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Structures Immunoreactive with Porcine NPY in the Caudal Neurosecretory System of Several Fishes and Cyclostomes

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ABSTRACT—Using a double immunostaining technique, we examined the relationships between neuropeptide Y (NPY)- and urotensin I (UI)- and/or urotensin II (UII)-positive structures in the caudal spinal cord of various species of fishes, particularly in the teleost *Oncorhynchus masou* and in elasmobranchs *Scyliorhinus torazame*, *Triakis scyllium*, and *Raja kenojei*. Primitive actinopterygians, i.e., *Acipenser transmontanus*, *Polypterus senegalus*, and *Lepisosteus productus*, and cyclostomes *Lethenteron japonica* and *Eptatretus atami* were also studied from the viewpoint of comparative endocrinology. In the teleost and elasmobranchs, NPY-positive fibers were demonstrated to be often in contact with UI- and/or UII-positive cells and fibers, suggesting a control of the caudal neurosecretory neurons by NPY or a related substance. Similar association was seen in the primitive actinopterygians. As to the cyclostomes, immunoreactivities for NPY and UII were found in the lamprey, but none of the immunoreactivity for these peptides was seen in the hagfish. In the lamprey, NPY-positive varicose fibers abutting on the UII-positive cells were also demonstrated.

INTRODUCTION

The caudal neurosecretory system designated by Enami (1955) consists of the neurosecretory cells, fibers, and neurohemal terminal complex, the urophysis. In spite of great structural variations, this system is generally found in the caudal spinal cords of bony fishes and cartilaginous fishes. However, it is exceptionally absent in dipnoans and holocephalans (Kobayashi *et al.*, 1986). On the other hand, in cyclostomes and cephalochordates, no structural elements of the caudal neurosecretory system were demonstrated (Sano, 1965; Kobayashi *et al.*, 1986).

Two major peptide hormones, urotensin I (UI) and urotensin II (UII), have been well characterized chemically and pharmacologically. UI has an amino acid sequence homologous with corticotropin-releasing factor (CRF) and sauvagine (Lederis *et al.*, 1982; Ichikawa *et al.*, 1982), whereas UII is partially homologous with somatostatin (Pearson *et al.*, 1980; Ichikawa *et al.*, 1984). Subsequent immunohistochemical studies using antisera raised against UI and UII demonstrated cellular and subcellular localization of these peptides in various fish species (Kobayashi *et al.*, 1986; Larson *et al.*, 1987; Oka *et al.*, 1989). In addition, the presence of UII-immunoreactive cerebrospinal fluid (CSF)-contacting neurons have been shown in the extraurophyseal region of the spinal cord (Yulis and Lederis, 1986, 1988; Oka *et al.*, 1989). It was also demonstrated, by *in situ* hybridization studies, that all caudal neurosecretory cells in the carp are able to synthesize both UI and UII (Ichikawa *et al.*, 1988). However, the manner in which differential synthesis and release are regulated remains unknown (Bern, 1990).

Concerning the control of the caudal neurosecretory system, many studies have shown that the neurosecretory cells receive synaptic contacts (Kobayashi *et al.*, 1986) and that the cells do not operate autonomously (O'Brien and Kriebel, 1982). The chemical nature of the neuronal input to the caudal neurosecretory system has been analyzed by various techniques including fluorescence histochemistry (Audet and Chevalier, 1981), electron microscopy (O'Brien and Kriebel, 1983; Miller and Kriebel, 1986a), and immunohistochemistry (Miller and Kriebel, 1986b; Onstott and Elde, 1986; Yulis *et al.*, 1990), and was demonstrated to be cholinergic, catecholaminergic (Kriebel *et al.*, 1985), serotoninergic (Onstott and Elde, 1986; Yulis *et al.*, 1990), and peptidergic (Miller and Kriebel, 1986b).

Brain centers that project to the caudal neurosecretory perikarya have also been demonstrated (Kriebel *et al.*, 1985). However, information on the peptidergic innervation of the caudal neurosecretory system is too meager to evaluate its contribution to the control mechanisms; one paper (Miller and Kriebel, 1986b) reported projection of gonadotropin-releasing

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hormone (GnRH)-immunoreactive fibers to the caudal neurosecretory system in a cyprinodont fish.

In light of the scanty information on peptidergic innervation, the present immunohistochemical study was designed to elucidate whether or not neuropeptide Y (NPY)-containing fibers might innervate the caudal neurosecretory system of various fish groups, mainly teleost and elasmobranchs, because NPY is known to be a regulatory peptide that occurs widely and abundantly in the central nervous system of higher vertebrates and to act as a neurotransmitter or neuromodulator (Colmers and Whalestedt, 1993). Our previous data on fish brains have supported this view (Chiba and Honma, 1992, 1994; Chiba and Oka, 1996; Chiba *et al.*, 1993).

MATERIALS AND METHODS

Four species of Osteichthyes, the yamame, *Oncorhynchus masou*; the Senegal bichir, *Polypterus senegalus*; the spotted gar, *Lepisosteus productus*; and the white sturgeon, *Acipenser transmontanus*, three species of Chondrichthyes, the banded dogfish, *Triakis scyllium*; the cloudy dogfish, *Scyliorhinus torazame*; and the thornback ray, *Raja kenojei*, two species of Agnatha, the lamprey *Lethenteron japonica* and the hagfish *Eptatretus atami* were used in this study (Table 1). Except for the Senegal bichir, the spotted gar, and the white sturgeon, which were obtained through a local dealer, the animals were collected from the coastal and inland waters of Niigata Prefecture. After having been anesthetized with MS-222, the animals were perfused through the heart with acid-free Bouin's fluid. Then the caudal spinal cords were dissected out, immersed in the same fixative, dehydrated with graded ethanols, and embedded in paraffin. Serial cross and sagittal sections were cut at a 10-μm thickness, mounted on gelatin-coated glass slides, and immunostained by the streptavidin-biotin method with a commercial kit (Nichirei, Tokyo). The following antisera at the specified dilution were used as the primary antibodies: anti-porcine NPY (1:3000; UCB Bioproducts, Belgium), anti-*Catostomus* UI (1:4000; Suess *et al.*, 1986), and anti-*Gillichthys* UII (1:3000; Bern *et al.*, 1985). For simultaneous demonstration of NPY and UI/UII immunoreactivities in the same section, double immunoenzymatic labeling was applied according to the recommendation of Nakane (1968) and Hsu and Soban (1982). Controls were prepared by replacement of the specific antiserum with (1) normal rabbit serum, (2) antiserum preabsorbed with homologous antigens, i.e., synthetic UI, UII (1 μM; Sigma Chemical Company, USA), and NPY (1 μM; Penninsula Laboratories, USA), or (3) antiserum pretreated with related peptides, i.e., human CRF (10 μM; Peptide Institute Inc, Japan), somatostatin-14 (10 μM; Funakoshi, Japan), synthetic avian pancreatic polypeptide (10 μM, Penninsula Lab.) and peptide YY (10 μM, Penninsula Lab.). Immunostaining was negative in all sections treated with the control procedures (1) and (2), but was not abolished in those treated with procedure (3).

RESULTS

NPY-like-immunoreactive fibers were demonstrated in the caudal spinal cord of most of the species examined, with the exception of the hagfish. The frequency and density of the fibers showed variations among the different species. The results of NPY immunohistochemistry are summarized in Table 1 together with those of UI and UII immunohistochemistry.

Osteichthyes

Sagittal sections of the caudalmost eight segments of the spinal cord of the yamame revealed fine-beaded fibers showing moderate or intense NPY immunoreactivity. No NPY-positive cells were found in this area, but the NPY-positive fibers were scattered throughout the entire spinal cord. In the median sections, some NPY-positive fibers were intermingled with UI- and/or UII-positive cells and their processes (Fig. 1). Occasionally, NPY-positive fibers approached closely and appeared to come in contact with UI/UII-positive fibers, but the formers were never found in the neurohemal terminal area. In the caudal spinal cords of the spotted gar and the white sturgeon, the distributional outline of NPY and UI/UII immunoreactivities was similar to that of the yamame. The Senegal bichir showed interesting features in the distribution of UI- or UII-

Table 1. Occurrence of NPY-positive (NPY+ve) fibers in the spinal cord of the animals examined and contact with urotensin-positive (U+ve) structures

Name of animals	Age	NPY+ve fibers	Contact with U+ve cells	Contact with U+ve fibers
Osteichthyes				
Oncorhynchus masou	Juv	+	UI/UII	UI/UII
Polypterus senegalus	Ad	++	UI or UII	UI or UII
Lepisosteus productus	Ad	+	UI/UII	UI/UII
Acipenser transmontanus	Juv	+	UI/UII	UI/UII
Chondrichthyes				
Triakis scyllium	Juv	++	UI/UII	UI/UII
Scyliorhinus torazame	Ad, Juv	++	UI/UII	UI/UII
Raja kenojei	Ad, Juv	++	UI/UII	UI/UII
Agnatha				
Lethenteron japonica	Ad	+	UII	UII
Eptatretus atami	Ad	–	–	–

Ad, adult; Juv, juvenile.
Score: ++, frequent; +, less frequent; –, not detected.
UI/UII: contact with UI and/or UII-positive structures.

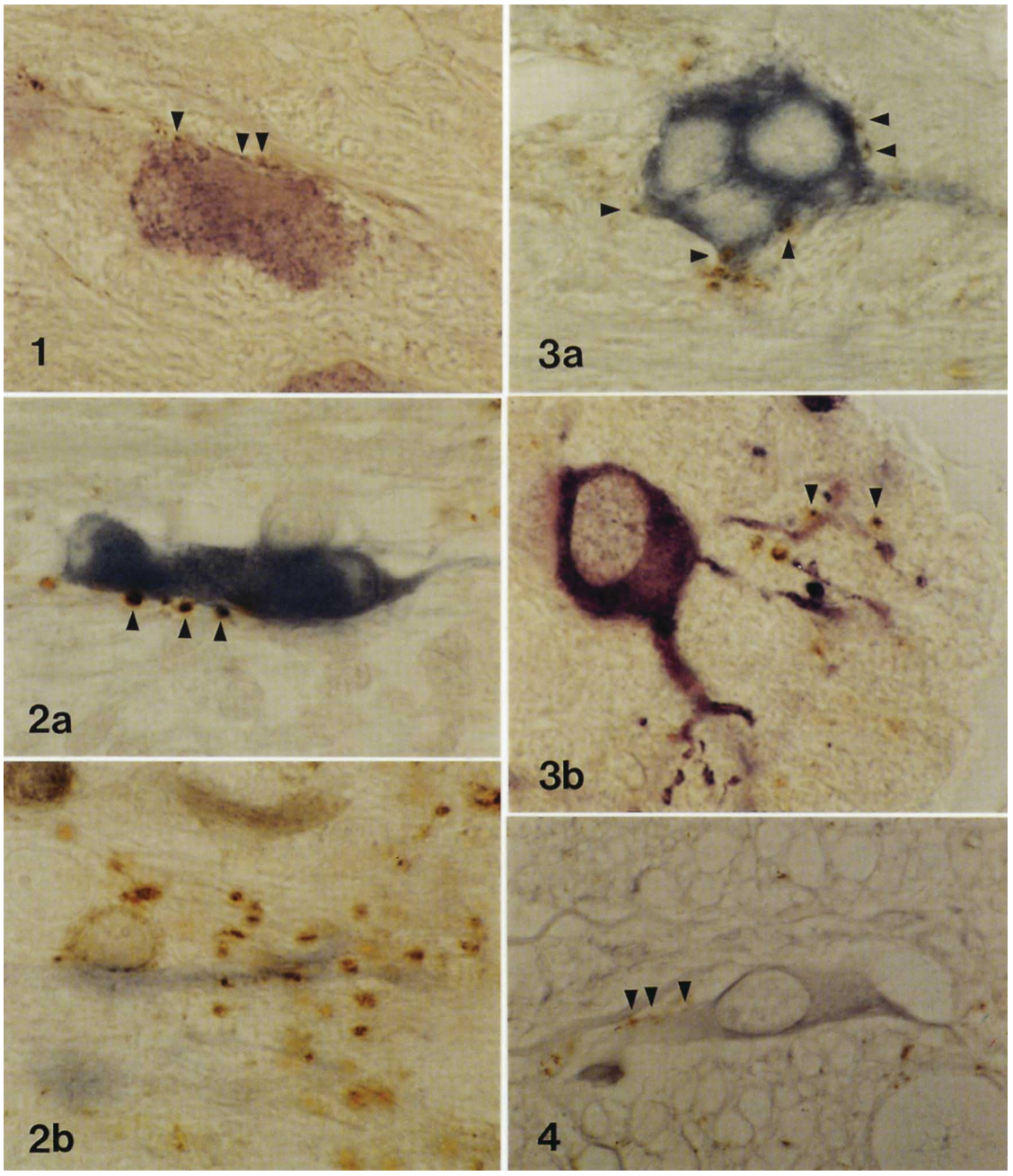

Fig. 1. Double immunohistochemical technique applied to the caudal spinal cord of the yamame, *Oncorhynchus masou*, showing close appositions of NPY-positive fibers (arrowheads: brown elements) to a UI-positive cell (Purple element). ×1000.
Fig. 2a. Double immunohistochemical technique applied to the anterior part of the spinal cord near the medulla oblongata of the bichir, *Polypterus senegalus*, showing contact of NPY-positive fibers (arrowheads: brown elements) with a UI-immunoreactive cell (dark blue element). ×1000.
Fig. 2b. Close relationship between NPY-positive cells and fibers (brown elements) and UI-positive fibers (dark blue elements) in the same specimen shown in Fig. 2a. ×1000.
Fig. 3a. Simultaneous demonstration of UI- and NPY-positive structures in the caudal spinal cord of the banded dogfish, *Triakis scyllium*. Note NPY-positive fibers (arrowheads: brown elements) abutting on a UI-positive cell (dark blue element). ×1000.
Fig. 3b. Double immunohistochemical technique applied to the caudal spinal cord of the dogfish, *Scyliorhinus torazame*, showing NPY-positive fibers (arrowheads: brown elements) in contact with UI-positive fibers (purple elements). ×1000.
Fig. 4. Caudal spinal cord of the lamprey, *Lethenteron japonica*, showing close apposition of UII-positive cell (grayish blue) and NPY-positive fibers (arrowheads: brown elements). ×1000.

cells and fibers; even in the anterior part of spinal cord near the medulla oblongata, UI-positive cells were often observed. There NPY-positive varicose fibers were often demonstrated to be abutting on the UI-positive cells (Fig. 2a). In addition, NPY-positive cells and fibers were often observed to be intermingled with UI-immunoreactive cells and fibers (Fig. 2b).

Contacts of UI- and NPY-positive structures were seen widely in the entire spinal cord except for the caudalmost segments. In the bichir, UII-positive cells and fibers were seen in a limited area, i.e., the caudalmost part of the spinal cord, where they were found in contact with NPY-positive fibers.

Chondrichthyes

In the banded dogfish, UI/UII-positive cells with a large lobulated nucleus were aligned laterally to the central canal. On the other hand, NPY fibers were found mainly in the dorsal area of the spinal cord; but some of the fibers coursed ventrally and tended to be concentrated in the areas ventrolateral to the central canal, where the UI/UII-immunoreactive neurosecretory cells were located. Contacts of NPY-positive fibers with the UI/UII-positive cells and processes were often observed (Fig. 3a). Nearly the same pattern was seen in the cloudy dogfish (Fig. 3b) and the thornback ray. Noticeably, in the ray, NPY-positive cells were rarely observed near the caudal neurosecretory cells, although NPY-positive processes were not traced. In the sharks and ray, as well as in the bony fishes, no NPY-positive fibers were seen in the neurohemal terminal area of the caudal neurosecretory system.

Agnatha

In the caudal spinal cord of the lamprey, UII-positive cells and fibers were barely recognized. NPY-positive fibers were seen in contact with the UII-positive structures (Fig. 4). On the other hand, in the hagfish, neither NPY- nor UI/UII-positive structures were detected in any spinal cord segment.

The present study gave no evidence for colocalization of NPY and urotensins at somal and fiber levels in any specimen examined.

DISCUSSION

The present study demonstrated that NPY-positive fibers are often in contact with UI/UII-positive cells and processes in most of the species that belong to representative groups of fishes, with the exception of the negative result for the hagfish. NPY is generally considered to be an essential peptidergic neurotransmitter (Colmers and Whalestedt, 1993), and its primary structure is highly conserved and its cDNA libraries of the goldfish and the ray, *Torpedo marmorata*, show strong conservation between the data of mammals (Blomqvist *et al.*, 1992). Moreover, NPY-related peptide has been isolated from the central nervous system of the lamprey (Rawitch *et al.*, 1992). In the light of such data, it is natural to consider that NPY or some related substance in the immunostained fibers may also serve as transmitter or modulator in the caudal neurosecretory system. However, further studies should be carried out to determine chemical nature of antigens that were demonstrated immunohistochemically in the present study.

Noteworthily, UII-positive cells were detected in the lamprey, and these cells were also in contact with NPY-positive fibers. It has been generally accepted that cyclostomes do not possess any elements of the caudal neurosecretory system, based on not only routine histological studies (Sano, 1965) but also UI/UII immunohistochemistry (Owada *et al.*, 1985). However, UI/CRF-immunopositive fibers were detected in the caudal spinal cord of the larval sea lamprey, *Petromyzon marinus* (Onstott and Elde, 1986). Moreover, UII was recently isolated from the brains of two species of lampreys (Waugh *et al.*, 1995). Thus, reinvestigation of the caudal neurosecretory system of lampreys would appear to be in order. In the hagfish, no NPY immunoreactivity was detected in the caudal spinal cord segments, although NPY-positive cells and fibers were demonstrated in various brain areas (Chiba *et al.*, 1993). Neither UI nor UII immunoreactivity was detected in the hagfish specimens. This result is consistent with earlier immunohistochemical observations on *Eptatretus stouti* (Onstott and Elde, 1986). However, it cannot be excluded that the hagfish contain unknown related substances that are structurally and/or immunologically different from urotensins of lampreys and fishes.

In the caudal neurosecretory system of the molly, *Poecilia sphenops*, it was reported that descending peptidergic innervation may arise from the dorsal tegmental magnocellular nucleus (Miller and Kriebel, 1986a) and that some neurons of the nucleus show GnRH immunoreactivity (Miller and Kriebel, 1986b). Moreover, at least some of the peptidergic terminals appear to arise from collaterals of the caudal neurosecretory system of the same species (O'Brien and Kriebel, 1983). According to previous mapping studies on fish brains, the distribution of NPY immunoreactivity in the dorsal tegmentum differed from species to species (Danger *et al.*, 1991; Vecino and Ekström, 1992; Chiba and Honma, 1992, 1994; Chiba and Oka, 1996). Additional experiments are required to determine the origin of NPY-positive fibers and to elucidate the functional significance of NPY or related substances in the caudal neurosecretory system.

In addition to NPY-positive fibers, in an earlier study (Oka *et al.*, 1993) we detected substance P- and FMRFamide (molluscan cardioexcitatory tetrapeptide) -positive fibers in contact with UI/UII-positive cells in the caudal neurosecretory system of some of the fishes examined in the present study. These data suggest a possibility of multipeptidergic regulation of caudal neurosecretory functions.

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