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Journal of Chemical Neuroanatomy 19 (2000) 197–210

Journal of CHEMICAL NEUROANATOMY

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Characterization of neuropeptide Y expression in the brain of a perciform fish, the sea bass (*Dicentrarchus labrax*)

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Received 1 March 2210; received in revised form 17 May 2000; accepted 17 May 2000

Abstract

The distribution of neuropeptide Y (NPY) gene expression was mapped in the brain of the sea bass (*Dicentrarchus labrax*) by in situ hybridization with ^{35}S-UTP labeled cRNA probes. Gene expression was mainly detected within the forebrain, although NPY mRNA transcripts were also localized in the tectum and tegmentum mesencephali and posterior brain. New NPY-expressing nuclei were found in the dorsal and ventral telencephalon, preoptic area, tuberal hypothalamus, synencephalon, tegmentum

Abbreviations: A, anterior thalamic nucleus; AC, anterior commissure; AP, accessory pretectal nucleus; C, caudal nucleus; CCe, corpus of the cerebellum; CP, central posterior thalamic nucleus; Dc1, subdivision 1 of the central part of the dorsal telencephalon; Dc2, subdivision 2 of the central part of the dorsal telencephalon; Dd, dorsal part of the dorsal telencephalon; Dld, laterodorsal part of the dorsal telencephalon; Dlv2, subdivision 2 of the lateroventral part of the dorsal telencephalon; Dm2, subdivision 2 of the medial part of the dorsal telencephalon; Dm3, subdivision 3 of the medial part of the dorsal telencephalon; Dm4, subdivision 4 of the medial part of the dorsal telencephalon; Dp, posterior part of the dorsal telencephalon; DP, dorsal posterior thalamic nucleus; DT, dorsal tegmental nucleus; E, entopeduncular nucleus; ECL, external cell layer of olfactory bulbs; GL, glomerular layer of olfactory bulbs; HaCo, habenular commissure; I, intermediate thalamic nucleus; ICL, internal cell layer of olfactory bulbs; IP, interpeduncular nucleus; LC, locus coeruleus; LT, lateral thalamic nucleus; NAPv, anterior periventricular nucleus; NAT, anterior tuberal nucleus; NC, nucleus corticalis; NCLI, central nucleus of the inferior lobe; NDLI, diffuse nucleus of the inferior lobe; NDLIl, lateral part of the diffuse nucleus of the inferior lobe; NDLIm, medial part of the diffuse nucleus of the inferior lobe; nFR, nucleus of the fasciculus retroflexus; Nga, anterior part of the nucleus glomerulosus; NGp, posterior part of the nucleus glomerulosus; NGS, secondary gustatory nucleus; NGT, tertiary gustatory nucleus; NHd, dorsal habenular nucleus; NHv, ventral habenular nucleus; nIV, trochlear nerve nucleus; NLTd, dorsal part of the lateral tuberal nucleus; NLTi, inferior part of the lateral tuberal nucleus; NLTl, lateral part of the lateral tuberal nucleus; NLTm, medial part of the lateral tuberal nucleus; NLTv, ventral part of the lateral tuberal nucleus; NLV, lateral valvula nucleus; nMLF, nucleus of the medial longitudinal fasciculus; NP, paracommissural nucleus; NPC, central pretectal nucleus; NPGa, anterior preglomerular nucleus; NPGc, commissural preglomerular nucleus; NPGl, lateral preglomerular nucleus; NPGm, medial preglomerular nucleus; NPOav, anteroventral part of the parvocellular preoptic nucleus; NPOpc, parvocellular part of the parvocellular preoptic nucleus; NPPv, posterior periventricular nucleus; NPT, posterior tuberal nucleus; nPVO, nucleus of the paraventricular organ; NRLD, dorsal part of the lateral recess nucleus; NRLl, lateral part of the lateral recess nucleus; NRLv, ventral part of the lateral recess nucleus; NRP, posterior recess nucleus; NSC, suprachiasmatic nucleus; NSV, nucleus of the saccus vasculosus; NTe, nucleus of the thalamic eminentia; Ob, olfactory bulb; OI, inferior olive nucleus; OLN, olfactory nerve layer; OT, optic tectum; P, pituitary; pgd, dorsal periglomerular nucleus; PGZ, periventricular gray zone of the optic tectum; PM, magnocellular preoptic nucleus; PPd, dorsal periventricular pretectal nucleus; PPv, ventral periventricular pretectal nucleus; Psi, intermediate superficial pretectal nucleus; PSm, magnocellular superficial pretectal nucleus; PSp, parvocellular superficial pretectal nucleus; PVO, paraventricular organ; RI, inferior reticular nucleus; RL, lateral reticular nucleus; RS, superior reticular nucleus; SCO, subcommissural organ; SOF, secondary olfactory fiber layer of olfactory bulbs; SR, raphes superior nucleus; SV, saccus vasculosus; Tla, nucleus of the lateral torus; Tlo, nucleus of the longitudinal torus; TPp, periventricular nucleus of the posterior tuberculum; TS, semicircular nucleus; VAO, ventral accessory optic nucleus; Vc, central part of the ventral telencephalon; VCe, valvula cerebelli; Vd, dorsal part of the ventral telencephalon; Vi, intermediate .

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PII: S0891-0618(00)00063-6

mesencephali and posterior brain. The profuse NPY gene expression within the main neuroendocrine areas of the teleost fish further supports a physiological role in the control of the pituitary secretion. In addition, NPY gene was expressed within the primary visual, olfactory and gustatory circuits of teleost which, subsequently, project to hypothalamic feeding center in teleost fish. Our results extend the NPY-expressing areas known in teleost species.

Keywords: NPY; PP; In situ hybridization; Neuroanatomy; Feeding behavior; Reproduction; Teleost

1. Introduction

The neuropeptide Y family consists of structurally-related 36-amino acid peptides, which includes neuropeptide Y (NPY), gut endocrine peptide YY (PYY), pancreatic polypeptide (PP) and fish pancreatic peptide Y (PY, reviewed by Cerdá-Reverter and Larhammar, 2000). Neuropeptide Y is expressed mainly in the central nervous system (CNS) of vertebrates (Tatemoto et al., 1982), despite mRNA transcripts have also been detected in the pancreas of dexamethasone-treated rat (Myrsén et al., 1995) and testis cells of the same species (Kanzaki et al., 1996). The networks of NPY neurons have been characterized well in mammals (reviewed in Hendry, 1993) and the hypothalamus appears to be the main synthesis site (Stanley, 1993). Roles on different physiological functions including blood pressure (Zukowska-Grojec and Wahlestedt, 1993), circadian rhythms (Leibowitz, 1991, 1992, 1995), food intake (Schwartz et al., 1992) and pituitary hormone secretion (McDonald and Koenig, 1993) have been proposed.

Neuropeptide Y is the most conserved peptide for its size suggesting a preserved function throughout evolutionary process (Larhammar, 1996). Several studies in teleost fish, including the sea bass, have shown a functional role in the control of the pituitary secretion (Peng et al., 1990; Cerdá-Reverter et al., 1999a) and the feeding behavior (López-Patiño et al., 1999). The central distribution of NPY immunoreactivity has been reported in several fish species and the ventral telencephalon is considered as a major component of the NPY neural system in teleost and non-teleost species (Kah et al., 1989; Pontet et al., 1989; Cepriano and Schreibman, 1993; Chiba and Honma, 1994; Chiba et al., 1996; Vallarino et al., 1995; Subhedar et al., 1996). In contrast, NPY gene expression has only been studied in the goldfish (*Carassius auratus*; Peng et al., 1994; Vecino et al., 1994). The cDNA encoding sea bass NPY (sbNPY) has been cloned recently and probes are now available for expression studies (Cerdá-Reverter et al., 1998, 1999b). Because NPY-related peptides cannot be distinguished reliably using immunological methods (Pieribone et al., 1992), we used in situ hybridization to study the distribution of NPY mRNA within the CNS of the sea bass. The objectives of this study were to investigate the NPY gene expression in the CNS of a non-tetrapod vertebrate for comparative studies.

2. Material and methods

2.1. Animals and chemicals

Three-year-old female sea bass (*Dicentrarchus labrax*), ranging from 30 to 32 cm body length, were used in these experiments. Fish were reared in 500-l tanks supplied with continuously aerated, running sea water under natural photoperiod and temperature conditions. The animals were hand-fed twice a day, ad libitum. Experiments were carried out throughout September, when sea bass is just starting the vitellogenic phase of the reproductive cycle (Mañanós et al., 1994). The animals were treated in accordance with the European Union animal care regulations. Unless otherwise indicated, all chemicals and compounds were purchased from Sigma (St. Louis, MO, USA).

2.2. Tissue preparation

Animals were anaesthetized in a small tank containing tricaine methasulfonate (MS 222, 0.02%), then perfused transcardially with 75 ml of physiological saline solution (NaCl 0.65%) and subsequently with the same volume of fixative containing paraformaldehyde (PAF, 4%) in phosphate buffer (PB, 0.1 M, pH 7.4). After decapitation, the brains were removed, post-fixed overnight in the same fixative at 4°C, dehydrated and embedded in paraffin (Sherwood). Transverse serial sections were cut at 6 μm using a rotary microtome. One section every 200 μm was mounted on 3-aminopropyltriethoxylane (TESPA)-treated slides and then airdried at room temperature (RT) overnight. Seven consecutive series covering the entire extension of the sea bass brain were done. Six series were used for hybridization with the sense and anti-sense probes. The last series was stained with cresyl-violet 0.1% for detailed identification of brain nuclei. Sections were stored at 4°C under dry conditions and used for hybridization within 1 week.

Before hybridization, sections were deparaffinized, rehydrated and post-fixed in 4% PAF for 20 min. Slides were then rinsed twice in PB for 5 min and treated with a Proteinase-K solution (20 pg/ml in 50 mM Tris–HCl, 5 mM EDTA, pH 8) for 5 min at RT. Slides were next washed in PB and post-fixed again in PAF for 5 min, subsequently rinsed in sterile water and acetylated in a triethanolamine (0.1 M, pH 8)/acetic anhydride solution. Sections were then dehydrated and dried at RT.

2.3. Riboprobe synthesis and labeling

Full length NPY cDNA (639 bp, Gene Bank accession number AJ005378) inserted into pPCR-Script Amp SK(+) plasmid (Stratagene, La Jolla, CA, USA) was used to prepare riboprobes. Anti-sense and sense RNA probes were synthesized in vitro by linearizing the plasmids with *Sst*I or *Pst*I, (Life Technologies Inc., Rockville, MD, USA) and in vitro transcription was carried out with T7 or T3 RNA polymerase, respectively. Both sense and anti-sense probes were labeled with 10 μl of ^{35}S-UTP (10 mCi/ml) using the riboprobe synthesis kit (Amersham Pharmacia Biotech, Pistcataway, NJ, USA) as described by the manufacturer. After in vitro RNA synthesis, samples were treated with RQ1-DNAse (Promega, Madison, WI, USA) for 15 min at 37°C in presence of 50 U of RNAsin (Promega, Madison, WI, USA) and then incubated at −20°C for 3 h with 10 μg/ml of yeast RNA type III in an 8% formamide solution. Probes were subsequently purified onto Sephadex G50 columns. The two fractions containing the highest radioactivity were pooled and precipitated in ethanol potassium acetate at −20°C. The labeled probes were then stored at −20°C and used within 1 week.

2.4. Hybridization

The ^{35}S-UTP riboprobes were pelleted and dissolved in an appropriate volume of 100 mM DTT to obtain 2×10^5 cpm/μl. After 5-min incubation at 80°C, ^{35}S-UTP riboprobes were diluted 1/10 (final concentration of probes, 10 MM DTT and 2×10^4 cpm/μl) in hybridization buffer containing 50% formamide, 300 mM NaCl, 20 mM Tris–HCI (pH 8), 5 mM EDTA (pH 8), 10% Dextran sulphate, 1 × Denhardt's solution and 0.5 μg/ml yeast RNA type III. Subsequently, 60 μl of hybridization solution was added to each pre-treated slide (see above) which were cover-slipped and incubated in a humidified chamber at 55°C overnight. The coverslips were removed next day by incubating slides into a solution containing 5 × standard saline citrate buffer (SSC, 1 × SSC contains 150 mM NaCl, 15 mM sodium citrate, pH 7), 10 mM DTT, for 30 min at 55°C. The slides were then rinsed in 2 × SSC, 50% formamide, 10 mM DTT for 30 min at 65°C and three times immersed into NTE buffer (500 mM NaCl, 10 mM Tris–HCl, 5 mM EDTA, pH 7.5) for 10 min at 37°C. After RNAse treatment (20 μg/ml RNAse in NTE) for 30 min at 37°C, slides were rinsed three times in NTE buffer for 10 min at 37°C, once in 2 × SSC, 50% formamide, 10-mM DTT for 30 min at 65°C, once in 2 × SSC for 15 min at RT and twice in 0.l × SSC for 15 min at RT. Slides were finally dehydrated in increasing graded ethanol solutions containing 0.3-M ammonium acetate and dried at RT.

2.5. Signal detection

After the hybridization process, slides were dipped in Ilford K5 emulsion and exposed under dry conditions at 4°C for 7–10 days, developed in Kodak D-19 and counter-stained with toluidine blue. Anatomical locations were confirmed by reference to a brain atlas of sea bass (Cerdá-Reverter et al., unpublished results). Nomenclature used to identify brain nuclei follows Bradford and Northcutt (1983), Muñoz-Cueto et al. (2000).

2.6. Southern blot hybridization

Genomic DNA from sea bass was extracted from blood (Martínez et al., 1998), digested with *Pst*I, (Life Technologies Inc., Rockville, MD, USA) electrophoresed in agarose gels and blotted onto Hybond-N nylon membrane (Amersham, Pharmacia Biotech, Pistcataway, NJ, USA). Probe encoding sbNPY was labeled with [α-^{32}P] dATP (Amersham Pharmacia Biotech, Pistcataway, NJ, USA) using the random primer labeling kit (Life Technologies Inc., Rockville, MD, USA). The hybridization was done at 42°C in 50% formamide, 6 × SSPE, 0.5% SDS, 5 × Denhardt's solution, containing 10 μg/ml yeast RNA type III and washes were finally performed in 0.1 × SSPE/0.1% SDS at 65°C for 90 min (l × SSPE contains 150 mM NaCl, 1 mM EDTA, 9 mM NaH_2PO_4, pH 7.4).

3. Results

Southern and northern blot hybridization with the sbNPY probe always resulted in a single band (data not shown). The in situ hybridization with the sense probe never generated specific signals in the sea bass brain (data not shown). In situ hybridization with sbcRNA probe showed that the neuropeptide Y was expressed within the forebrain mainly, although peptide expression was also detected within the posterior brain. Fig. 1 represents levels of the sections shown in Fig. 2, where distribution of NPY-expressing perikarya is schematically illustrated.

3.1. Telencephalon

The rostralmost NPY mRNA-containing cell population corresponds to the internal cell layer (ICL) of the olfactory bulb (Ob, Fig. 3A). Hybridization signals within this layer were detected throughout the whole rostro-caudal extent. A conspicuous population of NPY mRNA positive cells within the most rostral pole of the dorsal part of the ventral telencephalon (Vd) was located in the caudal end of the olfactory bulb. The NPY-expressing cells of the latter nucleus adopted cau-

dally a periventricular position bordering the whole extension of the telencephalic ventricle. A remarkably intense labeling was detected consistently in the ventral region of the dorsal part of the ventral telencephalon (Vd, Fig. 3B). On the same section and immediately dorsal to this latter nucleus, some scattered cells of the medial part of the dorsal telencephalon (subdivision 4; Dm4) were labeled (Fig. 3C). At the same level, some NPY mRNA-expressing perikarya within the medial part of the dorsal telencephalon (subdivision 2; Dm2) could also be detected (Fig. 3B). It is worth mentioning that NPY-positive neurons of Dm2 extend caudally to the vicinity of the positive cells of the dorsal part of the ventral telencephalon (Vd). Therefore, it may be that this extension of Dm2 actually constituted a laterally-migrated population of the sea bass Vd. Progressing caudally, the NPY mRNA cells of the Vd were displaced gradually from the periventricular position by the cells of the supracommissural part of the ventral telencephalon (Vs). The latter nucleus rostrally exhibited NPY mRNA labeled perikarya in a small cluster placed at the dorsal pole of the telencephalic ventricle. However somewhat more caudally, the NPY mRNA positive cells extended ventrolaterally resulting in an intense hybridization signal (Fig. 3D). At the same level, a strong hybridization signal corresponding to a small cell cluster of the central part of the dorsal telencephalon (subdivision 1; Dcl) was labeled with the NPY-cRNA probe. The cells of the latter cluster occupied a position lateral to those of the Vd and were clearly distinct according to their size (Fig. 3E). The highest level of expression within the telencephalon was localized in the lateral (VI) and central (Vc) part of the ventral telencephalon (Fig. 3F and G). The large NPY mRNA expressing perikarya of this latter nucleus appeared intermixed commonly with the fascicles from the lateral forebrain bundle (LFB, Fig. 3G). In addition, occasional positive perikarya were also localized in the ventral part (Vv) and postcommissural part (Vp) of the ventral telencephalon (Fig. 2).

3.2. Preoptic region and hypothalamus

Perikarya positive for the NPY cRNA-probe were localized in five preoptic subdivisions. Rostrally, some labeled cell bodies appeared just anterior to the aperture of the preoptic recess coinciding with the rostralmost aspect of the parvocellular part of the parvocellular preoptic nucleus (NPOpc). Slightly more caudal, NPY mRNA positive neurons were detected in the ventrolateral pole of the preoptic recess within the anteroventral part of the parvocellular preoptic nucleus (NPOav, Fig. 3H). A more important cell group was detected more caudally in the anterior periventricular nucleus (NAPv, Fig. 4A). Some labeled perikarya were also found at the level of the suprachiasmatic nucleus (NSC, Fig. 4B), but the highest hybridization signal was localized in the posterior periventricular nucleus (NPPv, Fig. 4C).

Within the sea bass hypothalamus, positive NPY-labeled neurons were evident in several divisions. The first NPY mRNA-expressing hypothalamic cells appeared in the ventral division of the lateral tuberal nucleus (NLTv, Fig. 4D) as well as in the anterior tuberal nucleus (NAT, Fig. 4C) and the medial part of the lateral tuberal nucleus (NLTm, Fig. 4D). Prior to the lateral aperture of the medial hypothalamic ventricle, some perikarya were observed in the dorsolateral extension of the ventral part of the lateral recess nucleus (NRLv, Fig. 4E). Caudally, a profuse NPY mRNA expressing population forming the nucleus of the posterior recess, coated the ventral aspect of the

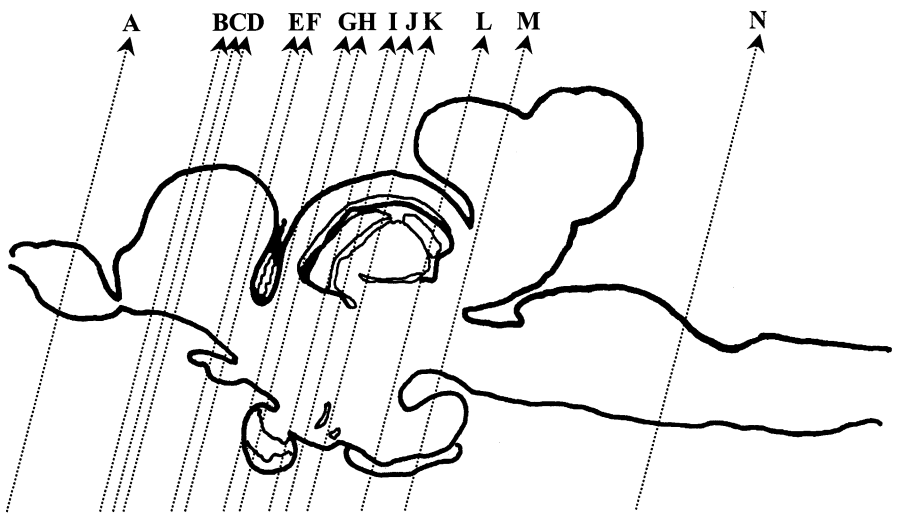


Fig. 1. Lateral view of the sea bass brain showing the levels of sections in Fig. 2; scale bar = 1 mm.

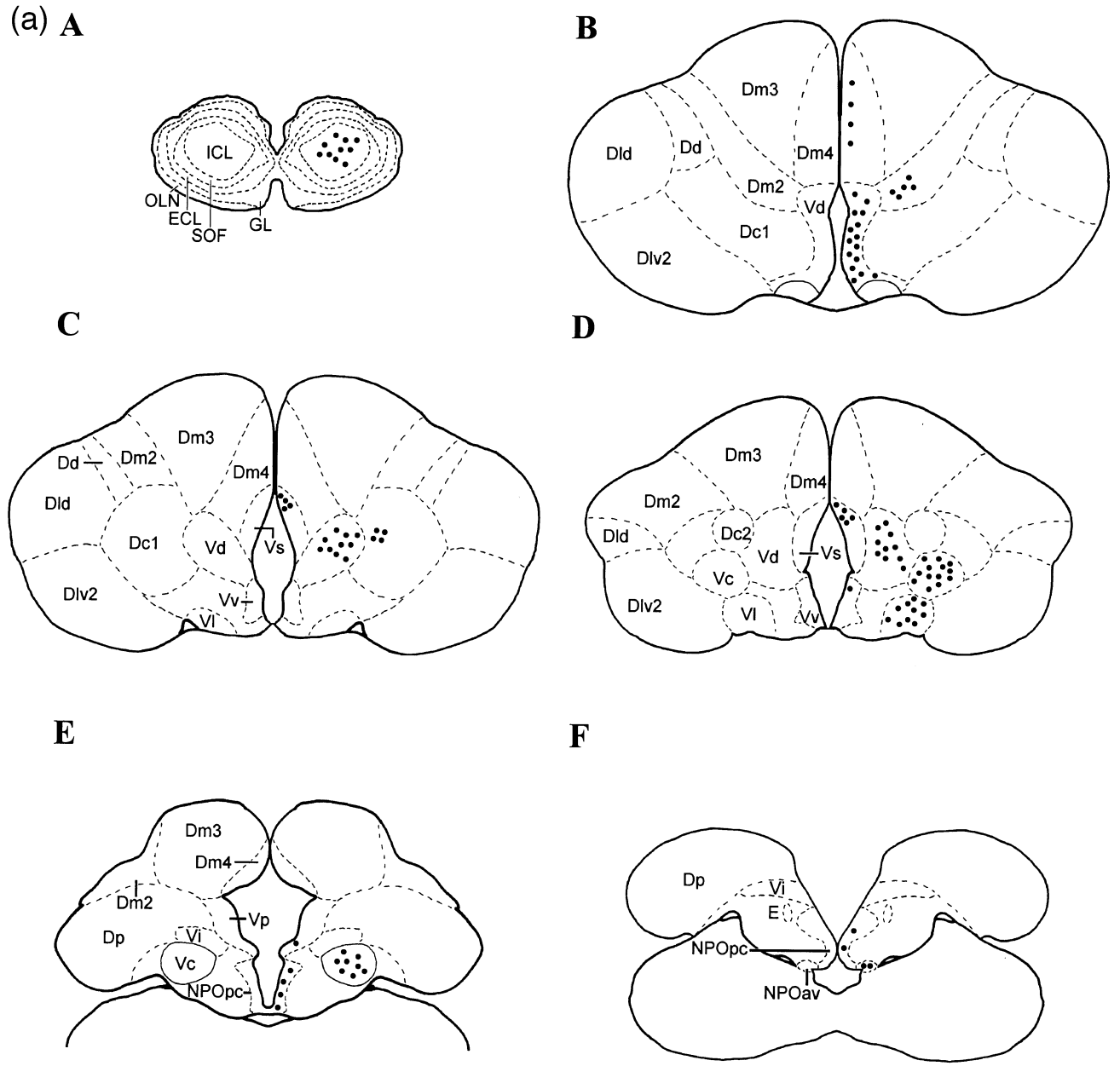


Fig. 2. A–N, schematic drawings of successive rostrocaudal transverse sections of the sea bass brain showing the distribution of NPY-expressing perikarya, (black circles); scale bar = 1 mm.

posterior recess (NRP, Fig. 4F). Finally, some dispersed NPY mRNA-expressing cells were observed in the diffuse nucleus of the inferior lobe (NDLI, Fig. 2).

3.3. Synencephalon and thalamus

In the synencephalon, the NPY gene expression was restricted to the dorsal part of the periventricular pretectal nucleus (PPd, Fig. 5A) although some labeled neurons were also visible in the ventral part of the periventricular pretectal nucleus (PPv). The rostralmost NPY mRNA-labeled perikarya within the thalamus surged medioventrally to the nucleus of the thalamic eminentia (nTe), in the rostral pole of the ventromedial nucleus (VM, Fig. 4A). More caudally after the habenular dissipation, the intermediate thalamic nucleus (I) exhibited numerous NPY mRNA expressing perikarya (Fig. 5B). At the same level, some positive neurons were present in the caudal pole of the ventromedial nucleus. In the dorsal thalamus, positive NPY mRNA cell bodies were evident in the ventrolateral extension of the anterior nucleus (A, Fig. 5B), although some NPY gene-expressing neurons were also patent in the ventrolateral extension of the nucleus central posterior (CP, Fig. 5C).

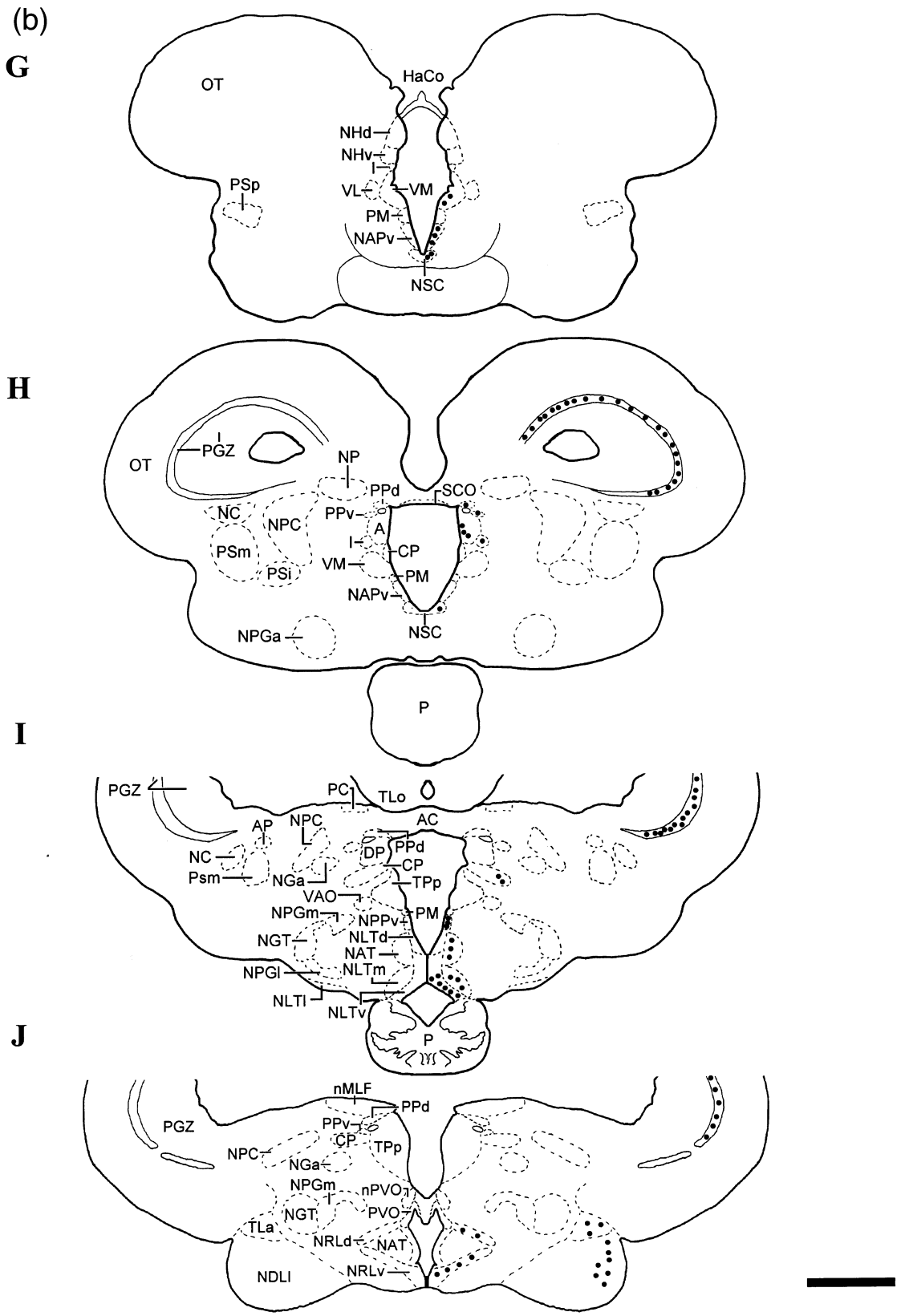
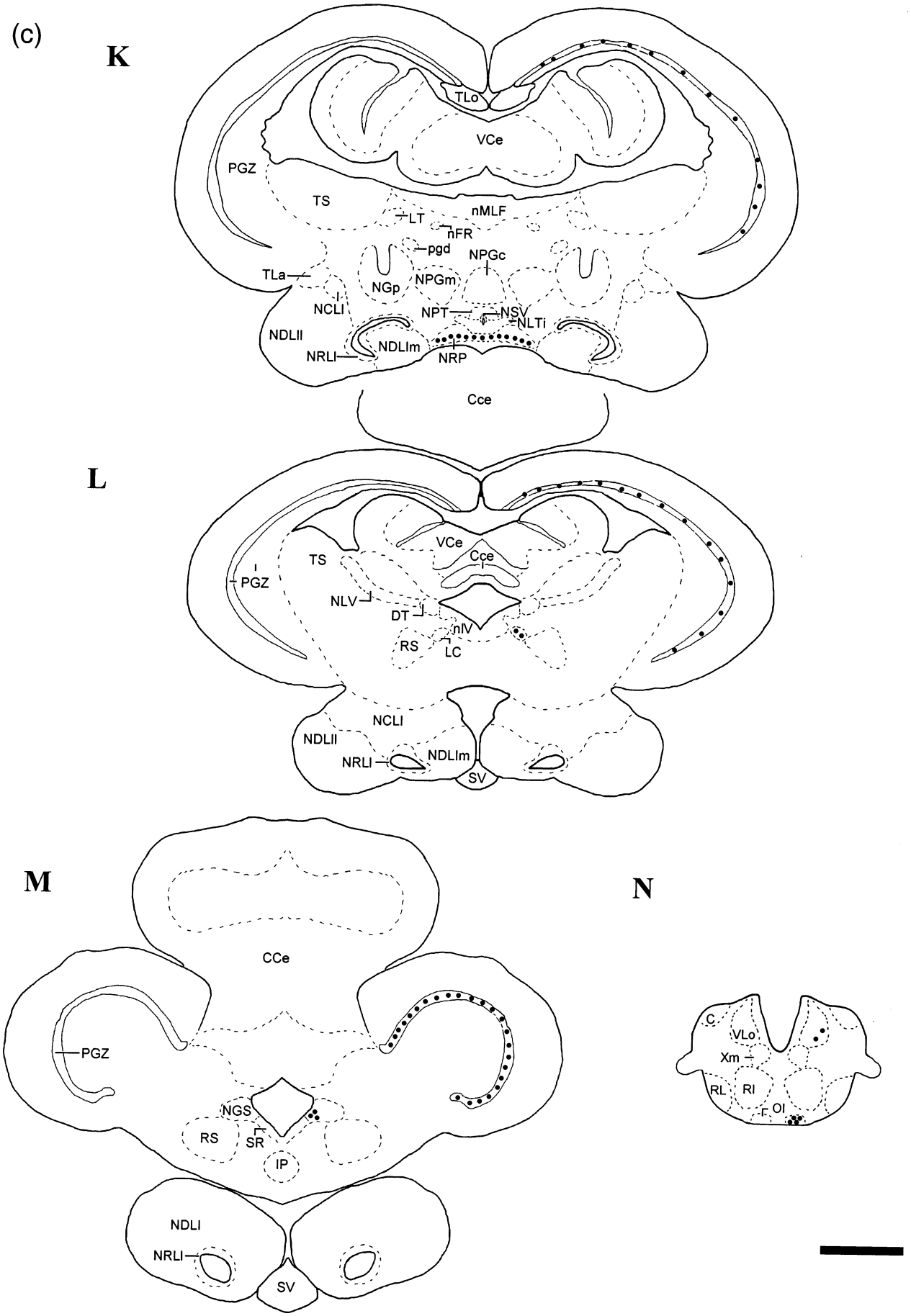


Fig. 2. (*Continued*)



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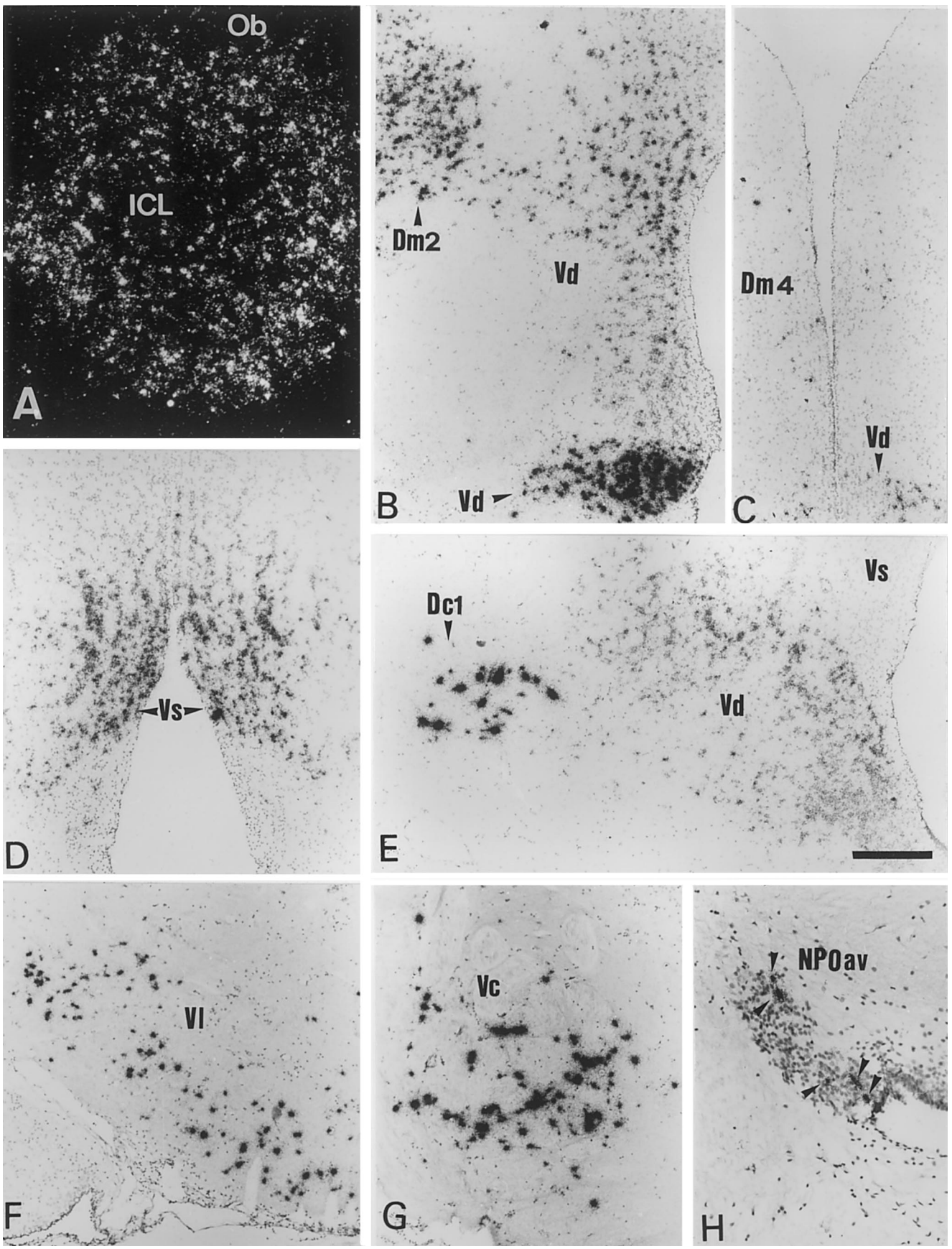

Fig. 3.

3.4. Mesencephalon and hindbrain

The NPY-expressing cells were further found in the outer layer of the periventricular gray zone (PGZ) of the mesencephalic tectum (Fig. 5E) and the secondary gustatory nucleus (NGS, Fig. 5G) at the medial zone of the mesencephalic tegmentum. Within the posterior tuberculum, NPY mRNA expression was confined exclusively to the nucleus of the lateral torus (TLa, Fig. 5F). Some scattered NPY mRNA cell bodies were detected in the locus coeruleus (LC, Fig. 5D) as well as in the caudal brainstem lateral to the vagal lobe (Vlo, Fig. 2). A distinct population of NPY labeled neurons was also found within the oliva inferior nucleus (OI, Fig. 2).

4. Discussion

By in situ hybridization with cRNA probes, we studied the central distribution of NPY expression in a perciform species. Several lines of evidence support the specificity of the sbNPY probe, (1) southern blot hybridization with cDNA probes encoding the mature region of the sea bass NPY always resulted in unique band, (2) northern blot hybridizations with sbNPY probe encoding exon 2 were shown to generate unique band (Cerdá-Reverter et al., 1999b). Specificity of sbNPY cRNA probe in neural tissue was tested by hybridization studies with sense probe. No hybridization signal with sense probe was detected consistently in parallel in situ hybridization experiments.

Our results on the distribution of NPY expression in the CNS of the sea bass show that the ventral telencephalon, preoptic area and tuberal hypothalamus are prominent synthesis places. Messenger RNA was further detected within the dorsal and ventral thalamus, synencephalon, tectum and tegmentum mesencephali and hindbrain. The distribution of NPY-expressing neurons in the sea bass resembles that reported in other non-tetrapod vertebrates, but new NPY-expressing nuclei were localized within the telencephalon, preoptic area, tuberal hypothalamus, synencephalon, mesencephalon and posterior brain (Kah et al., 1989; Pontet et al., 1989; Cepriano and Schreibman, 1993; Chiba and Honma, 1994; Chiba et al., 1996; Peng et al., 1994; Vecino et al., 1994; Vallarino et al., 1995; Subhedar et al., 1996). Peptide immunoreactivity or NPY transcripts have been described in the external cell layer (ECL) of the olfactory bulbs and nucleus olfactoretinalis (Kah et al., 1989; Pontet et al., 1989; Peng et al., 1994; Chiba et al., 1996; Subhedar et al., 1996). In contrast, NPY expression within the olfactory bulbs of the sea bass was localized in the internal cell layer (ICL). The ventral telencephalon is considered as a principal constituent of the central NPY network in teleost and non-teleost fish and both entopeduncular nucleus and the lateral part of the ventral telencephalon (Vl) have been reported as major synthesis sites in the goldfish (Peng et al., 1994; Vecino et al., 1994). Expression or NPY immunoreactivity has also been found within the dorsal and ventral parts of the ventral telencephalon of the goldfish (Pickavance et al., 1992; Peng et al., 1994) and killifish (*Fundulus heteroclitus*; Subhedar et al., 1996), respectively. Additionally, the sea bass showed a profuse expression in the supracommissural part and some scattered perikarya were also found in the postcommissural part. The presence of NPY transcripts in the dorsal telencephalon has not been reported previously in non-tetrapod vertebrates, although NPY immunoreactivity within the central part has been found in the goldfish (Pickavance et al., 1992), killifish (Subhedar et al., 1996) and dogfish (*Scyliorhinus torazame*; Chiba and Honma, 1994).

Similar to the goldfish, NPY transcripts were detected within both parvocellular (NPOpc) and anteroventral (NPOav) parts of the parvocellular preoptic nucleus. Both parts match the periventricular preoptic nucleus (NPP) of other authors, but we could not detect any expression within the magnocellular preoptic nucleus as previously reported in the goldfish (Peng et al., 1994). However, other studies failed to demonstrate NPY expression in the entire preoptic area of the goldfish (Vecino et al., 1994). Additionally, the sea bass showed NPY expression in the anterior and posterior periventricular nucleus (NAPv, NPPv) and some scattered perikarya within the suprachiasmatic nucleus (NSC). Consistently, peptide immunoreactivity has also been detected in the posterior periventricular nucleus of the goldfish (Kah et al., 1989).

Almost all subdivisions of the tuberal hypothalamus of the sea bass showed NPY expression, which was particularly intense within the anterior tuberal nucleus (NAT), ventral part of the tuberal lateral nucleus (NLTv) and nucleus of the posterior recess (NRP). The

Fig. 3. Dark and bright field photomicrographs of transverse sections of the sea bass at the level of telencephalon (A–G and rostral preoptic area (H). A, numerous small positive cells within the internal cell layer of the olfactory bulb. B, positive hybridization signal at the level of rostral telencephalon. Note the intense labeling at the ventral zone of the dorsal part of the ventral telencephalon (Vd). C, at the same level some disperse perikarya appear at the medial part of the dorsal telencephalon (Dm4). D, strong positive signal at dorsal zone of the paracommissural part of the ventral telencephalon (Vs). E, strongly positive large cells forming a cluster of the central part of the dorsal telencephalon. F, positive neurons within the lateral part of the ventral telencephalon (Vl). G, large NPY-labeled cell bodies of the central part of the ventral telencephalon (Vc) intermixed with the fascicles of the lateral forebrain (LFB). H, positive NPY-neurons within the anteroventral part of the parvocellular preoptic nucleus (NPOav). Scale bar = 200 μm (A–G) and 100 μm for H.

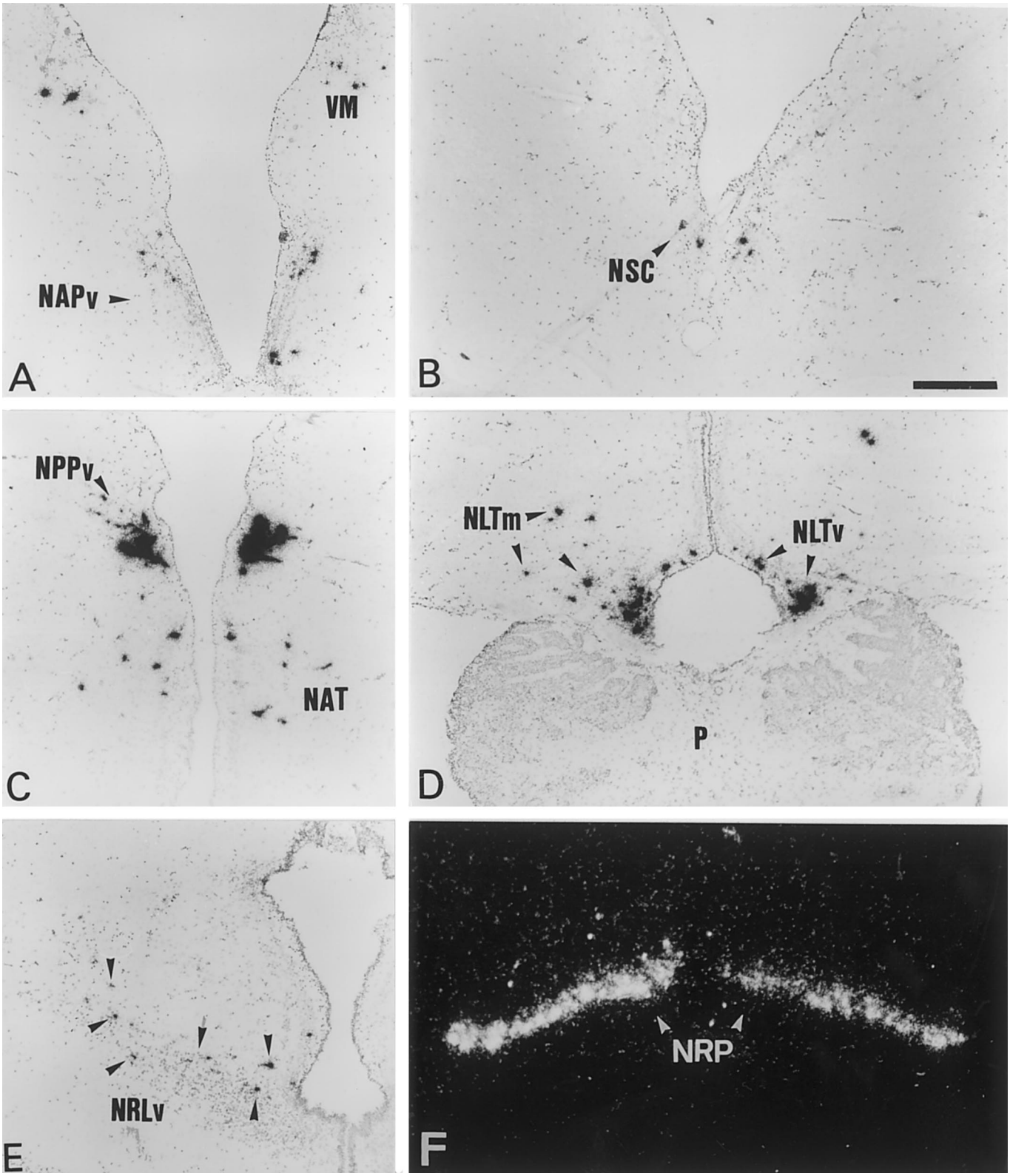

Fig. 4. Dark and bright field photomicrographs of transverse sections of the sea bass at the level of preoptic area (A–C), rostral ventral thalamus (A) and tuberal hypothalamus (C–F). A, positive hybridization at the level of anterior periventricular nucleus (NAPv). Note the presence of scattered positive cells at rostral pole of ventromedial nucleus (VM). B, some labeled somata within the caudal pole of the suprachiasmatic nucleus (NSC). C, strong hybridization signal at the level of caudal preoptic area (posterior periventricular nucleus, NPPv) and positive cell bodies within the anterior periventricular nucleus (NAT). D, intense labeling within the ventral part of the lateral tuberal nucleus (NLTv) and scattered positive perikarya within the medial part of the lateral tuberal nucleus (NLTm). E, positive hybridization signal (arrowheads) in the ventral part of the lateral recess nucleus (NRLv), just prior lateral aperture of the tuberal ventricle. F, dark field photomicrograph showing a strong hybridization signal coating the ventral aspect of the posterior recess nucleus (NRP). Scale bar = 200 μm.

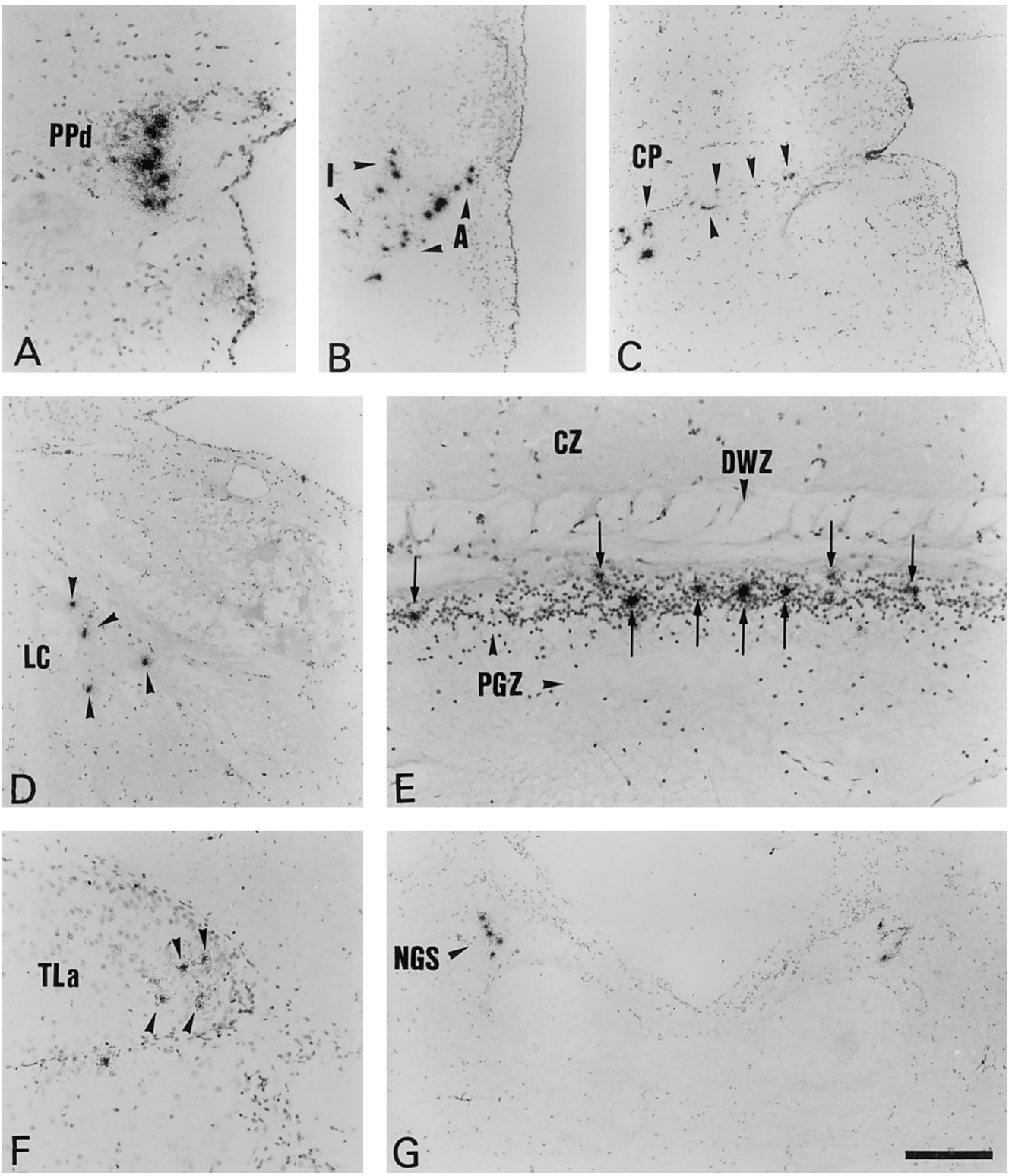

Fig. 5. Bright field photomicrographs of transverse sections of the sea bass at the level of synencephalon (A), ventral (B) and dorsal (C) thalamus, locus coeruleus (D), optic tectum (E), posterior tubercle (F) and rhombencephalon (G) processed by in situ hybridization for localization of preproNPY mRNA. A, strongly positive neurons in the dorsal part of the periventricular pretectal nucleus (PPd). B, positive neurons within the intermediate nucleus (I) and ventrolateral extension of the anterior nucleus (A). C, positive cell bodies (arrowheads) in the central posterior nucleus (CP). D, disperse positive perikarya in the locus coeruleus (LC) at the level of trochlear nerve nucleus. E, positive hybridization signal within the periventricular gray zone (PGZ) of the optic tectum. F, photomicrography showing positive hybridization signal (arrowheads) within the lateral torus (TLa). Positive NPY-cells at the rostral pole of the secondary gustatory nucleus (NGS). Scale bar = 200 μm for B–D and G and 100 μm for A, E, and F.

diffuse nucleus of the inferior lobe was shown to be the only NPY-expressing region of the goldfish hypothalamus (Peng et al., 1994; Vecino et al., 1994). However, NPY-like gene expression has been found in the posterior hypothalamus of coho salmon (*Oncorhynchus kisutch*; Silverstein et al., 1998). Likewise, peptide immunoreactivity has been reported within the lateral tuberal nucleus of the killifish (Subhedar et al., 1996), ayu (*Plecoglossus altivelis*, Chiba et al., 1996), goldfish (Pickavance et al., 1992), dogfish (Chiba and Honma, 1994) and lungfish (*Protopterus annectens*, Vallarino et al., 1995).

The expressions within the thalamus and posterior tuberculum agree well with that reported for the goldfish (see Bradford and Northcutt, 1983 for equivalent nuclei). However, NPY expression was detected in both dorsal and ventral parts of the periventricular pretectal nucleus of the sea bass synencephalon. The ventral part (PPv) is included within the dorsomedial thalamic nucleus, but the dorsal part (PPd) exhibits no equivalent in the nomenclature used in the goldfish (Peter and Gill, 1975; Bradford and Northcutt, 1983 for equivalencies).

Neuropeptide Y expression in the mesencephalon was much lower than that found in the forebrain and was detected within the optic tectum and secondary gustatory nulceus. In teleost, the optic tectum is a laminated structure consisting of five main layers. Peptide expression was restricted to the periventricular gray zone as reported previously in the goldfish (Peng et al., 1994). Within the hindbrain, NPY expression was localized in the locus coeruleus, vagal lobe and inferior olive nucleus. Locus coeruleus has extensively been reported as an NPYergic area and NPY immunoreactivity has been found in the vagal lobe of the goldfish and trout (Pickavance et al., 1992; Castro et al., 1999). However, peptide expression within the secondary gustatory nucleus and inferior olive nucleus is newly reported here.

In mammals, NPY has been suggested to be a substrate of the central-effector anabolic pathways of feeding. Intracerebroventricular NPY injections increase food intake in satiated rats whereas fasting induces NPY expression in the arcuate nucleus. Peptide release into the paraventricular nucleus, a key area for the control of energy balance in mammals, correlates with enhanced food-intake (Kalra et al., 1991; Schwartz et al., 1992; Stanley, 1993; Kaiyala et al., 1995). Terminals containing NPY in the paraventricular nucleus arise mainly from both arcuate nucleus and nucleus of the solitary tract. The NPY neurons of the brainstem projecting to paraventricular nucleus seem to be involved in the feeding-associate circadian rhythm whereas arcuate neurons projecting to the latter hypothalamic nucleus are implied in the fasting response (Yoshihara et al., 1996a,b). The neural circuits involved in the feeding behavior of teleost fish are poorly understood. It is generally accepted that the hypothalamic inferior lobe, particularly areas placed closely to lateral recess, as well as the ventro-posterior hypothalamus are involved in the control of feeding behavior. The latter areas form the hypothalamic feeding center where food intake is modified according to the visceral and sensorial incoming information (reviewed by Peter, 1979). Therefore, the inferior lobe seems to be the terminal field for incoming feeding-related information. However, the latter area is part of the widespread neural system as electrical stimulation of the ventromedial telencephalon and nucleus rotundus (corresponding actually to glomerular nucleus, Bradford and Northcutt, 1983), evoke feeding and aggressive responses in the bluegill (*Lepomis macrochirus*, Demski and Knigge, 1971). The involvement of NPY in the neural system controlling feeding behavior in teleost species is unknown largely. It has been demonstrated recently that intracerebroventricular injections of NPY increase food intake in the goldfish and the Y1-receptor antagonists block feeding after fasting (López-Patiño et al., 1999). Data reported in the goldfish indicate that NPY is involved in the compensatory-feeding response after fasting in teleost. The lateral tuberal nucleus has been proposed to be the teleostean homologous of the arcuate nucleus in mammals (Peng et al., 1994), but increased NPY-like expression after fasting was detected within the preoptic area of the coho salmon (Silverstein et al., 1998). Our results confirm that NPY expression is associated directly with the hypothalamic feeding center, since almost all subdivisions of the tuberal hypothalamus of the sea bass, including diffuse nucleus of the inferior lobe, lateral tuberal nucleus and posterior recess nucleus, synthesize the peptide. However, NPY expression was localized further within the main gustatory (Wulliman, 1988; Kanwal and Finger, 1991), olfactory (Kanwal and Finger, 1991; Becerra et al., 1994; Anadón et al., 1995) and visual (Butler and Saidel, 1993) centers projecting to the hypothalamic feeding center in teleost fish. It suggests further that peptide expression may be involved in the sensorial modulation of feeding behavior.

The direct innervation of teleostean pituitary has allowed characterization by retrograde tracing the main hypophysiotropic areas of the fish brain. Studies in several teleost species show that the preoptic area and tuberal hypothalamus are the main hypophysiotropic areas of the brain, although the thalamus and the ventral telencephalon contribute to a lesser extent (Kah et al., 1993; Prasada Rao et al., 1993; Holmqvist and Ekström, 1995). The present results show that NPY is expressed profusely within the main neuroendocrine areas of the teleost brain. The teleostean adenohypophysis receives NPY-containing terminals (Kah et al., 1989; Moons et al., 1989; Chiba et al., 1996). Although the origin of the NPY-containing fibers within the adenohypophysis is unknown, seasonal changes in

NPY immunoreactivity within the pituitary and the lateral tuberal nucleus seem to be correlated in the ayu (Chiba et al. 1996). Studies in fish including the sea bass, have shown that central or peripheral NPY administration stimulates both growth hormone (GH) and luteinizing hormone (LH) secretion either directly on the gonadotropic cells (Breton et al., 1989, 1991; Kah et al., 1989; Peng et al., 1990, 1993a,b,c; Danger et al., 1991; Cerdá-Reverter et al., 1999a) via Y1-like receptors or through presynaptical stimulation of GnRH release via Y2-like receptors (Danger et al., 1991; Peng et al., 1993a). The abundant NPY expression within the preoptic area and tuberal hypothalamus of the sea bass brain further supports a physiological role for the reported NPY-induced LH secretion (Cerdá-Reverter et al., 1999a).

In conclusion, this study represents the first detailed mapping of NPY expression in the brain of a perciform species, extending the knowledge of the NPY-expressing areas known in teleost species. The profuse NPY expression in the feeding hypothalamic center points towards a major role of NPY in the control of food intake. In addition, NPY was also expressed in the most important neuroendocrine brain areas supporting a physiological role for the NPY-induced LH secretion reported previously in the sea bass (Cerdá-Reverter et al., 1999a).

Acknowledgements

This work has been supported by CICYT grant, CYTMAR MAR 95-1888-C03-01 and EU FAIR grant, PL96-1410. During part of this work, José Miguel Cerdá-Reverter was a recipient of a fellowship from Balaguer-Gonel Foundation.

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