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Immunohistochemical detection of acth and msh cells in the hypophysis of the hermaphroditic teleost, *Diplodus sargus*

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SUMMARY

Hypophyseal ACTH and MSH cells were immunohistochemically characterised in the teleost fish, *Diplodus sargus*, using anti-ACTH (1-24) and anti α-MSH polyclonal antisera. ACTH cells were found both in the pars distalis and in the pars intermedia. In the former region, they appeared small, round-shaped and clustered; in the latter, they were either small or large and elongated. Moreover, a few ACTH-immunoreactive cells resembling microglia were present in the neurohypophysis. Conversely, MSH cells were found only in the pars intermedia, and were similar to the larger ACTH cells of the same region. In the pars intermedia, co-localisation of ACTH and MSH immunoreactivity in the same cell was revealed by double immunostaining, though the two hormones were also observed in distinct cell types. The distribution of ACTH cells appeared quite uniform, without any marked difference between the specimens tested. Conversely, MSH cell amount varied according to the stage of the sexual cycle of this teleost fish, which is characterised by protandrous hermaphroditism. In fact, a lower amount of MSH cells were observed in females, whereas no significant difference was found between immature and male specimens.

INTRODUCTION

ACTH (adrenocorticotropic hormone) and MSH (melanotrope-stimulating hormone) are two hormones secreted by the cells in the anterior and intermediate pituitary lobes, respectively, and arise from a common precursor, proopiomelanocortin (POMC). POMC follows different processing pathways in the ACTH and MSH cells of some vertebrates (Dores, 1990; Ottaviani *et al.*, 1997). ACTH (1-39) is a major terminal product in corticotropic cells, whereas, in MSH cells, it gives rise, through a proteolytic cleavage, to two other peptidic hormones: α-MSH (1-13) and the corticotropin-like intermediate lobe peptide (CLIP, 18-39) (García-Hernández *et al.*, 1997; Ottaviani *et al.*, 1997). ACTH is involved in the regulation of stress response and cortisol release in fish (Balm and Pottinger, 1995); α-MSH regulates colour changes (Baker, 1993) and adaptation to the external environment (Malo-Michèle, 1977; van Eys, 1980) in teleosts. These cells have been immunohistochemically characterized in the hypophysis of some teleosts (Follenius *et al.*, 1974, 1976, 1978; Oliverau *et al.*, 1976 a, b; Malo-Michele *et al.*, 1976; ; Munro, 1985; Batten, 1986; Quesada *et al.*, 1988; Toubeau *et al.*, 1991; García-Hernández *et al.*, 1996, 1997; Rendon *et*

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al., 1997). The aim of the present study was to analyse the occurrence and distribution of ACTH and MSH cells, relating it for the first time to the stage of sexual maturity in the hypophysis of *Diplodus sargus*, a protandrous hermaphroditic teleost of the Mediterranean Sea. In this species, showing sexual inversion, the gonad consists of a dorsal ovarian zone and a ventral testicular zone that never undergo maturation at the same time. Generally, the testicular lobe matures first and the ovarian lobe develops at a subsequent stage (female stage) simultaneously with the progressive regression of the testicular lobe (Micale *et al.*,1987, 1994). The study was performed using the immunohistochemical technique of ABC (avidin biotin complex) and anti ACTH (1-24) and anti α-MSH polyclonal antisera.

MATERIALS AND METHODS

Tissue

A total of fourteen hermaphroditic specimens of *Diplodus sargus* (80-350 g in body weight) was collected throughout the year from the Bay of Naples (Italy). The animals were provided by the Zoological Station "A. Dohrn" of Naples. The experiments were performed under the approval of institutional committees; all efforts were made to avoid animal suffering and to minimise the number of the animals used. The specimens were killed by decapitation, after anaesthesia with 62.5 mg/l of MS222 (Sigma, USA). The brains with the hypophysis were immediately taken and fixed in Bouin's solution for 48 hours. To compare the stage of sexual maturation of each of the specimens, the gonads were also taken and fixed in Bouin's solution for 48 hours. All the pieces were then dehydrated, embedded in vacuum-paraffin (Carlo Erba, Italy), serially sectioned at 5-7 μm in either the sagittal or the transverse plane and mounted on glass slides for histological and immunohistochemical staining.

Histological Staining

Some sections of each of the gonads were stained with the Mallory trichromic stain to establish the functional stage of each animal, since this species is characterised by protandrous hermaphroditism. The sections of the hypophyses were stained with the Mallory trichromic stain and the Periodic Acid Schiff (PAS). These stains were generally performed on sections adjacent to immunostained sections to compare the ACTH-immunoreactive (ir) or MSH-ir cell types with their staining properties.

Immunohistochemistry

For the characterisation of the two hormones, we used the anti ACTH(1-24) fraction and anti α-MSH antisera. The former (1-24) was chosen since it allows the detection of the most conserved fraction of the whole ACTH molecule (1-39) in vertebrates; moreover, it avoids probable superimposition with CLIP, another product of the ACTH cleavage, corresponding to sequence 18-39. Anti α-MSH antiserum is the most frequent form present in the pars intermedia of vertebrates**.** Deparaffined, dehydrated sections were immunostained using the avidin-biotin-peroxidase complex (ABC) technique (Hsu *et al.*,1981) and performed using single and double immunostaining. In the single immunostaining, the sections were treated with 3% hydrogen peroxide for 10 minutes to prevent endogenous peroxidases, rinsed in distilled water and treated with 1% bovine serum albumin in 0.01M PBS to avoid unspecific links. The sections were then incubated with the primary antisera developed in rabbit against synthetic anti ACTH(1-24) (Biogenesis, England) at a working dilution of 1:2000 and anti α-MSH (Biogenesis) at a working dilution of 1:1000 for 24 hours at 4°C. After each step, the sections were washed in PBS for 3x5 minutes, incubated with the secondary biotinylated antiserum of Vectastain ABC Kit Elite (Vector Laboratories, USA) for 1.5 hours at room temperature, and then treated with the ABC complex of Vectastain ABC Kit Elite for 30 minutes. Immunolabelling was revealed using 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma), 3 mg in 10 ml PBS and 500 μl of 3% H_2O_2. Then the sections were contrasted with Mayer's hemalum for 30 seconds, dehydrated and mounted. The specificity of each of the antisera was demonstrated by treating some sections with the antiserum, which had been preincubated with an excess of the appropriate antigen for 24 hours, and by omitting the primary antiserum. Positive controls were performed by incubation of anti ACTH, which had been preincubated with an excess of α-MSH (Peninsula Laboratories, Eng-

land), and by incubation of anti α-MSH, which had been preincubated with an excess of ACTH 1-24 (Peninsula Laboratories).

In the double immunostaining, the first part was performed as in the simple immunostaining with the exposition to anti α-MSH revealed by DAB producing a brown colour. The sections were not contrasted. They were then treated with acid glycocoll buffer for 30 minutes and washed in 0.4% triton PBS at 4°C overnight. In the second part of immunostaining, the sections were incubated with anti ACTH as primary antibody and processed as in the first part. In this case, the chromogen used was 4-chloro-1-naphthol (Sigma) at a dilution of 4mg in 100 μl absolute ethanol and 10 ml PBS, which produced a blue violet colour. The sections were not contrasted with hemalum, but directly luted without dehydration. The images were examined with a Kontron Elektronic Imaging System KS300 (Zeiss, Germany).

RESULTS

General morphology

In *D. sargus*, the hypophysis appeared as an ovoid body, slightly flattened dorso-ventrally, with an anterior narrower end; it was attached to the brain base by a thin stalk (Fig. 1). In sexually mature specimens, weighing about 250 g, the mean size of the gland was 1.0(±0.15)x1.5(±0.2) mm; in immature specimens, weighing about 80 g, the mean size of the gland was instead 0.7(±0.05) x0.9(±0.07)mm. Sagittal sections revealed the typical organisation of the teleost hypophysis, where the adenohypophysis consists of an anterior rostral pars distalis (RPD), a medium proximal pars distalis (PPD) and a posterior pars intermedia (PI) (Fig. 2). The neurohypophysis interdigitated not only with the PI but also with the pars distalis (PD) of the adenohypophysis (Figs. 2, 3). Using Mallory's trichromic stain, the RPD of sexually immature specimens appeared to consist of chromophobic and scanty acidophilic cells (Fig. 3), whereas the PPD was formed by numerous acidophilic cells and, in the ventral zone of the gland, by a few basophilic cells (Fig. 3). These basophilic cells, which were PAS-positive, were also observed around the PI in the caudal end (Fig. 3). Numerous chromophobic cells were present in the PI (Fig. 3). These cells were also PAS-negative. In sexually mature specimens, there was an increase in the number of the basophilic and PAS-positive cells (Fig. 4) localised around the PI. They did not correspond to the anti ACTH and anti MSH immunoreactive cells, which, instead, were chromophobic and PAS-negative.

The study of the gonads conducted on 14 animals revealed 3 sexually immature specimens weighing

Fig. 1 - Left–lateral view of *D.sargus* brain, lightly oblique to show the hypophysis (↙). On the left, the rostral end.

Fig. 2 - Mid-sagittal section of sargus hypophysis showing the lobulation and the various regions of the gland: the rostral pars distalis (RPD), the proximal pars distalis (PPD) and the pars intermedia (PI). The neurohypophysis (NH) interdigitates with all the regions of the gland. Tot. Enl. 80X.

<80 g, 5 in the male stage (animals weighing 100-140 g), and 6 in the female stage (animals weighing >150 g).

ACTH-Immunoreactivity

Numerous ACTH cells were found in the hypophysis of all the specimens, both in the PD and in the PI (Fig. 5). In the PD, they were mostly present in the RPD; however, a few of them were also observed in the PPD (Fig. 5a). They were clustered, forming intensely ir-cell cordons (Fig. 5a) and, in a few regions, some scattered ir-cells were also detected. They were small, round in shape with a mean diameter of 5.5 μm (± 0.7). In the PI, two different ACTH cell types were observed: a type similar in shape and size to those of the PD, and a more abundant type, with a mean diameter of 11(±1.5)x7(±0.8)μm, which appeared larger and elongated in shape (Figs. 5b, 7). The latter were generally confined to the gland periphery. Unlike the PD ones, the PI ACTH cells were scattered (Fig. 7). In serial sections of the PI, ACTH ir cells (Fig. 10) were observed to be MSH ir as well (Fig. 11). ACTH immunoreactivity was also found in the neurohypophysis, where the cells appeared irregular in shape, resembling microglial cells (Figs. 12,13). No particular difference in the presence and distribution of ACTH cells was observed between sexually immature fishes and male and female mature specimens (Figs. 14, 15, 16).

MSH-Immunoreactivity

In the PD of the *D. sargus* hypophyses tested, there were no MSH cells (Fig. 6), which, unlike ACTH cells, were confined to the PI (Fig. 6). They were 12(±1.5)x7(±0.9)μm in mean size and appeared elongated in shape, with a slightly granular content, an eccentric nucleus and an evident nucleolus (Figs. 6a, 8). Most ACTH cells were also MSH-ir in adjacent sections (Figs. 10, 11). Unlike ACTH cells, MSH cells showed a different distribution according to the stage: a significant decrease in MSH cells was observed in females (Fig. 19), whereas there was no significant difference between immature (Fig. 17) and male (Fig. 18) specimens.

Double immunostaining

In double immunostained sections, most ir-cells of the PI showed co-localisation of the two hormones (Fig. 9); however, a few cells were ir either to the anti-MSH antiserum (stained in brown) or to the anti-ACTH antiserum (stained in blue-violet).

Table I summarises the results obtained.

DISCUSSION

The general morphology of the *Diplodus sargus* hypophysis is consistent with that reported for other teleost fish, where the neurohypophysis interdigitates with the other regions of the gland by its lobes (Sage and Bern, 1971; Schreibman *et al.*, 1973, 1999; Batten, 1986; Peter *et al.*, 1990; Toubeau *et al.*, 1991). Sagittal sections showed an anterior narrower end, the RPD, followed by the PPD. In these regions, trichromic stain mostly revealed strongly acidophilic cell cordons, which very likely com-

Fig. 3/9 - 3) Mid-sagittal section of an immature sargus hypophysis. Mallory trichromic stain. In the RPD note the chromophobic and scanty acidophilic cells. The PPD contains numerous acidophilic cells and, in the ventral zone of the gland, few basophilic cells that are also present around the PI in the caudal end. Numerous chromophobic cells are present in the PI. The neurohypophysis interdigitates with all the regions of the gland. Tot. Enl. 82X. **4)** Sagittal section of a mature *D.sargus* hypophysis. PAS reaction. Note the PAS-positive cells in the ventral zone of the PD and around the PI. The cells of the RPD and the PI are PAS-negative. Tot. Enl. 45X. **5)** Horizontal section of a male hypophysis. ABC technique. ACTH-ir. Note the presence of ACTH cells both in the PD (left) and in the PI (right). The insets indicate the areas reported in Figs. 5a and 5b. Tot. Enl. 70X. **5a)** Detail of Fig. 5, inset of left. Note the numerous small-sized and clustered ACTH cells in the RPD and few ACTH cells in the PPD. Tot. Enl. 700X. **5b)** Detail of Fig. 5, inset of right. Note the presence of two types of ACTH cells: small cells similar in shape and size to those of PD (↘) and a larger type elongated in shape (↘). Tot. Enl. 400X. **6)** Horizontal section of a male hypophysis adjacent to that of fig.5. ABC technique. MSH-ir. Note the presence of MSH cells only in the PI of the gland. The insets indicate the area reported in fig. 6a. Tot. Enl.70X. **6a)** Detail of Fig. 6. Note the elongated shape of MSH cells. Tot. Enl. 570X. **7)** Detail of the PI of a *D. sargus* hypophysis. ABC. ACTH-ir. Note the presence of a small type (↘) and a large type of ACTH cells (↖). Tot. Enl. 1100X. **8)** Detail of the PI of a *D. sargus* hypophysis. ABC. MSH-ir. Note the elongated MSH-cells with a slightly granular content, an eccentric nucleus and an evident nucleolus. Tot. Enl.1100X. **9)** Detail of the PI of male hypophysis. ABC technique. Double immunostaining: MSH-ir in brown, ACTH in blue-violet. Note the co-localisation of ACTH and MSH in the same cell (←) and the presence of only ACTH cells (↖). Tot. Enl. 830X.

Fig. 10/13 - Detail of the PI of a *D. sargus* hypophysis. ABC. ACTH ir. Note the ACTH cells to be compared with the MSH cells of Fig. 11 (↘). Tot. Enl. 650X. **11)** Adjacent section to that of fig.10. MSH-ir. Note the superimposition of the two hormones in the PI of *D. sargus* hypophysis between Figs. 11 and 10 (↘). Tot. Enl. 650X. **12**) Detail of *D. sargus* neurohypophysis. ABC. ACTH-ir. Some ACTH-ir microglial cells are observed (→). Tot. Enl.1100X. **13)** Detail of *D. sargus* neurohypophysis. ABC. ACTH-ir. A group of microglial cells that are immunoreactive to anti-ACTH. Tot. Enl. 1000X.

prise mammotropic (in the RPD) or somatotropic (in the PPD) cells, and chromophobic cell cordons, as has already been reported in *Spaurus aurata* (Quesada *et al.*, 1988), in three sciaenid fish (Yan and Thomas, 1991) and in *Seriola dumerilii* (García-Hernández *et al.*, 1996). A few basophilic and

Figs.14/19 - 14/16) Comparison of ACTH cell distribution among the hypophyses of three specimens at different sexual stages: Fig. 14, in a sexually immature specimen; Fig. 15, in a male specimen; and Fig. 16, in a female specimen. Significant quantitative variations in this cell type are not observed among the different stages. Tot. Enl.280X. **17/19)** Comparison of MSH cell distribution among the hypophyses of three specimens at different sexual stages: Fig. 17, in a sexually immature specimen; Fig.18, in a male specimen; and Fig. 19, in a female specimen. Unlike ACTH cells, MSH cells are decreased in female specimens. Tot. Enl. 280X.

Table I
Schematic summary of results

Sexual stage	**RPD**	**PPD**	**PI**	**NH**
ACTH				
Immature	++	+	++	+
Male	++	+	++	+
Female	++	+	++	+
MSH				
Immature	-	-	++	-
Male	-	-	++	-
Female	-	-	+	-

- No ir-cells; + Few ir-cells; ++ Several ir-cells;
RPD:rostral pars distalis;
PPD:proximal pars distalis;
PI: pars intermedia; **NH**:neurohypophysis.

PAS-positive cells, probably gonadotropes or thyrotropes, were found in the PPD and around the PI in immature fish, becoming more numerous in mature fish, consistent with what has been observed in salmon (Olsen and Walther, 1993). The PI of *D. sargus* is more developed, being generally constituted by chromophobic and PAS-negative cells as in sciaenid fish (Yan and Thomas, 1991).

Generally, the immunohistochemical characterisation of ACTH and α-MSH cells in the hypophysis of *D. sargus* resembles that reported for other teleosts. ACTH cells, as revealed by the anti-ACTH antibody (1-24), were present both the PD and the PI of the *D. sargus* hypophysis. This result is in agreement with what has been reported for *Dicentrarchus labrax* (Cambré *et al.*, 1986), three sciaenid fish (Yan and Thomas, 1991) and *Seriola dumerilii* (García-Hernández *et al.*, 1996), but in contrast to data on *Barbus barbus* (Toubeau *et al.*, 1991), where ACTH (1-24) was identified only in the PD but not in the PI. In the PD, ACTH cells are clustered in cellular cordons; they are round in shape and 5.5 μm in mean diameter, hence smaller than those characterised in the sciaenid fish (7-9 μm) (Yan and Thomas, 1991). Unlike what has been reported in the other teleosts, we detected two ACTH cell types in the PI: cells resembling those of the PD, and larger cells similar to the MSH ones. Immunostaining on adjacent sections and double immunostaining showed that, in these cells, there is often co-localisation of the two hormones; although either ACTH-ir or MSH-ir cells, with an average diameter of 10 μm, were also observed. The use of an anti-ACTH antiserum (1-24) avoiding possible cross-reaction with CLIP, the demonstrated lack of reactivity with α-MSH by positive controls of preabsorption of anti ACTH with α-MSH and of anti α-MSH with ACTH, as well as the negative controls of preabsorption, are indicative of the specificity of the signal, and hence of the presence of ACTH cells in the PI, unlike what has been reported for other species (Yan and Thomas, 1991). Moreover, the negative PAS reaction in these two chromophobic cell types, which has also been reported by other investigators (Holmes and Ball, 1974; Hansen, 1983; Joss *et al.*, 1990), confirms that teleosts are unable to glycosylate POMC (Iturriza and Estivariz, 1986; Quesada *et al.*, 1988) and show different pathways of ACTH and MSH production from the common precursor. The positive PAS reaction in the basophilic cells surrounding the PI demonstrates the presence of gonadotropic cells in this region. This result rules out any correlation with the possible presence of POMC, which is related to PAS-positive cells in all the other vertebrates but teleosts, *D. sargus* included.

The presence of ACTH immunoreactivity in the neurohypophysis is not an exception; in fact, it has also been found in the teleost, *Poecilia latipinna* (Batten,1986), where, reportedly, it is due to "blebs" of adenohypophyseal ACTH cells that protrude towards the neurohypophysis. Conversely, immunoreactivity was observed by us inside the neurohypophysis. In view of their irregular shape, these immunoreactive cells appear as microglial cells, which, having macrophage functions, may contain the ACTH molecule, as has been reported in fish leukocytes (Ottaviani *et al.*, 1997). Unlike *Seriola dumerilii* (García-Hernández *et al.*, 1996), which show MSH cells also in the PD, in *D. sargus*, as in the other teleosts, the MSH cells are confined to the PI. They are chromophobic and PAS-negative, and appear elongated with an eccentric nucleus, very similar in size (12x7μm) and shape to the ACTH cell type present in the PI. Unlike ACTH cells, which do not show considerable differences among the animals sampled, MSH cells are more abundant in the PI of sexually immature specimens, and more in that of males than of females. These data show that the amount of MSH

cells is related to the sexual stage of this protandrous hermaphroditic fish. This is partly in agreement with the results obtained by Schreibman *et al.* (1973), who reported that MSH cells undergo variation in teleost fish during the gonadal maturation period. This decrease of MSH cells in the females of *D. sargus* is probably related to the role played by the MSH hormone in colour changes by the dispersion of melanin pigment, as has already been observed in other species (Baker *et al.*, 1984).

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