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Development of melanin-concentrating hormone-immunoreactive elements in the brain of gilthead seabream (*Sparus auratus*)

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Abstract. The development of the hypothalamic melanin-concentrating hormone (MCH) system of the teleost *Sparus auratus* has been studied by immunocytochemistry using an anti-salmon MCH serum. Immunoreactive perikarya and fibers are found in embryos, larvae, and juvenile specimens. In juveniles, most labeled neurons are present in the nucleus lateralis tuberis; some are dispersed in the nucleus recessus lateralis and nucleus periventricularis posterior. From the nucleus lateralis tuberis, MCH neurons project a conspicuous tract of fibers to the ventral hypothalamus; this penetrates the pituitary stalk and reaches the neurohypophysis. Most fibers end close to the cells of the pars intermedia, and some reach the adenohypophysial rostral pars distalis. Immunoreactive fibers can also be seen in extrahypophysial localizations, such as the preoptic region and the nucleus sacci vasculosi. In embryos, MCH-immunoreactive neurons first appear at 36 h post-fertilization in the ventrolateral margin of the developing hypothalamus. In larvae, at 4 days post-hatching, perikarya can be observed in the ventrolateral border of the hypothalamus and in the mid-hypothalamus, near the ventricle. At 26 days post-hatching, MCH perikarya are restricted to the nucleus lateralis tuberis. The neurohypophysis possesses MCH-immunoreactive fibers from the second day post-hatching. The results indicate that MCH plays a role in larval development with respect to skin melanophores and cells that secrete melanocyte-stimulating hormone.

Key words: Melanin-concentrating hormone – Immunocytochemistry – Development, ontogenetic – *Sparus auratus* (Teleostei)

Introduction

Melanin-concentrating hormone (MCH) is a 17-amino-acid peptide purified by Kawauchi et al. (1983) from pituitary glands of chum salmon, *Oncorhynchus keta*. This peptide induces the aggregation of melanin granules in melanophores from several species of teleosts (Kawauchi et al. 1983; Wilkes et al. 1984; Baker et al. 1986; Baker 1991). The MCH hypothalamo-hypophysial system has been studied by immunocytochemistry in teleosts (*O. keta* and *O. mykiss*: Naito et al. 1985; *Poecilia latipinna*: Batten and Baker 1988), cartilaginous fishes (*Scyliorhinus canicula*: Vallarino et al. 1988), and cyclostomes (Baker 1991). MCH immunoreactivity has also been detected in the hypothalamus of other vertebrate classes, such as amphibians (Andersen et al. 1986), reptiles (Cardot et al. 1994), and mammals (rats: Skofitsch et al. 1985; Zamir et al. 1986; Naito et al. 1988; humans: Pelletier et al. 1987).

In adult teleost fishes, the MCH system consists of hypothalamic neurons in the nucleus lateralis tuberis (NLT) projecting to the neural lobe of the hypophysis. MCH activity in the hypothalamus of fishes has also been confirmed by in vitro assay (*O. mykiss*: Baker and Rance 1983; *O. keta:* Kawazoe et al. 1987), and radioimmunoassay (*O. keta*: Kawazone et al. 1987).

It has been shown that the MCH system of the chum salmon (*O. keta*) differentiates before hatching (Naito et al. 1993), thus supporting a role of MCH in early development. In this species, embryonic development lasts for 7 weeks. In the present study, we describe the embryonic and larval development of the MCH hypothalamo-hypophysial system in a species having a rapid embryonic development (48 h), viz., the teleost *Sparus auratus*. For comparison, the distribution of MCH-immunoreactive (IR) perikarya and nerve fibers has also been analyzed in the brain of juvenile specimens.

Materials and methods

Eggs and larvae from naturally spawned, cultured gilthead seabream *Sparus auratus* were kindly supplied by a commercial marine fish farm, CUPIMAR (Cultivos Piscícolas Marinos, S.A., San Fernando, Cádiz, Spain.). The fish were supplied with underground salt water (salinity 36‰ and temperature 18±0.5° C) in an open-circuit system with aeration. Larvae were maintained at LD:

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12:12 and were fed rotiphers (*Brachionus plicatilis*) and crustaceans (*Artemia salina*). *S. auratus* larvae hatched from eggs 48 h after fertilization. Embryo samples were taken at 24, 36, and 48 h. Larvae were taken at 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 23, 26, 31, 40, 50, and 60 days after hatching. Six specimens were examined at each stage. Embryos and larvae were placed in Bouin's fluid for 24 and 48 h, respectively, dehydrated, and embedded in paraffin.

Seven juvenile (about 100–150 g body weight) fish were also used in this study. They came from sea water (salinity 39‰) and were kindly supplied by a fish culture experimental center, "El Toruño" (PEMARES, El Puerto de Santa María, Cadiz, Spain). Fish were anesthesized with phenoxyethanol (0.2‰, P-1126 Sigma, St. Louis, Mo., USA) dissolved in the water, and killed by decapitation. The concentration of the anesthetic was the lowest possible to avoid additional stress. The brains were dissected out and placed in Bouin's fluid for 48 h; they were then dehydrated and embedded in paraffin.

Sagittal and transverse (8 µm thick) sections of embryos, larvae and juvenile brains were hydrated and immunostained according to the unlabeled enzyme method of Sternberger (1986), using an anti-salmon MCH (Naito et al. 1985) as the primary antiserum at a dilution of 1:10000 (kindly provided by Prof. H. Kawauchi, Kitasato University, Japan). Some brain sections at the level of the hypophysis were stained with an anti-human adrenocorticotrophic hormone (ACTH; 1:1500) (Peninsula Laboratoire, Calif., USA).

All sections were incubated for 18 h at 22° C in the primary antiserum. The second antiserum (anti-rabbit IgG developed in goat in our laboratory) was used at a dilution 1:10 for 30 min at 22° C, and the PAP complex (1:100) (DAKO, Copenhagen, Denmark) for 30 min at 22° C; 3,3′ diaminobenzidine tetrahydrochloride (Sigma) was used as the electron donor. All antisera and the PAP complex were diluted in 0.05 M TRIS buffer, pH 7.8, containing 0.7% non-gelling seaweed gelatin, lambda carrageenan (Sigma), 0.5% Triton X-100 (Sigma), and 0.02% sodium azide. Coplin jars were used for incubations in the first and the second antisera, whereas the PAP incubation was carried out in a moist chamber.

The specificity of the anti-salmon MCH antiserum was tested by immunoabsorption with synthetic salmon MCH (Peninsula Laboratoires, St. Helens, Merseyside, UK). Aliquots of the antiserum, diluted 1:10000, were mixed separately with the synthetic MCH at concentrations of 10 and 20 µg/ml. Immunoadsorbed anti-MCH did not stain any structures in the sections. In order to control the immunoreactive procedure, contiguous sections were stained according to the described protocol, but incubation in the primary antisera was omitted. No positive structures or cells were found in these sections.

Results and discussion

Distribution of MCH-IR perikarya and fibers in juvenile specimens

The nomenclature used for brain regions and nuclei of *Sparus auratus* was according to the stereotaxic studies in *Fundulus heteroclitus* (Peter et al. 1975) and *Oncorhynchus mykiss* (Billard and Peter 1982). The most prominent group of MCH-IR perikarya was found in the ventrolateral border of the hypothalamus, the pars lateralis of the nucleus lateralis tuberis (NLT) (Fig. 1A, B). Neurons were large, round or fusiform, and showed strong immunoreactivity (Fig. 1D); they extended medially to the subependymal region of the infundibular recess (Fig. 1B), and caudally to a dorsolateral position in continuity with the nucleus recessus lateralis. A few isolated strongly MCH-IR fusiform neurons were also found in other hypothalamic locations, such as the nucleus periventricularis posterior (Fig. 1C).

From the NLT, immunoreactive fibers extended rostrally to the preoptic region and caudally to the pituitary. A conspicuous tract of MCH-IR beaded fibers penetrated the pituitary stalk (Fig. 1B) and terminated in the neural lobe of the hypophysis close to the cells of the pars intermedia that were immunostained with anti-ACTH (Fig. 1E, F). Some fibers penetrated the rostral pars distalis of the adenohypophysis.

The hypothalamo-hypophysial MCH system of *S. auratus* is similar to those described for other teleost species (*O. keta* and *O. mykiss*: Naito et al. 1985; *Poecilia latipinna*: Batten and Baker 1988; Batten et al. 1990). Pharmacological and morphological studies have shown that, in teleosts, MCH may be involved in the control of the activity of MSH and ACTH cells (see Baker 1991). Accordingly, in *S. auratus*, the presence of MCH-IR endings close to the pars intermedia cells (immunostained by the anti-ACTH antiserum) and in the adenohypophysial rostral pars distalis, where ACTH cells exist (Quesada et al. 1988; Mancera et al. 1993), also suggests a control of MCH on the release of MSH and ACTH.

In *S. auratus*, the posterolateral hypothalamus and the preoptic area appear to be innervated by MCH fibers. Moreover, the nucleus sacci vasculosi receives MCH fibers. These same areas have been reported to receive MCH fibers in other teleosts (Naito et al. 1985; Batten and Baker 1988; Batten et al. 1990). In the cartilaginous fish *Scyliorhinus canicula*, the nucleus sacci vasculosi has been reported to contain MCH-positive perikarya in addition to MCH-IR fibers (Vallarino et al. 1989). In other fishes, extrahypothalamic areas, such as the olfactory bulb, pretectal thalamus, optic tectum, and midbrain, have been reported to be innervated by MCH-IR fibers (Naito et al. 1985; Batten and Baker 1988; Batten et al. 1990). In the present study, extrahypothalamic MCH-IR fibers have not been detected in *S. auratus*. MCH could play a role as a central neurotransmiter or neuromodulator in addition to its role as a hypophysiotropic factor. In *S. auratus*, this central function would be restricted to the hypothalamus.

Embryonic and larval development of the MCH system

The embryonic development of *S. auratus* lasted for 48 h from fertilization, at a culture temperature of 18° C. From 36 h post-fertilization (12 h before hatching), MCH-IR perikarya were identified in the marginal region of the developing hypothalamus (Fig. 1G). The number of immunoreactive neurons increased slowly in this location with age. At day 4 post-hatching, MCH-IR perikarya were seen in two locations: (1) the ventrolateral margin of the hypothalamus, and (2) close to the ventricle in the medial zone of the hypothalamic region (Fig. 1H). As larval development progressed, the number of MCH-IR neurons in the hypothalamic midline decreased, and those in the ventrolateral margin increased. This region corresponded to the localization of the NLT in juvenile specimens. At 26 days post-hatching MCH-IR perikarya were only seen in the NLT. The neurohypophysis

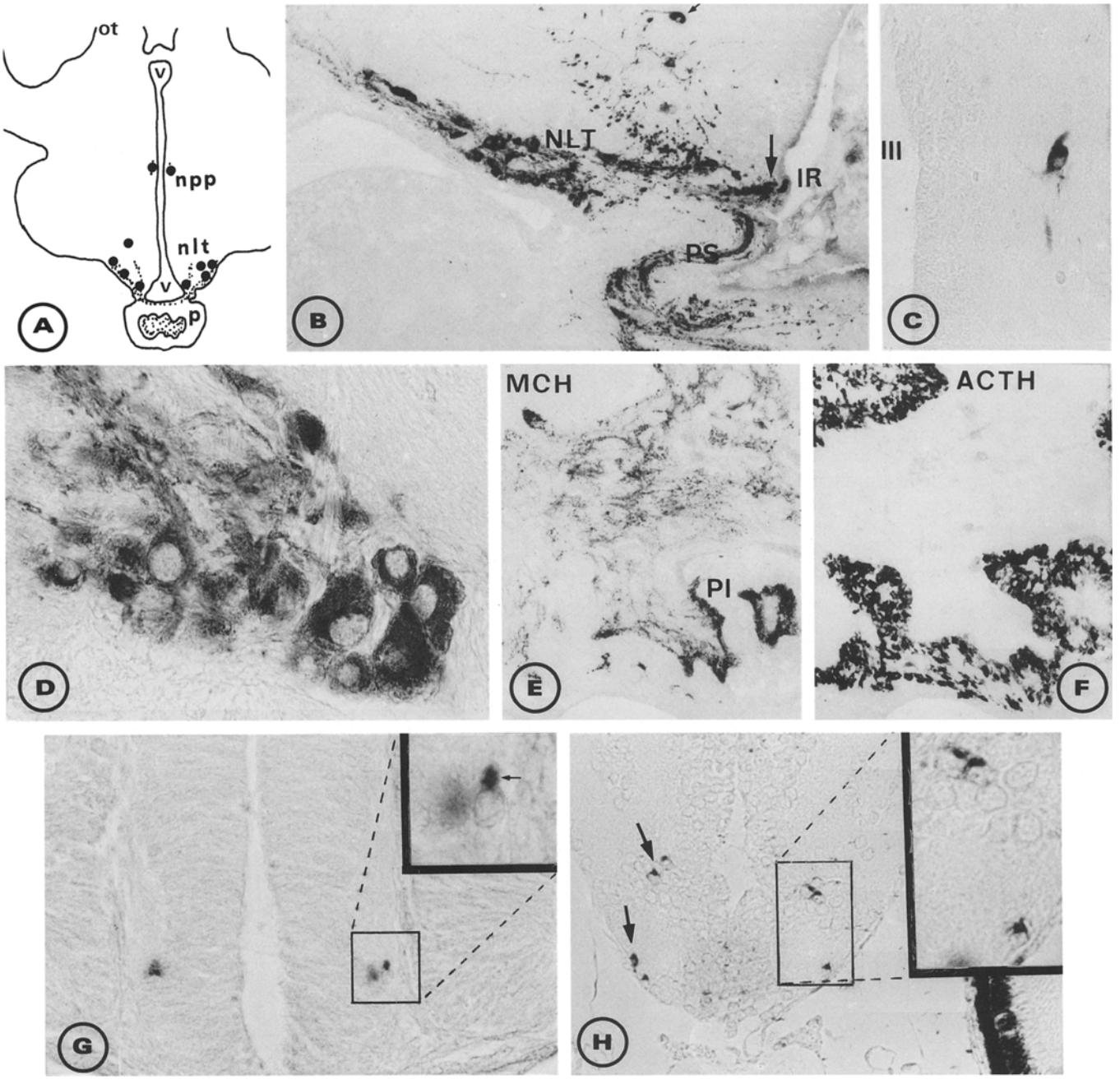

Fig. 1. **A** Schematic drawing of a transverse section through the brain of a juvenile *Sparus auratus* showing the localization of MCH-immunoreactive perikarya (*dark circles*) and fibers (*dots*). *nlt* Nucleus lateralis tuberis; *npp* nucleus periventricularis posterior; *ot* optic tectum; *p* pituitary; *v* third ventricle. **B** Transverse section through the pars lateralis of the nucleus lateralis tuberis (*NLT*) of a juvenile *Sparus auratus*. Note MCH-immunoreactive fibers entering the pituitary stalk (*PS*). Some perikarya are seen in the medial region near the infundibular recess (*IR*) (*thick arrow*). Isolated neurons are found throughout the hypothalamus (*small arrow*). ×135. **C** Isolated MCH-immunoreactive neuron in the region of the nucleus preopticus periventricularis. *III* Third ventricle. ×270. **D** Detail of the MCH-immunoreactive neurons of the nucleus lateralis tuberis. ×675. **E, F** Adjacent transverse sections through the neurointermediate lobe of the hypophysis of a juvenile specimen immunostained with antisera against MCH (**E**) and ACTH (**F**). MCH-immunoreactive fibers end close to the cells of the pars intermedia (*PI*), which are immunostained with the anti-ACTH serum. ×135. **G** Transverse section through the brain of a larva at 48 h post-fertilization (hatching). Note two MCH-immunoreactive cells in the developing hypothalamus. *Insert*: Detail of the enclosed zone showing immunoreactivity in a cell pole (*arrow*). ×320. *Insert*: ×950. **H** Transverse section through the hypothalamus of a larva, 4 days after hatching. Note two groups of MCH-immunoreactive cells (*arrows*). *Insert*: Detail of the enclosed zone showing immunoreactive cells in the medial and the marginal hypothalamus. ×320. *Insert*: ×800

displayed MCH-IR fibers from day 2 post-hatching. The number of fibers progressively increased with age.

Embryonic and larval development of the MCH-IR system has been described in the chum salmon *O. keta* (Naito et al. 1993). The embryonic development of this species lasts for 7 weeks. By the time of hatching, the MCH hypothalamic system is fully developed, with perikarya appearing in the NLT at 5 weeks, and fibers in the

neurohypophysis at 6 weeks. In *S. auratus*, embryonic development is as rapid as 48 h (see above), and immunoreactivity to anti-salmon MCH also appears before hatching. However, unlike in *O. keta*, MCR-IR fibers do not appear in the neurohypophysis until 2 days post hatching (a time equivalent to the duration of the whole embryonic period). Naito et al. (1993) have suggested that a fully developed MCH system in hatching salmon is essential for the control of skin melanophores, which, in this species, increase in number immediately after hatching. The delay in the appearance of the MCH fibers in the neurohypophysis of *S. auratus* compared with salmon indicates that the control of skin melanophores does not occur until after hatching in this species. This observation could be related to the behavior of seabream larvae; indeed, newly hatched *S. auratus* larvae remain floating upward at the surface of the water. In this situation, darkening of the skin would be disadvantageous because of predation.

In *S. auratus*, cells immunostained with anti-ACTH appear in the pars intermedia of the pituitary gland of 8-day-old larvae (Power and Canario 1992). Since we have found MCH-IR fibers in the neurohypophysis before this time, a role of MCH in the differentiation of melanocyte stimulating hormone (MSH) cells seems feasible in *S. auratus*.

In *S. auratus*, from day 4 to 23 days post-hatching, a group of MCH-IR perikarya occur close to the ventricle in the medial zone of the hypothalamic region. Later, these cells cannot be identified. This can be interpreted in two ways: (1) these neurons originate in the ependymal layer, migrate to the region of the NLT, but express MCH before reaching their definitive location, or (2) these are local neurons that express MCH and that play some role in development. The existence of MCH-IR perikarya and fibers in the early development of larvae of *S. auratus* thus argues in favor of a role of this hormone in the development of, for example, the control of skin melanophores (Kawauchi et al. 1983; Powell and Baker 1988), the control of MSH and ACTH secretions (see Batten and Baker 1988; Baker 1991), and the early development of MSH cells (Naito et al. 1993).

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