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The hypothalamo-hypophyseal system of the white seabream *Diplodus sargus*: Immunocytochemical identification of arginine-vasotocin, isotocin, melanin-concentrating hormone and corticotropin-releasing factor

G. Duarte[1,2], M.M. Segura-Noguera[1], M.P. Martín del Río[1] & J.M. Mancera[1,*]
[1]*Departamento de Biología Animal, Facultad de Ciencias del Mar, Universidad de Cádiz, Puerto Real 11510, Cádiz, Spain*
[2]*Departamento de Zoología e Genética, Instituto de Biología, Universidade Federal de Pelotas, Brasil*

[*]Author for correspondence

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Summary

The distribution of the neurosecretory hormones vasotocin, isotocin and melanin-concentrating hormone and the hypophysiotropic hormone corticotropin-releasing factor was studied in the hypothalamo-hypophyseal system of the white seabream (*Diplodus sargus*) using immunocytochemical techniques. Magnocellular and parvocellular perikarya immunoreactive for arginine-vasotocin and isotocin were present in the nucleus preopticus. Perikarya immunoreactive for arginine-vasotocin extended more caudally with respect to isotocin-immunoreactive perikarya. Parvocellular perikarya were located at rostroventral levels and magnocellular perikarya in the dorsocaudal portion of the nucleus. Arginine-vasotocin and isotocin did not coexist in the same neuron. Fibres immunoreactive for arginine-vasotocin and isotocin innervated all areas of neurohypophysis and terminate close to corticotropic and melanotropic cells. Perikarya immunoreactive for melanin-concentrating hormone and corticotropin-releasing factor were observed in the nucleus lateralis tuberis, with a few neurons in the nucleus periventricularis posterior. In addition, melanin-concentrating hormone immunoreactive perikarya were detected in the nucleus recessus lateralis. The preoptic nucleus did not show immunoreactivity for these antisera. Fibres showing melanin-concentrating hormone and corticotropin-releasing factor immunoreactivity ended close to the melanotropic and somatolactotrophic cells of the *pars intermedia*, and close to the corticotrophic cells of the rostral *pars distalis*.

Introduction

In teleosts, neurons of the preoptic nucleus (NPO) and the nucleus lateralis tuberis (NLT) project to the neurohypophysis and release hormones such as arginine-vasotocin (AVT), isotocin (IST) and melanin-concentrating hormone (MCH) into the systemic blood. Other neurons of the same nuclei project to the neurohypophysis and release hormones such as corticotrophin-releasing factor (CRF), gonadotrophin-releasing hormone or somatostatin that exert a direct neuromodulator control on the activity of adenohypophyseal cells. Neurons of these nuclei also project to extrahypothalamic areas (Anglade *et al.* 1993, Holmqvist & Ekström 1995, Batten *et al.* 1999).

In fish, the hypothalamo-neurohypophyseal system has been studied in teleosts (Goosens *et al.* 1977, Cumming *et al.* 1982, van Dungen *et al.* 1982, Batten 1986, Batten *et al.* 1990, Holmqvist & Ekström 1995) and elasmobranchs (Vallarino *et al.* 1990, Meurling *et al.* 1996). AVT and IST are synthesized in neurons of the NPO and the axons form the hypothalamo-hypophyseal tract that runs along the ventral hypothalamus, pituitary stalk and ends in the neurohypophysis where AVT and IST are released into the systemic blood. In fish, AVT and IST play a physiological role in osmoregulation and vascular function (Balment *et al.* 1993, Warne & Balment 1997). Moreover, recent studies on mRNA expression have revealed a participation of these hormones in the physiology of seasonal changes, stress and reproduction (Gilchriest *et al.* 2000, Godwin *et al.* 2000, Warne *et al.* 2000). A role of AVT on the secretion of adenohypophyseal hormones has also been shown (Fryer & Lederis, 1986, Batten *et al.* 1999), in agreement with the presence of AVT receptors in the *pars distalis* and the *pars intermedia* (Moons *et al.* 1989b, Mahlmann *et al.* 1994). Projections of AVT and IST fibres have been found in extrahypophysiotropic areas, where AVT receptors have been detected (Moons *et al.* 1989a, Batten *et al.* 1990, 1999, Holmqvist & Ekström 1995).

MCH is a neuropeptide involved in the skin adaptation to light backgrounds by inducing aggregation of melanin granules in melanophores (Baker 1991). A role of MCH as a central neurotransmitter or neuromodulator has also been proposed (Balm & Gronoveld 1998) and supported by the wide distribution of MCH receptors (Saito *et al.* 1999, Sone *et al.* 2000). A MCH hypothalamo-neurohypophyseal system appears to be present in teleosts (Naito *et al.* 1985, Batten & Baker 1988, Batten *et al.* 1990, Mancera & Fernández-Llebrez 1995b), elasmobranchs (Vallarino *et al.* 1989a) and cyclostomes (Baker 1991). The MCH gene has been cloned

and mRNA expression analysed in teleosts (Groneveld *et al.* 1995, Suzuki *et al.* 1995, Francis *et al.* 1997). The most prominent group of MCH-producing neurons was found in the NLT and projections of these neurons ran via the diencephalic floor to end in the neurohypophysis close to the melanotropin and somatolactin (SL) cells of the *pars intermedia* (PI) and adrenocorticotrophin cells of the rostral *pars distalis* (RPD). In addition, MCH-immunoreactive fibres have been observed in other extrahypothalamic areas (Naito *et al.* 1985, Batten & Baker 1988, Batten *et al.* 1990).

CRF is a hypothalamic peptide with hypophysiotrophic activity, stimulating the release of adrenocorticotropin (ACTH) and β-endorphin (Rivier & Plotsky 1986). In addition, CRF is considered as a neurotransmitter and neuromodulator (Sawchencko *et al.* 1993). Several studies on the anatomical localization of CRF-immunoreactive perikarya and nerve fibres have been performed in teleosts (Yulis *et al.* 1986, Olivereau & Olivereau, 1988, 1990, Batten *et al.* 1990, Zupanc *et al.* 1999) and elasmobranchs (Vallarino *et al.* 1989b). In most teleosts, CRF-immunoreactive perikarya exist in the NPO and the NLT and project to the neuro hypophysis, where neurosecretory axons end close to the ACTH and MSH cells. At variance, in the sparid gilthead seabream *Sparus aurata*, CRF-immunoreactive perikarya have only been found in the NLT but not in the NPO (Mancera & Fernández-Llebrez 1995a). Recently, CRF cDNA has been cloned and sequenced and mRNA expression studied under different physiological conditions (Ando *et al.* 1999, Bernier *et al.* 1999, van Enckevort *et al.* 2000), and CRF receptor has been characterized (Perrin & Vale 1999, Arai *et al.* 2001). These studies revealed an involvement of CRF in several physiological process, such as stress, osmoregulation, energy metabolism, locomotor control and reproduction (Wendelaar Bonga 1997, Lovejoy & Balment 1999).

The white seabream (*Diplodus sargus* L.) is a commercial teleost, increasingly cultured in recent years (Divanach *et al.* 1982, Mordenti *et al.* 1996). In a previous study, we described pituitary cells using histological and immunocytochemical approaches (Segura-Noguera *et al.* 2000). In the present study, we analysed the hypothalamo-hypophyseal system of this species focusing on the distribution of perikarya and fibres containing the neurosecretory hormones AVT, IST, MCH and the hypophysiotrophic hormone CRF.

Material and methods

Immature specimens of white seabream, *Diplodus sargus* L., ($n = 10$, 100–150 g body weight) were provided by a fish-culturing centre (El Toruño, Consejería de Agricultura y Pesca, Junta de Andalucía, El Puerto de Santa María, Cádiz, Spain). Fish were transferred to the wet laboratories of the Faculty of Marine Science (Puerto Real, Cádiz), where they were kept for 1 month under natural photoperiod and temperature until full acclimatization (April–May 1998). The fish were housed in 1500 litre tanks with permanent water turnover and oxygen supply. They were fed twice a day with *Illex* sp.

Table 1. Primary antisera used in this study.

Antisera raised against	Source	Dilution
Rat CRF	Dr. E. M. Rodríguez[a]	1 : 500
Human ACTH	Peninsula INC[b]	1 : 3000
AVT	Dr. R.M. Buijs[c]	1 : 1000
IST	Dr. R.M. Buijs[c]	1 : 1000
Salmon MCH	Dr. H. Kawauchi[d]	1 : 10,000
Bovine α-MSH	Dr. S.E. Wendelaar-Bonga[e]	1 : 3000

[a]Valdivia, Chile. [b]California, USA. [c]Amsterdam, Holland. [d]Kitasato, Japan. [e]Nijmegen, Holland.

The fishes were anaesthetized with 2-phenoxyetanol dissolved in the water (1 millilitre per litre water) and killed by decapitation. The brains were dissected out, placed in Bouin's fluid for 48 h, and then dehydrated and embedded in paraffin wax. Sagittal and transverse (8 µm-thick) sections were obtained. The sections were stained with haematoxylin–eosin. For immunocytochemistry, tissue sections were immunostained according to the unlabelled enzyme method of Sternberger (1968). The primary rabbit antisera and working concentrations shown in Table 1 were used. Sections were examined by bright field microscopy.

The antisera against anti-AVT and anti-IST were kindly provided by Dr. R.M. Buijs, Amsterdam, Holland (van den Dungen *et al.* 1982). The anti-salmon MCH was kindly provided by Prof. Dr. H. Kawauchi, Kitasato University, Japan (Naito *et al*. 1985). The anti-rat CRF was kindly provided by Dr. E.M. Rodríguez, Valdivia, Chile. The anti-human ACTH was provided by Península Laboratories, California, USA. The anti-bovine mono-acetyl α-melanocyte-stimulation hormone (MSH) was kindly provided by Dr. S.E. Wendelaar Bonga, Nijmegen, Holland (van Zoest *et al*. 1989).

All sections were incubated for 18 h at 22 °C in the primary antiserum. The second antiserum (anti-rabbit IgG, raised in goat and kindly provided by Dr. P. Fernández-Llebrez, Málaga, Spain) was used at a dilution of 1 : 40 for 60 min at 22 °C. 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) was used as electron donor. All antisera and the PAP complex (peroxidase–antiperoxidase–peroxidase soluble complex with antibodies developed in rabbit, Sigma P-2026) were diluted in TRIS buffer, pH 7.8, containing 0.7% non-gelling seaweed gelatin, O-carrageenan (Sigma), 0.5% Triton X-100 (Sigma), and 0.02% sodium azide. Coplin jars were used for incubation in the first and second antisera, whereas PAP incubation was carried out in a moist chamber.

The specificity of the anti-salmon MCH antiserum and anti-rat CRF was tested by immunoabsorption with synthetic salmon MCH and rat CRF (Mancera *et al*. 1991, Mancera & Fernández-Llebrez 1995b). Immunoabsorbed antisera did not stain any structures in the sections of the white seabream brain. Anti-AVT and anti-IST were previously tested by van den Dungen *et al.* (1982). In order to control the immunoreactive procedure, contiguous sections were stained according to the described protocol, but incubation in the

primary antisera was omitted. In addition, non-immune rabbit serum was used as primary antiserum. No positive structures or cells were found in these sections. The nomenclature used for nuclei of *Diplodus sargus* was carried out according to that used by other authors in other species (*Fundulus heteroclitus*: Peter *et al.* 1975, *Oncorhynchus mykiss*: Billard & Peter 1982, *Dicentrarchus labrax*: Cerdá-Reverter *et al.* 2001).

Results

Distribution of AVT- and IST-immunoreactive perikarya and fibres

Figures 1 and 2 show schematic drawings of sagittal (Figure 1) and transverse sections (Figure 2) through the brain showing the distribution of AVT- and IST-immunoreactive perikarya and fibres. The pattern of distribution of perikarya and fibres for both peptides was very similar, but AVT-immunoreactive perikarya extended more caudally with respect to IST-immunoreactive perikarya.

AVT- and IST-immunoreactive perikarya were found in the NPO (Figure 3A and B). This nucleus showed a rostroventral portion containing mainly parvocellular perikarya and a dorsocaudal portion with magnocellular perikarya (Figure 3C). Bipolar or pear-shaped magnocellular neurons showed strong immunoreactivity for both antisera (Figure 3E) as did round or pear-shaped parvocelullar neurons (Figure 3F). The study of consecutive sections showed that AVT and IST did not coexist in the same neurons. Immunoreactive fibres arising from AVT and IST perikarya formed a conspicuous tract that ran along the diencephalic floor and pituitary stalk (Figure 3D). In the neurohypophysis, AVT-immunoreactive fibres were more numerous and showed stronger immunoreactivity with respect to IST-immunoreactive fibres. The neurohypophyseal lobe showed an irregular shape with extensions that penetrated the RPD and the PI. In this way the AVT and IST axonal ending intermingled with and ended close to the adenohypophysial cells of RPD and PI (Figure 3G). No extrahypothalamic fibres were observed in the brain of *Diplodus sargus*.

Distribution of MCH- and CRF-immunoreactive perikarya and fibres

Figures 4 and 5 show schematic drawings of sagittal (Figure 4) and transverse sections (Figure 5) through the

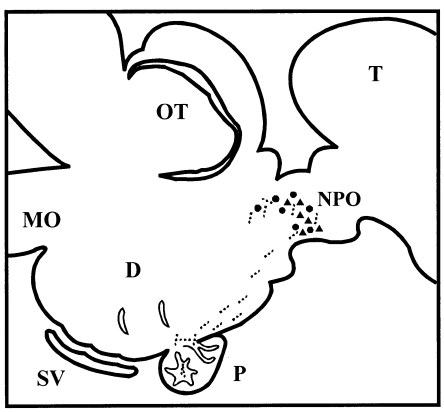

Figure 1. Schematic drawing representing a mid-sagittal section showing the distribution of AVT- (circles), IST-immunoreactive (triangles) perikarya and AVT-, IST-immunoreactive (dots) fibres. For abbreviations, see Table 2.

Table 2. Abbreviations.

D	diencephalon
HYP	hypothalamus
MO	medulla oblongata
NH	neurohypophysis
NLT	nucleus lateralis tuberis
NPO	nucleus preopticus
NPOpm	nucleus preopticus *pars magnocellularis*
NPOpp	nucleus preopticus *pars parvocellularis*
NPP	nucleus periventricularis posterior
NRL	nucleus recessus lateralis
OC	optic chiasm
OT	optic tectum
P	pituitary
PI	*pars intermedia*
RPD	rostral *pars distalis*
SCO	subcommisural organ
SV	saccus vasculosus
T	telencephalon

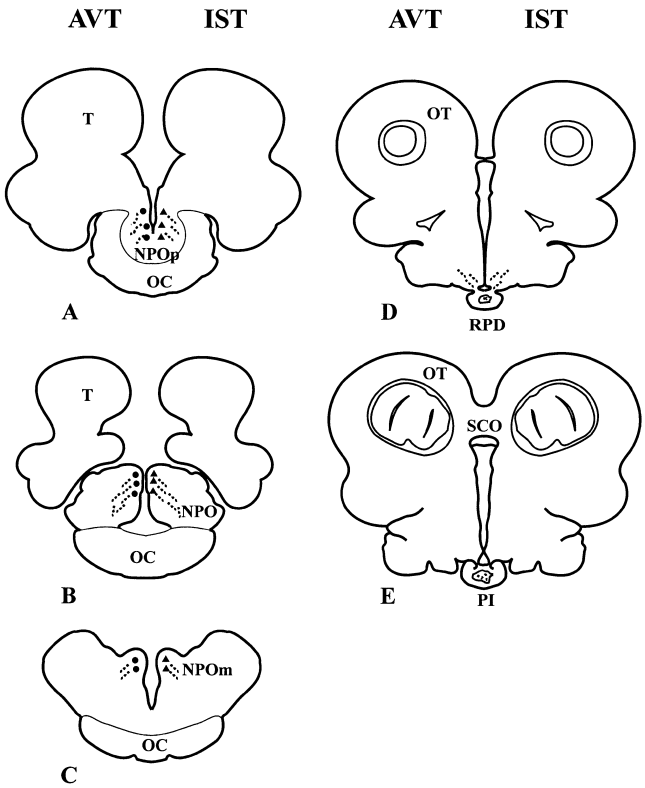

Figure 2. Schematic drawings of transverse sections from rostral (A) to caudal (E) levels through the hypothalamus of *Diplodus sargus*. AVT-immunoreactive perikarya (circles) and fibres (dots) are shown on the left side and IST-immunoreactive perikarya (triangles) and fibres (dots) on the right side. For abbreviations, see Table 2.

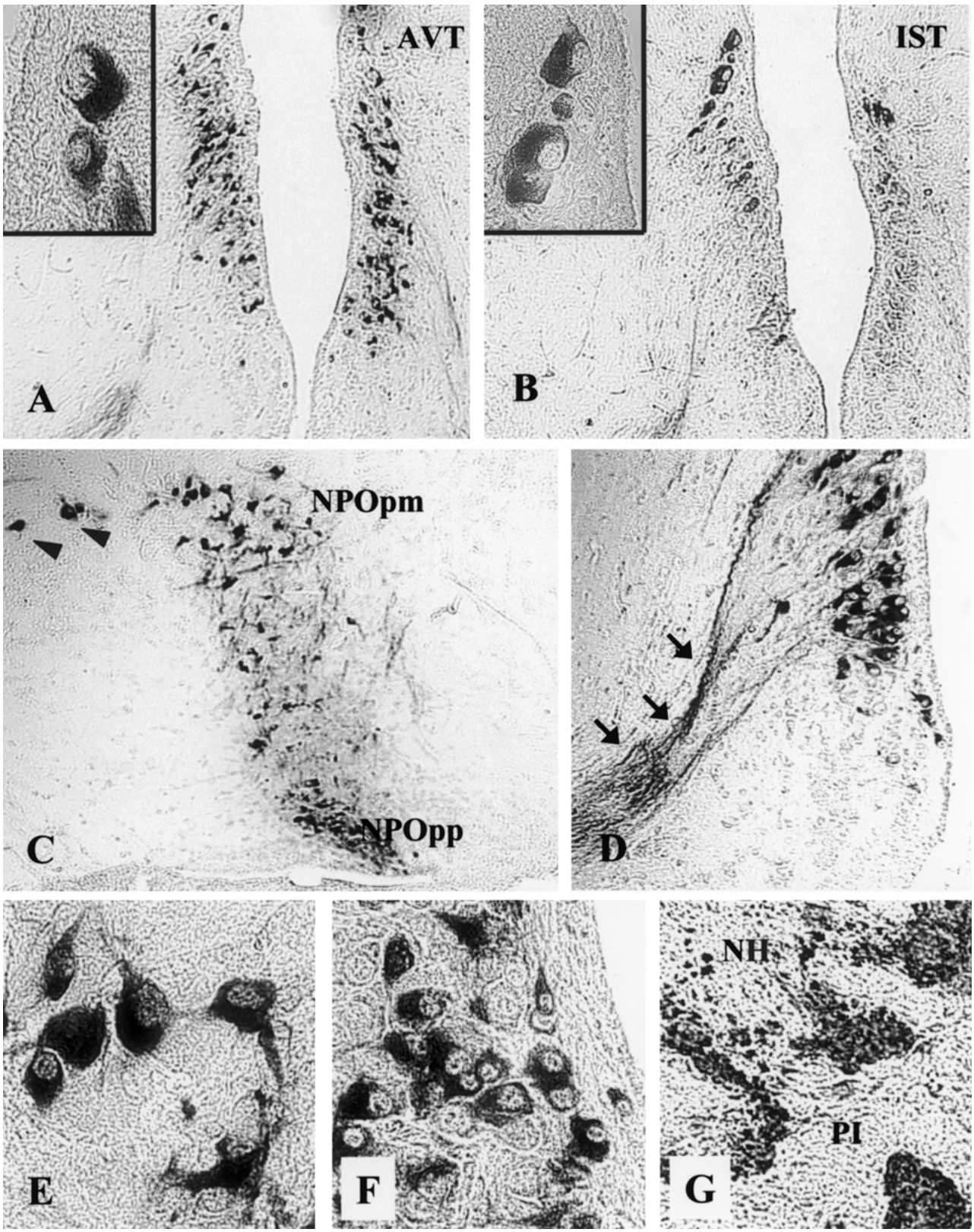

Figure 3. (A and B) Adjacent transverse sections through the NPO immunostained with anti-AVT (A) and anti-IST (B). ×80. Inset: Detail of AVT-immunoreactive and IST-immunoreactive magnocelullar perikarya. ×700. (C) Sagittal section of the NPO immunostained with anti-AVT. The rostroventral portion of the nuclei showed mainly parvocellular perikarya, while the dorsocaudal portion displayed mainly magnocellular perikarya (arrowheads). ×75. (D) Transverse section through the NPO immunostained with anti-AVT antiserum. The projections of AVT-immunoreactive perikarya form a compact bundle of fibres that run dorsoventrally (arrows). ×250. (E and F) Details of NPO showing magnocellular (E) and parvocellular (F) perikarya immunostained with anti-AVT. ×700. (G) AVT-immunoreactive fibres in branches of the neurohypophysis that penetrated the PI. ×150.

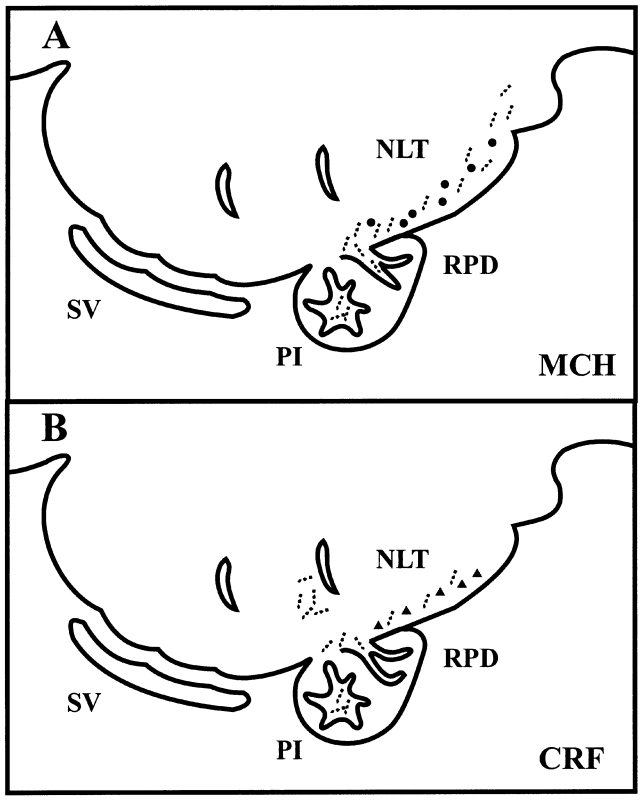

Figure 4. Schematic drawing of mid-sagittal section showing the distribution of MCH-immunoreactive (A) and CRF-immunoreactive (B) perikarya and fibres in the hypothalamus of *Diplodus sargus*. For abbreviations, see Table 2.

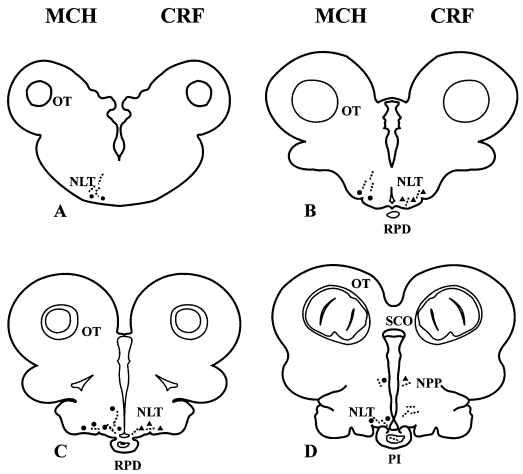

Figure 5. Schematic drawings of transverse sections from rostral (A) to caudal (D) levels through the hypothalamus of *Diplodus sargus*. MCH-immunoreactive perikarya (circles) and fibres (dots) are shown on the left side and CRF-immunoreactive perikarya (triangles) and fibres (dots) on the right side.

hypothalamus with the distribution of MCH- and CRF-immunoreactive perikarya and fibres. MCH- and CRF-immunoreactive neurons presented a similar distribution, but some differences were observed.

MCH-immunoreactive was detected mainly in the *pars lateralis* of the NLT (Figure 6A and C). These neurons were large, with round or pear-shaped perikarya and showed strong immunoreactivity (Figure 6B). In addition, fewer, small, weakly MCH-immunoreactive perikarya were found in the nucleus periventricularis posterior (NPP) and in the nucleus recessus lateralis (NRL) close to the lateral ventricle. The NPO did not show immunoreactivity for either of these antisera. A conspicuous bundle of MCH-immunoreactive fibres arose from NLT and ran along the diencephalic floor and pituitary stalk to end close to the MSH cells of the PI (Figure 6D–E), and the ACTH cells of the RPD. In addition, some MCH-immunoreactive fibres extended through the hypothalamic preoptic area. MCH-immunoreactive perikarya of the NPP and NRL did not show hypophysiotropic projections.

CRF-immunoreactive perikarya were found in the NLT, mainly in the *pars ventralis* and *pars lateralis*, but not in the NPO (Figure 7A). These neurons showed medium size, round or pear-shaped form and strong immunoreactivity (Figure 7B). In addition, a few small and weakly immunoreactive perikarya were observed in the NPP. CRF-immunoreactive fibres were seen in the diencephalic floor and pituitary stalk. They ended in the neurohypophysis adjacent to ACTH cells of the RPD (Figure 7C and D) and MSH cells of the PI (not shown). A dense network of CRF-immunoreactive fibres was observed ventrally to the recessus posterior in the basal hypothalamus. In this region scarce small and strongly CRF-immunoreactive cells were detected (Figure 7E). However, extrahypothalamic CRF-immunoreactive fibres were not detected in *Diplodus sargus*.

Discussion

In this study, we described the hypothalamo-hypophyseal system of the white seabream, *Diplodus sargus*, focusing on distribution of AVT-, IST-, MCH- and CRF-immunoreactive perikarya and fibres. The results are similar to those for the other teleosts studied previously, with AVT- and IST-immunoreactive perikarya located in the NPO and MCH- and CRF-immunoreactive perikarya in the NLT. A interesting feature of this species is the lack of immunoreactivity for anti-CRF antiserum in the NPO. At hypophyseal level, the close topographical proximity between ACTH and MSH adenohypophyseal cells and AVT, IST and MCH-immunoreactive fibres suggested an hypophysiotropic role for these peptides in addition to its well known systemic role. In this same way, the proximity of MSH cells of PI and CRF-immunoreactive fibres also suggested a role of this hormone in the release of MSH in *Diplodus sargus*.

Distribution of AVT- and IST-immunoreactive perikarya and fibres

The vasotocinergic and isotocinergic system observed in *Diplodus sargus* is similar to that reported previously for other teleosts (Goosens *et al.* 1977, Schreibman & Halpern 1980,

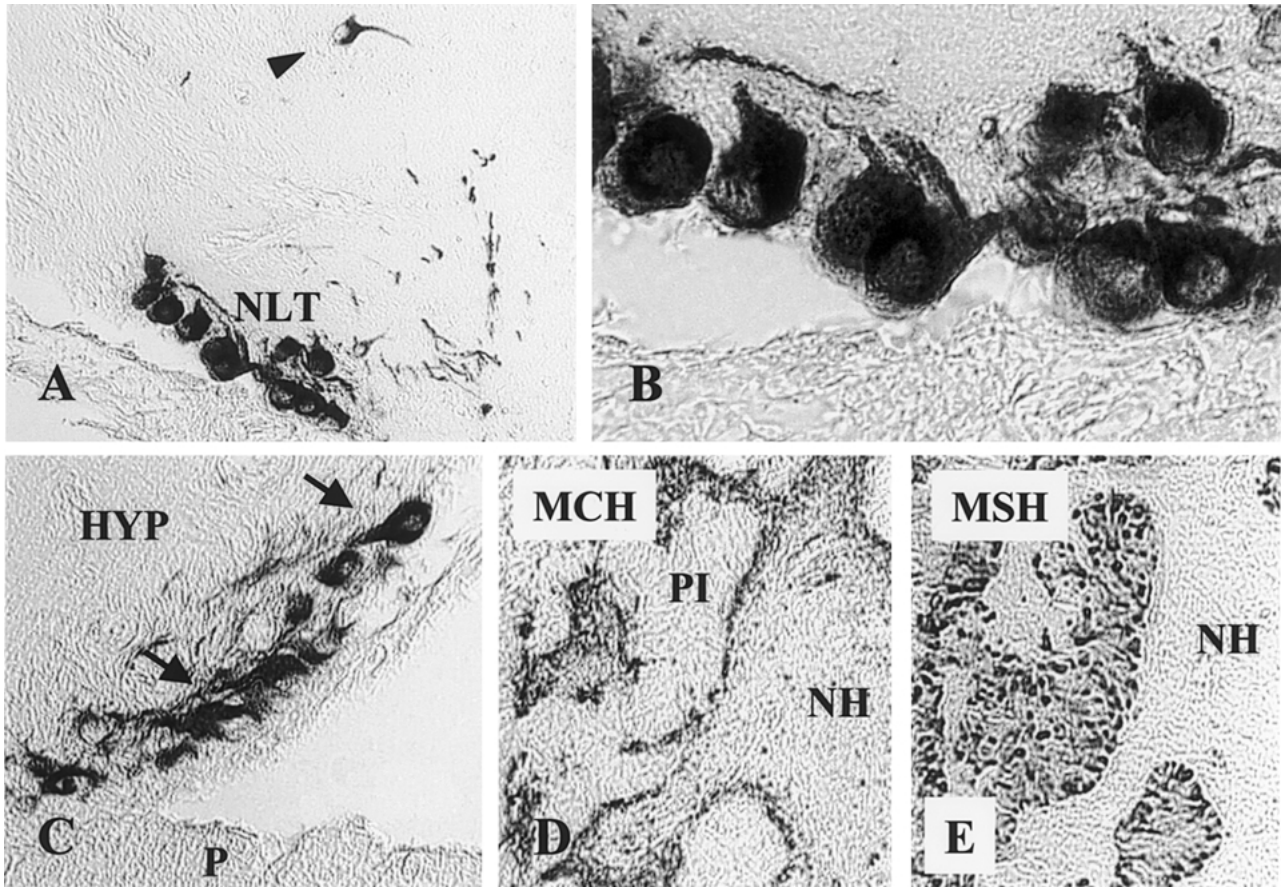

Figure 6. (A) Transverse section through the *pars lateralis* of the NLT showing MCH-immunoreactive perikarya and fibres. Also isolated-immunoreactive cells were observed in the basal hypothalamus (arrow head). ×250. (B) Detail of MCH-immunoreactive perikarya in the NLT. ×800. (C) Sagittal section of the basal hypothalamus (HYP) and pituitary (P) at level of NLT showing MCH-immunoreactive cells (arrows). ×400. (D and E) Consecutive transverse sections through the PI immunostained with anti-MCH (D) and anti-MSH (E). MCH-immunoreactive fibres terminate close to MSH-immunoreactive cells. ×150.

Cumming *et al.* 1982, van Dungen *et al.* 1982, Yulis & Lederis 1987, Batten *et al.* 1990, Holmqvist & Ekström 1995, Foran & Bass 1998). In addition to AVT and IST, several studies have demonstrated the presence of other neuropeptides in the NPO. Perikarya of these nuclei present hypophysial and non-hypophysial projections (van den Dungen *et al.* 1982, Batten *et al.* 1990, Peter *et al.* 1990, Anglade *et al.* 1993, Holmqvist & Ekström 1995). The anatomical and neurochemical organisation of NPO is complex and the use of tracing and other immunocytochemical studies would be useful to determine the NPO organisation in *Diplodus sargus*.

Several studies have shown changes in the pattern of AVT-immunoreactive perikarya and mRNA AVT expression in fish with sex changes (Foran & Bass 1998, 1999, Godwin *et al.* 2000). *Diplodus sargus* is a protandrous hermaphrodite species and in this work we analysed the vasotocinergic system in immature male fish. Therefere, it will be interesting to study the AVT system in male and female specimens and during the sexual inversion process.

A role of AVT in the regulation of ACTH secretion has been proposed (Fryer & Lederis 1986). The presence of AVT receptors in the *pars distalis*, where ACTH cells are localized (Moons *et al.* 1989b, Mahlmann *et al.* 1994) and the strong relationship between AVT-immunoreactive fibres and ACTH cells (Moons *et al.* 1989a, Batten *et al.* 1990, 1999) also support this role. Our results in *Diplodus sargus* showed this relationship and thus support this hypophysiotropic role for AVT. In teleost, the presence of AVT/IST-immunoreactive fibres in the neurohypophysis close to the PI and AVT/IST receptors in the PI have also been reported. A role in the control of MSH and/or SL releases has been proposed for these hormones (Moons *et al.* 1989b, Batten *et al.* 1990, 1999, Mahlmann *et al.* 1994). The presence of AVT and IST in the neurohypophysis close to the PI of *Diplodus sargus*, where MSH and SL are present (Segura-Noguera *et al.* 2000), also supports this assumption.

The wide distribution of AVT/IST immunoreactivity and AVT receptors in the brain of many teleosts indicates a role of these peptides as neuromodulators in several physiological processes (Moons *et al.* 1989a, Holmqvist & Ekström 1995, Batten *et al.* 1990, 1999). The absence of AVT- and IST-immunoreactive fibres in other brain areas suggests that, in *Diplodus sargus*, this function as neuromodulator could be limited to the hypothalamus. The use of other methodological approaches such as vibratome or cryostat sections, other fixatives (i.e. van Den Dungen *et al.* 1982, Batten *et al.* 1990,

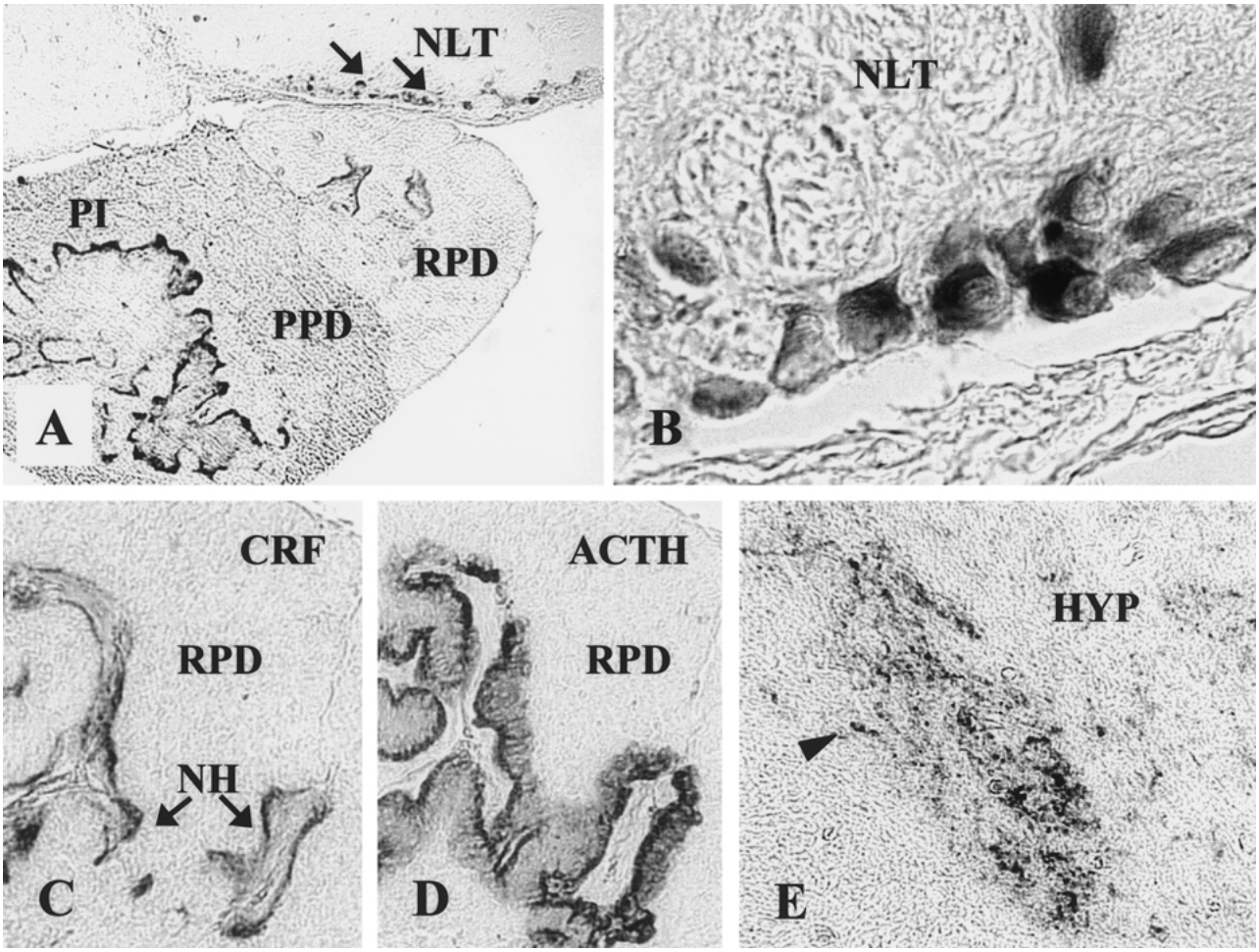

Figure 7. (A) Sagittal section of the basal hypothalamus and pituitary. CRF-immunoreactive perikarya were observed in the NLT (arrows). In the pituitary, CRF-immunoreactive fibres end close to the RPD and PI. ×60. (B) Detail of the NLT showing strong CRF-immunoreactive perikarya. ×700. (C and D) Consecutive sagittal sections through the RPD immunostained with anti-CRF (C) and anti-ACTH (D). ×150. (E) Detail of the posterior recessus in the basal hypothalamus showing a dense network of CRF-immunoreactive. Small CRF-immunoreactive perikarya were also detected (arrowhead). ×150.

Holmqvist & Ekström 1995) and other antisera (i.e. Meurling *et al.* 1996) could be useful to corroborate the absence of AVT- and IST-immunoreactive fibres in extrahypothalamic areas of *Diplodus sargus*.

Distribution of MCH-immunoreactive perikarya and fibres

In *Diplodus sargus*, the pattern of the MCH system in the hypothalamus is very similar to those described previously for other teleosts (Naito *et al.* 1985, Batten & Baker 1988, Batten *et al.* 1990, Mancera & Fernández-Llebrez 1995b). Magnocellular MCH-immunoreactive perikarya of the NLT send axons to the different regions of the neurohypophysis. In addition, *Diplodus sargus* also showed a few MCH-immunoreactive parvocellular perikarya in the NPP and NRL. The anatomical segregation of magnocellular perikarya at NLT and parvocellular perikarya at NRL has also been described for *Poecilia latipinna*, *Oreochromis mossambicus* and *Sparus aurata* (Batten & Baker 1988, Groneveld *et al.* 1995, Mancera & Fernández-Llebrez 1995b). In tilapia, MCH-immunoreactive perikarya have been reported in the neurointermediate lobe (Groneveld *et al.* 1995). However, in *Diplodus sargus* there were no MCH-immunoreactive present in this area.

In *Diplodus sargus*, similarly to other teleosts, a close topographical proximity of MCH-immunoreactive fibres to ACTH and MSH cells is observed (Naito *et al.* 1985, Batten & Baker 1988, Batten *et al.* 1990, Mancera & Fernández-Llebrez 1995b). Several studies have suggested that MCH controls the release of ACTH and MSH (Baker 1991, Balm & Groneveld 1998). In this way, our results also support this hypophysiotropic role of MCH. However, further studies are required to uncover the different subpopulations of MCH-immunoreactive perikarya that innervate ACTH and MSH cells. The use of different experimental design for the specific activation of ACTH or MSH cells (colour background, stress) and studies of mRNA expression would be useful to clarify this point.

In other teleosts, extrahypothalamic areas also receive an innervation by MCH-immunoreactive fibres (Naito *et al.* 1985, Batten & Baker 1988, Batten *et al.* 1990).

MCH receptors present a wide distribution in the brain (Saito *et al.* 1999, Sone *et al.* 2000) and it is generally accepted that, in fish, MCH, in addition to its hypophysiotrophic role, could function as a neuromodulator (Baker 1991, Balm & Groneveld 1998). However, neither in *Diplodus sargus* nor in another sparid, the gilthead seabream *Sparus aurata* (Mancera & Fernández-Llebrez 1995b), have we found extrahypothalamic MCH-immunoreactive fibres, thus suggesting that MCH functions as a neuromodulator only in the hypothalamus.

Distribution of CRF-immunoreactive perikarya and fibres

The CRF system has been previously analysed in several teleosts (Yulis *et al.* 1986, Olivereau & Olivereau 1988, 1990, Batten *et al.* 1990, Zupanc *et al.* 1999). The NPO and NLT were found to be the main sources of CRF-immunoreactive hypophyseal fibres. Our results in *Diplodus sargus* showed no immunoreactive perikarya in the NPO, while the NLT was the only source of CRF-immunoreactive fibres projecting to the neurohypophysis. The results agree with those reported for the sparid gilthead seabream *Sparus aurata* (Mancera & Fernández-Llebrez 1995a). In the eel, Olivereau *et al.* (1988) have shown that CRF and AVT coexist in the same magnocellular perikarya of NPO and both are increased by osmotic stress. In *Oncorhynchus nerka*, expression of mCRF in the NPO has only been observed in fish stressed by confinement (Ando *et al.* 1999). It could be of interest to study the CRF system in *Diplodus sargus* under different stress conditions.

In teleost, fibres from NPO directly innervate RPD and PI (Olivereau & Olivereau 1988, 1990, Batten *et al.* 1990, Zupanc *et al.* 1999). However, in the white sucker, *Catostomus commersoni*, the RPD is innervated by fibres coming from the NLT and the PI by fibres from the NPO (Yulis & Lederis 1986). According to our results, in *Diplodus sargus*, CRF-immunoreactive fibres ended close to ACTH cells in the RPD and to MSH cells in the PI. Thus, in *Diplodus sargus* the CRF fibres innervating RPD and PI arise from the NLT. The hypophysiotropic role of CRF in releasing ACTH is well known. In teleosts, CRF has also been demonstrated to stimulate MSH release (Rivier & Plosky 1986, Wendelaar Bonga 1997). The close relationship between CRF-immunoreactive fibres and MSH cells in our work, also suggest this melanotrophic role of CRF in *Diplodus sargus*.

As in other teleosts (Olivereau & Olivereau 1998, Mancera & Fernández-Llebrez 1995a), in *Diplodus sargus* we have found CRF-immunoreactive perikarya in the NPP. Our results suggest that CRF neurons of this nucleus do not project to the hypophysis but to the dorsal hypothalamus. CRF receptors have been located at different hypothalamic and extrahypothalamic areas of the teleost brain (Perrin & Vale 1999, Arai *et al.* 2001) and thus a role of CRF as a neuromodulator has been suggested (Sawchenko *et al.* 1993, Lovejoy & Balment 1999). Since in *Diplodus sargus*, there are no extrahypothalamic fibres, CRF could act as a neuromodulator only in the hypothalamus.

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