serum albumin at 4°C ON. Later, they were incubated at RT for 3 h with rabbit anti-saSL (1:2,000; this antiserum dilution had been previously established for *C. dimerus*; Cánepa et al. 2006). After three washes in TBST, membranes were incubated with an anti-rabbit IgG peroxidase-conjugated streptavidin (1:5,000) at RT for 45 min and again washed. The reaction was visualized on the nitrocellulose by an enzyme-linked enhanced chemiluminescence detection system (Amersham Biosciences), and the image was captured with a Luminescent Image Analyzer LAS-1000 plus (Fuji Photo Film). In all cases, blotting and developing conditions were repeated. In order to determine whether this semi-quantitative method was sensitive enough to detect significant differences, pituitary culture medium was serially diluted (5, 10, 15, 20 μl) and further Western blots were performed.

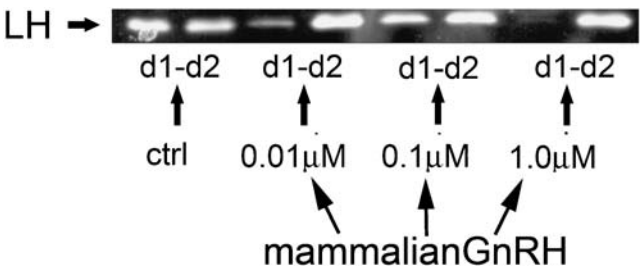


Fig. 1 Mammalian GnRH control tests. SDS–polyacrylamide gel electrophoresis analyses of βLH released; Western blotting. Representative βLH release from pituitary culture (*d1* day 1, *d2* day 2) performed with increasing concentrations of mammalian GnRH (0.01–1 μM)

SL release was semi-quantified by densitometric analysis and normalized against a 148-kDa protein present only in culture medium in order to avoid possible loading errors in the SDS-PAGE. This protein was visualized by Ponceau-S in the nitrocelulose membrane, digitalized, and semi-quantified. Quantification of culture media proteins could not be achieved, since pituitary hormones are released into the surrounding medium, which itself contains several proteins.

The optical density of both the 28-kDa (data not shown) and 32-kDa SL-ir bands and the 148-kDa band was measured by using Image Gauge Version 3.12 (Fuji Photo Film) Software. Values are expressed in arbitrary units as means±SEM ($P<0.05$).

SL release from each pituitary was evaluated as follows: day 1 and 2 culture media from each treatment were loaded into adjacent lanes for SDS-PAGE. Day 1 was considered as an indicator of the release state of the gland, and hence, SL of day 2 was normalized against that of day 1. This normalization allowed us to compare values obtained from different pituitaries; these values may have been different because of non-controlled variables present in the animals.

Viability control was performed as follows: pituitaries of both sexes, after incubation with medium containing salmon MCH or mammalian GnRH, were randomly selected, the medium was removed, and fresh medium was added. On the third day of incubation (24 h), the medium was again removed and stored frozen. Day 3 culture media was obtained in order to check that SL release returned to the basal levels observed in control treatments, and that the stimulatory effect of both peptides was not a non-specific activation. Additionally, pituitaries after 1, 2, and 3 days of culture were examined by immunohistochemistry and light counterstaining with hematoxylin in order to verify the morphological structure of SL cells and the morphology of the gland after treatment with mammalianGnRH or salmonMCH.

Five pituitaries were obtained from adult *C. dimerus* and were individually homogenized in 100 μl 50 mM TRIS-HCl buffer (pH 7.4). Western blot was performed as before in order to compare the relationship between the optical densities of the SL bands obtained from pituitary content and culture medium, for both the 32-kDa-ir and 28-kDa-ir bands. Since the relationship between the 32-kDa and 28-kDa bands was similar for both pituitary content and culture medium (see Results), only the results obtained for the 32-kDa band are presented.

Statistical analysis

Statistical analysis of data was performed by using an analysis of variance (ANOVA) followed by Tukey's test. When data did not meet the homogeneity of variances assumption, a log data transformation was applied. Statistical significance was established at the $P<0.05$ level. Linear regression analysis was performed by applying $\log_{10}$(concentration + 1) data transformation. When no data transformation was possible, the Kruskal Wallis test was used. Data are presented as mean±SEM.

Results

Double-label immunofluorescence

Double-label immunofluorescence showed a clear morphological association between SL-ir cells and both MCH-ir and GnRH-ir fibers (Fig. 2a,c). In detail, high densities of MCH-ir fibers were found reaching SL-ir cells, and in particular, MCH staining in the pars intermedia showed the strongest immunoreactivity close to SL-ir cells (Fig. 2b). On the contrary, GnRH-ir fibers were mostly found in the dorsal region of the pars intermedia, and only a minor quantity of them reached the ventral region (Fig. 2c). When compared with MCH-ir fibers, a smaller number of GnRH-ir fibers reached SL-ir cells of the ventral region of the pituitary (Fig. 2d), even though a close association of GnRH fibers with SL-ir cells was also observed in this region. On the other hand, we observed differences in the distribution of GnRH and MCH fibers, so we performed double-label immunofluorescence against GnRH and MCH in order to compare the said distribution in the pars intermedia. We observed that MCH was mainly located in the ventral region of the neurohypophysis, whereas GnRH fibers were predominant in the dorsal region (Fig. 2e). In this last assay, we found a morphologically close association between MCH projections and perikarya and GnRH-ir fibers in the hypothalamus (Fig. 2e–g). Both MCH and GnRH fibers apparently projected toward the pituitary, as previously observed in *C. dimerus*. These MCH-ir neurons were magnocellular and were localized in the nucleus lateralis tuberis as previously described (Pandolfi et al. 2003).

Release of SL from *in vitro* cultures of pituitaries

Analysis by immunoblots showed that pituitaries, incubated with 100 μl minimal essential medium for 1 day (24 h), released SL at detectable levels. This assay also showed that the pituitary of *C. dimerus* released two SL-ir forms with molecular weights of 32 and 28 kDa. These estimated molecular weights of SL-ir bands from culture media corresponded to those obtained from pituitary homogenates that were previously described for *C. dimerus* (Cánepa et al. 2006). Moreover, the relationship between the optical density of the 32 and 28 kDa bands (32 kDa/28 kDa) from pituitary homogenates and culture media of pituitary cultures showed similar values. This indicated that both forms of SL (32 and 28 kDa) were released at similar rates (Fig. 3; 1.118±0.036 homogenate vs 1.136±0.055 SL released; relative optical density). For this reason, we only analyzed the 32-kDa band to semi-quantify the amount of SL released from pituitaries.

A 148-kDa protein only present in the culture medium, as evidenced by Ponceau-S staining, was used as a loading control (Fig. 4a). Analysis by immunoblot of SL from serially diluted pituitaries culture media showed that this method was sensitive enough to detect differences between treatments (Fig. 4b).

SL released from male pituitaries increased in a dose-dependent manner after incubation in culture medium containing salmon MCH (day 2; linear regression, $P=0.025$; ANOVA, $P=0.031$; Fig. 5a,b). However, female pituitaries incubated with increasing salmon MCH concentrations did not show significant differences in SL release between treatments (Fig. 5c). Male relative optical density (ROD) values were control, 0.678±0.108; 0.1 μM, 0.859±0.010; 1.0 μM, 0.881±0.087; 10.0 μM, 1.398±0.238. Female ROD values were control, 0.831±0.063; 0.1 μM, 0.870±0.047; 1.0 μM, 0.849±0.016; 10.0 μM, 0.964±0.192.

Incubation with mammalian GnRH showed similar results to those of the salmon MCH assay; SL released from male pituitaries increased significantly in a dose-dependent manner (linear regression, $P=0.046$; ANOVA, $P=0.021$; Fig. 6a,b). Although female pituitaries did not show any evidence of significant differences between treatments, we observed a noticeable increasing tendency (Fig. 6c). Male ROD values were control, 0.648±0.109; 0.1 μM, 0.972±0.112; 1.0 μM, 1.403±0.249; 10.0 μM, 1.761±0.509). Female ROD values were control, 0.921±0.059; 0.1 μM, 1.212±0.106; 1.0 μM, 1.238±0.514; 10.0 μM, 1.430±0.440.

Immunohistochemistry of pituitaries used in the *in vitro* culture assays showed normal general histology and normal morphology of SL-ir cells (Fig. 7a). After 3 days of *in vitro* culture, the amount of SL released from pituitaries returned to similar basal levels to those observed in control treatments (Fig. 7b), even after incubation with mammalian GnRH or salmon MCH. This established the validity of this system, since the pituitaries used in this sort of assay did not lose their response capacity.

Discussion

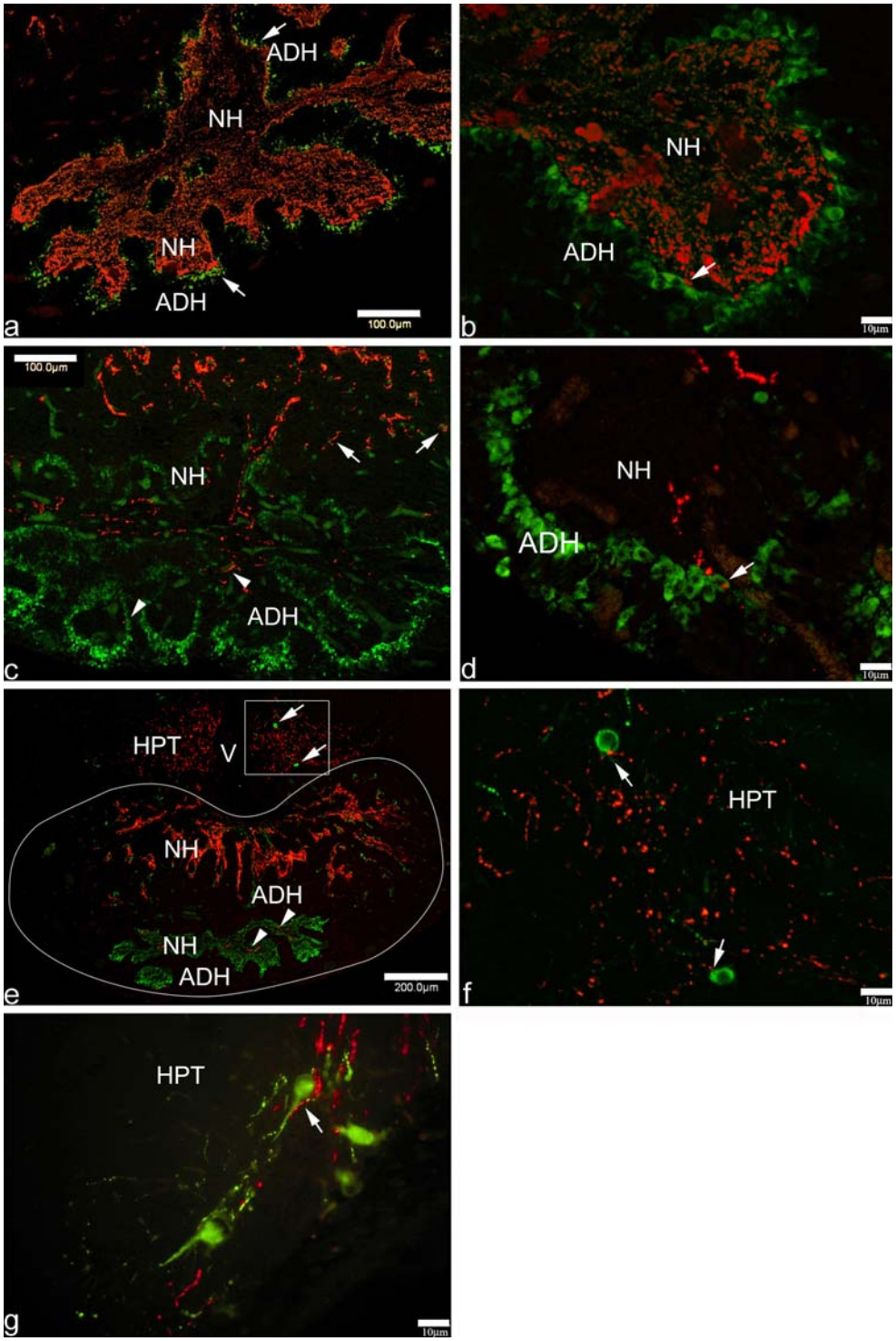
Based on the results of the present study, we propose the existence of a physiological relationship between pituitary SL cells, MCH, and GnRH neurons of the hypothalamus. We have confirmed this by two different techniques.

As previously reported for *C. dimerus* pituitaries, MCH-ir fibers are distributed throughout the neurohypophysis, mainly at the pars intermedia level, and are confined to it (Pandolfi et al. 2003). SL-ir cells are localized in the pars intermedia closely surrounding the neurohypophysis (Pandolfi et al. 2001a). In the present work, we have demonstrated that SL-ir cells are in close contact with MCH-ir fibers as suggested by previous studies. MCH magnocellular neurons of the nucleus lateralis tuberis send their axons toward the pars intermedia (Pandolfi et al. 2003); these magnocellular neurons have been implicated in background color adaptation (Baker and Bird 2002). Particularly in *C. dimerus*, MCH-ir neurons of the nucleus lateralis tuberis and pituitary MSH-ir cells have been related to background color adaptation, together with SL-ir cells (Cánepa et al. 2006) and MCH-ir neurons of the lateral recessus nucleus (M. Cánepa, unpublished). This work shows an opposite response of SL-ir cells and MCH-ir neurons in background color adaptation. Whereas SL-ir cells are larger and more numerous in animals adapted to black tanks than in those adapted to white tanks, MCH-ir neurons are more active in animals adapted to white tanks. Baker and Bird (2002) have suggested that MCH would have acquired a pituitary regulatory role early in vertebrate evolution; recently, MCH-R2 transcript has been detected at a low expression rate in pituitaries from the barfin flounder *Verasper moseri* (Takahashi et al. 2007). Although molecular cloning of MCH receptors and the further tissue distribution of MCH have not been carried out in *C. dimerus*, the close association of SL cells and MCH fibers suggests that they might interact through specific receptors located in SL cells.

Several studies have provided evidence for the relation between SL cells and GnRH. In the present work, we have found a close association of SL-ir cells and GnRH-ir fibers by double-label immunofluorescence. At the pars intermedia level, GnRH-ir fibers are mainly located in the dorsal region of the neurohypophysis, where SL-ir cells have also been visualized, and in the same region in which cells immunoreactive for follicle-stimulating hormone and LH have been located (Pandolfi et al 2006). In this region, SL-ir cells stain more weakly than SL-ir cells from the ventral region. The close association has mostly been observed in the dorsal region. However, GnRH-ir fibers have also been seen in close contact with SL-ir cells in the ventral region of the neurohypophysis, where a SL-MCH close association has also been found. This close association is in accordance with studies carried out on other teleost fish species and showing co-localization of the GnRH receptor and SL-ir cells. In the Pejerrey, *Odontesthes bonariensis*, co-localization of GnRH-binding sites and SL-expressing pituitary cells has been found by using a pituitary primary cell culture system (Stefano et al. 1999); a close association between GnRH fibers and SL expressing cells has also been reported for this species (Vissio et al. 2002). In the tilapia, *Oreochromis niloticus*, a cichlid fish like *C. dimerus*, GnRH receptors co-localize with SL-ir cells (Parhar et al. 2005). The existence of a close contact between GnRH-ir fibers and SL-ir cells in *C. dimerus* suggests that SL cells are also regulated by GnRH in this species.

Since a close association between salmon MCH and SL cells was detected by double-label immunofluorescence, *in vitro* culture of intact pituitaries followed by Western blot analysis of culture media was performed in order to investigate a possible releasing or inhibiting function of salmon MCH on SL cells. We found that salmon MCH stimulated SL release from pituitaries of male *C. dimerus* in a dose-dependent manner; no such an effect was observed in females. Only intact pituitaries were used for the pituitary culture assay; for this reason, we chose 10 μM salmon MCH as the highest concentration, to insure that it reached SL cells localized in the inner portion of the pituitary. In a previous work by Kakizawa et al (1997), 0.1 μM salmon MCH stimulated SL release from in vitro

Fig. 2 Close association of SL-MCH, SL-GnRH, and MCH-GnRH ▶ detected by double-label immunofluorescence in coronal sections at the hypothalamus-pituitary level of *Cichlasoma dimerus* (*ADH* adenohypophysis, *NH* neurohypophysis, *V* ventricle). **a** Confocal micrograph of double-label immunofluorescence for MCH (*red*) and SL (*green*) showing high quantities of MCH-ir fibers in close association with SL-ir cells at the pars intermedia level (*white arrows* examples of close association). **b** Higher magnification of SL and MCH association. Strong immunofluorescence for MCH (*red*) surrounding SL cells (*green*) can be seen (*white arrow*). **c** Confocal micrograph of double-label immunofluorescence for GnRH (*red*) and SL (*green*) showing some close morphological association in the ventral region (*arrowhead*) but mainly in the dorsal region (*arrows*) of the pars intermedia. **d** Detail of SL-ir cell in close association with GnRH-ir fiber (*white arrow*). **e** Confocal micrograph of double-label immunofluorescence for MCH and GnRH comparing the distribution of both peptides in the pituitary and hypothalamus (*HPT*). MCH-ir fibers (*green*) are located throughout the pars intermedia, but the strongest immunofluorescence is observed in the ventral region. GnRH-ir fibers (*red*) are mainly located in the dorsal region, and as described above, a few GnRH fibers are also located in the ventral region (*white arrowheads*). In the hypothalamus, close association between MCH somata and GnRH fibers is observed (*white arrows*). A *line* was drawn in order to facilitate the analysis of pituitary morphology. **f** Detail of nucleus lateralis tuberis (NLT) area (*boxed area* in **e**) in which MCH magnocellular neurons (*green*) are in close association with GnRH-ir fibers (*red*, *arrows*). **g** Fluorescence micrograph showing MCH (*green*) magnocellular NLT neurons in contact (*arrow*) with GnRH (*red*) fibers



cultures of *Oncorhynchus mykiss* pituitaries without dose-dependence. Moreover, in *O. mykiss*, MCH at a final concentration of 1 μM stimulated SL release from *in vitro* cultures of pituitaries (Balm and Gröneveld 1998). In the two mentioned studies, SL levels were quantified by radioimmunoassay, a more sensitive method than the one used in the present work, but both presented results following the use of only one MCH concentration. The concentrations used in this work might be similar to the concentrations found in the vicinity of MCH terminals and SL cells. We assumed that 10 μM salmon MCH did not stimulate SL release in males unspecifically, since no effect in females was observed when we use the same concentration.

SL cells are located in the pars intermedia together with pituitary MSH-ir cells. Both hormones are thought to be involved in background color adaptation in *C. dimerus*

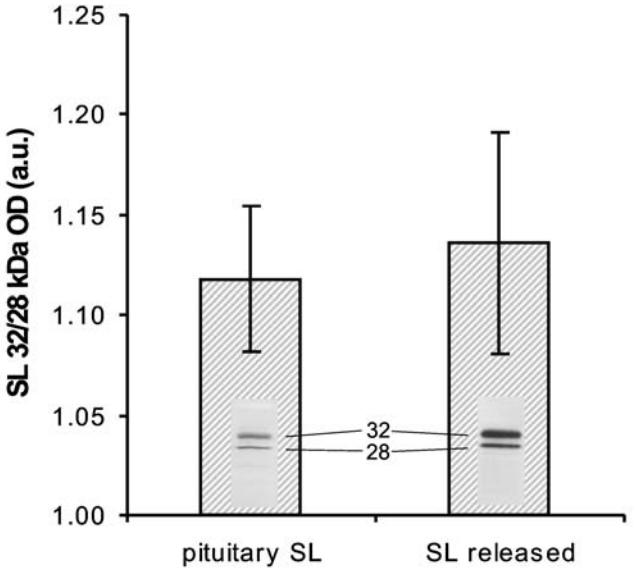


Fig. 3 Relationship of optical densities (*OD*) in arbitrary units (*a.u.*) between the 32-kDa and 28-kDa SL forms. No significant differences were found between pituitary content and amount released into culture medium

Male *C. dimerus* show a particular coloration display during courtship, spawning, and territorial and dominance behavior (Alonso et al. 2007); in females, this display is not so evident, which could explain why female pituitaries show no increase in SL release when stimulated with

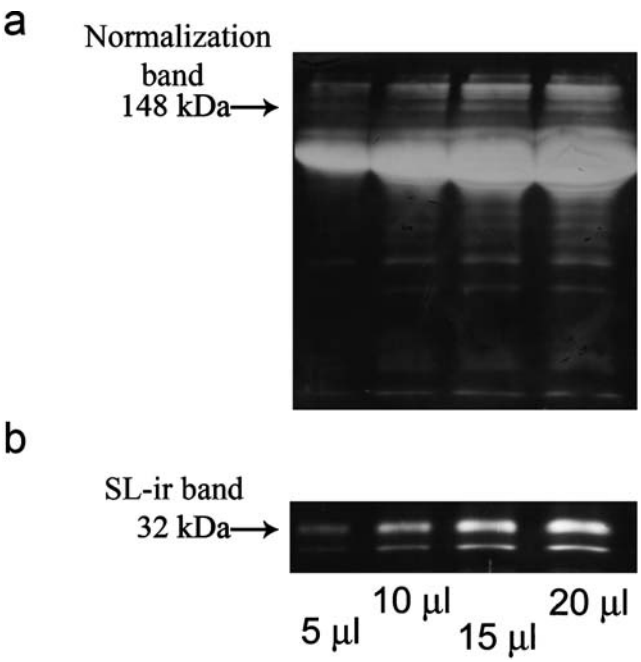


Fig. 4 Serially diluted culture medium from single pituitary cultures showing the reliability of Western blot as a sensitive method. **a** Total protein visualized by Ponceau-S showing the 148-kDa band, only present in culture medium, in increasing dilutions of pituitary culture media. **b** Intensity of SL immunoblots in increasing dilutions of media pituitary culture

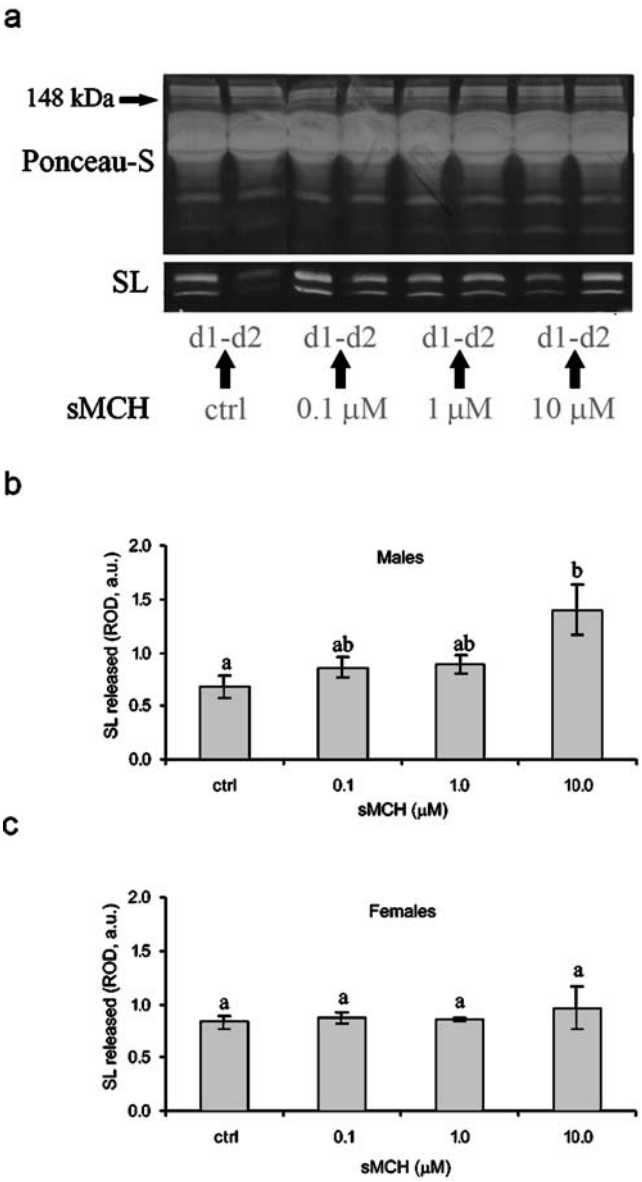


Fig. 5 **a** Representative immunoblots of SL from male pituitary culture media of day 1 (*d1*) and after treatment, at day 2 (*d2*), with increasing concentrations of salmon MCH (*sMCH*). **b**, **c** Analysis of SL released from pituitaries of male and female *C. dimerus*, respectively, following treatment with increasing concentrations of salmon MCH (0.1–10 µM). Values are expressed in arbitrary units (*a.u.*) as means±SEM of optical density of SL released on day 2/ optical density of SL released on day 1 (*ROD* relative optical density). A stimulatory effect of salmon MCH on SL release from male pituitaries was detected; no effect was detected for females. Linear regression revealed significant differences in the male assays ($P<0.05$; *different letters* significant differences between groups)

salmon MCH in the *in vitro* culture assay. In the tilapia, *Oreochromis mossambicus*, MCH affects α-MSH release from the pituitary, showing a biphasic response: incubation with MCH from 10 nM to 1 µM results in a concentration-dependent inhibition, whereas at final concentrations of 1 µM and 35 µM, α-MSH release increases (Gröneveld

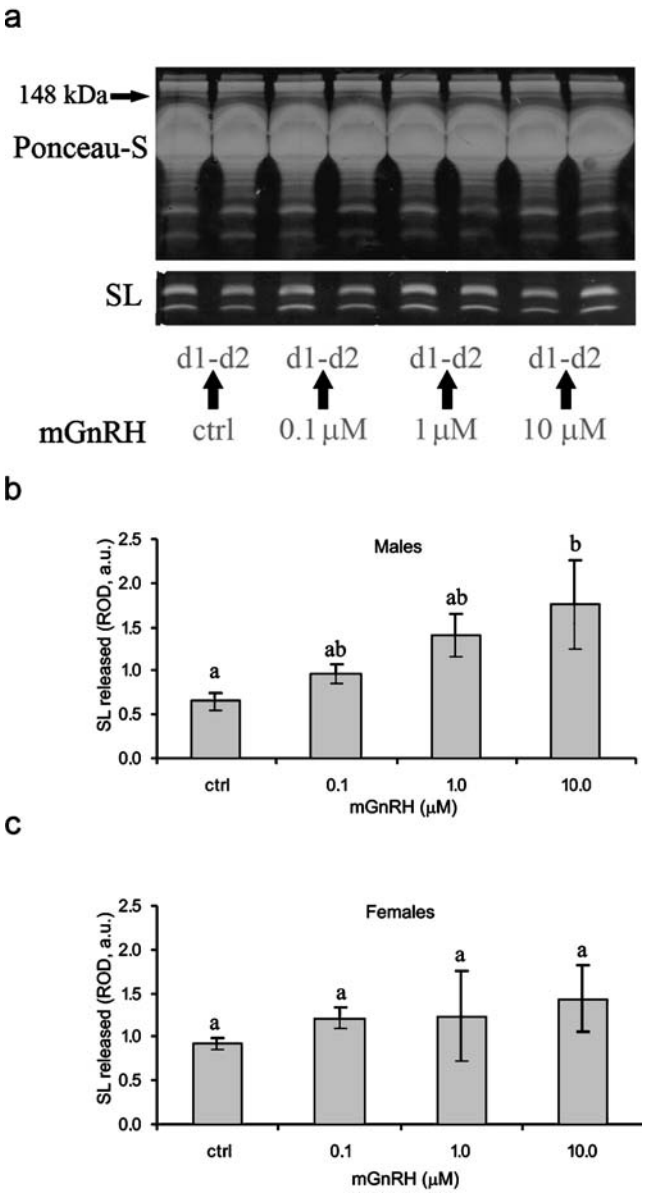


Fig. 6 **a** Representative immunoblots of SL from male pituitary culture media of day 1 (*d1*) and after treatment, at day 2 (*d2*), with increasing concentrations of mammalian GnRH (*mGnRH*). **b**, **c** Analysis of SL released from male and female pituitaries, respectively, following treatment with increasing concentrations of mammalian GnRH (0.1–10 µM). SL released from male pituitaries (**b**) increased in a dose-dependent manner (linear regression analysis revealed significance; $P<0.05$). SL released from female pituitaries (**c**) did not show significant differences, although a clear incresing tendency in a dose-dependent manner was observed. Values are expressed in arbitrary units (*a.u.*) as means ± SEM (*ROD* relative optical density). *Different letters* indicate significant differences between groups

et al. 1995). Although the in vitro culture assay has shown unequivocal results concerning the effect of MCH on SL release, we are currently undertaking molecular approaches in order to locate a MCH receptor in SL cells of *C. dimerus* by *in situ* hybridisation.

The effect of mammalian GnRH on SL release was assessed by an *in vitro* pituitary culture assay. We demonstrated that GnRH affected SL release *in C. dimerus*, as observed in other teleosts, and we also validated these results by double-label immunofluorescence. In this assay, we used mammalian GnRH belonging to the GnRH type I group and seabream GnRH. In *C. dimerus*, seabream GnRH fibers were mainly observed innervating the pituitary, and so, if SL cells possessed GnRH receptors, we should have observed an effect on SL release in pituitary cultures. In the present work, pituitaries of male *C. dimerus* incubated with mammalian GnRH gave a dose-dependent response, and an increasing tendency was also seen in females. Our use of intact pituitaries necessitated a high concentration of mammalian GnRH to ensure that it

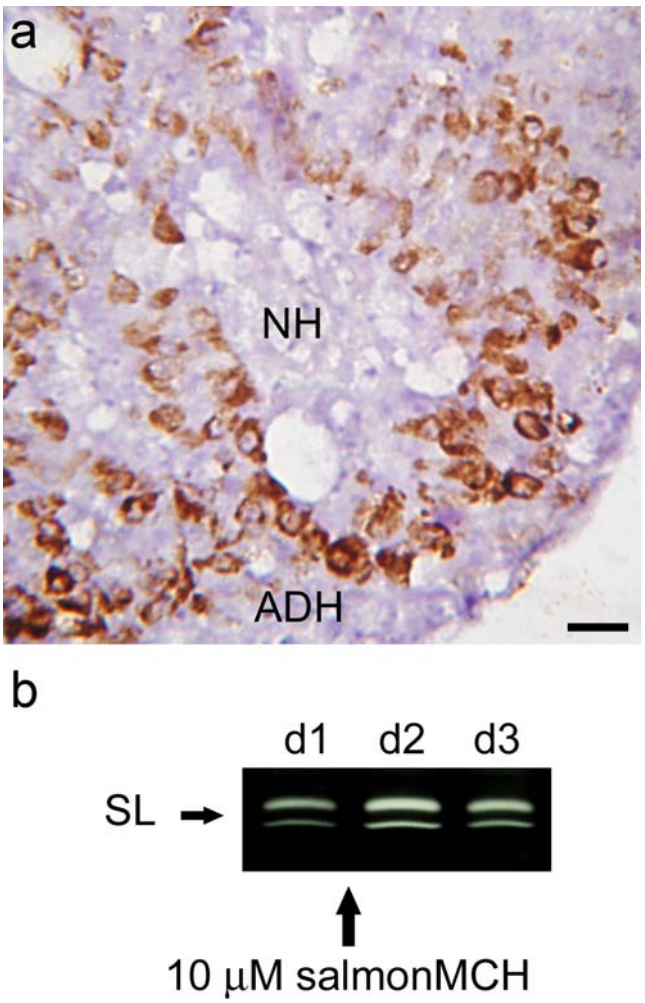


Fig. 7 Three-day pituitary cultures demonstrating the validity of the system. **a** Representative immunohistochemistry showing the morphology of SL-ir cells (*NH* neurohypophysis, *ADH* adenohypophysis). *Bar* 10 µm. **b** Representative Western blotting demonstrating SL released into culture medium after the first 24 h of treatment to give basal levels (*d1* day 1), after incubation with 10 µM of salmon MCH (*d2* day 2), and at the end of treatment returning to basal levels (*d3* day 3)

reached the SL cells located in the internal portion of the organ. These results were in accordance to results from rainbow trout, *Oncorhynchus mykiss*, where similar assays were performed, i.e., GnRH at 10, 100, and 1000 nM stimulated SL release, as measured by radioimmunoassay, in a dose-dependent manner (Kakizawa et al. 1997). In sockeye salmon, *Oncorhynchus nerka*, a capsule of an GnRH analog was implanted in the dorsal muscle of maturing animals, causing an increase of SL mRNA (Taniyama et al. 2000). Our results clearly demonstrate that mammalian GnRH stimulates SL release, thus validating the double-label immunofluorescence result. However, whether this increase occurs together with an increase in the expression of the SL gene is unclear. Molecular studies are planned in order to elucidate changes in SL mRNA expression.

The distribution of GnRH-fibers has been well described in *C. dimerus*. Salmon and mostly seabream GnRH fibers innervate the pituitary at the pars intermedia level together with MCH fibers (Pandolfi et al. 2003, 2005). We have compared the distributions of GnRH-ir fibers and MCH-ir fibers at the pars intermedia level. By double-label immunofluorescence, we have observed that, in the ventral region of the pars intermedia, MCH-ir fibers exhibit stronger immunoreactivity and are more numerous than GnRH-ir fibers. Since two populations of SL-expressing cells have recently been described in zebrafish (Zhu et al. 2004), this finding, together with the MCH-SL and GnRH-SL double-label immunofluorescence and the *in vitro* pituitary system results, might also be related to the different regulation of two possible populations of SL cells in *C. dimerus*.

Interestingly, we have observed a close association between GnRH-ir fibers and MCH-ir perikarya and fibers in the nucleus lateralis tuberis by double-label immunofluorescence. This finding is consistent with results obtained in the barfin flounder, where chicken GnRH-II-ir fibers lie in close contact with MCH-ir cell bodies in the nucleus lateralis tuberis of the hypothalamus (Amiya et al. 2008). In *C. dimerus*, chicken GnRH-II-ir, salmon GnRH-ir, and seabream GnRH-ir fibers are widely distributed in the brain, including the nucleus lateralis tuberis (Pandolfi et al. 2005). In the barfin flounder, chicken GnRH-II-ir levels in fish adapted to black tanks are higher than in those adapted to white tanks (Amiya et al. 2008). The results of Amiya et al (2008) together with the previously described MCH-ir levels related to background color adaptation in *C. dimerus* (Cánepa et al. 2006) and the double-label immunofluorescence results of the present work suggest that MCH and GnRH interact in the brain and, as a result, regulate body color display.

In conclusion, our data show that SL, like other pituitary hormones, might be under hypothalamic control and involved in diverse physiological processes, including background adaptation and reproduction. This conclusion is supported by two independent results: (1) double immunofluorescence showing a close association between MCH fibers and SL cells in the pars intermedia, and between GnRH fibers and SL cells; (2) salmon MCH and mammalian GnRH stimulate SL release from male *C. dimerus* pituitaries in a dose-dependent manner. Additionally, this study has shown that the *in vitro* culture of a single *C. dimerus* pituitary is a feasible method for studying the control of SL release and of other pituitary hormones in this fish species.

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