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Authors: Oka, Shunya, Chiba, Akira, and Honma, Yoshiharu

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Ontogenetic Development of the Caudal Neurosecretory System in the Chum Salmon, *Oncorhynchus keta*, with Regard to Its Ultrastructural Changes and with Relation to Neuropeptide Y-immunoreactive Fibers

Shunya Oka[1]*, Akira Chiba[1] and Yoshiharu Honma[2]

[1]*Department of Biology, Nippon Dental University School of Dentistry at Niigata, Niigata 951-8580, Japan and*
[2]*Department of Anatomy, Niigata University School of Medicine, Asahimachi 1, Niigata 951-8510, Japan*

ABSTRACT—The ontogeny of the caudal neurosecretory cells (Dahlgren cells) in the caudal spinal cord of the chum salmon, *Oncorhynchus keta*, was examined by conventional electron microscopy and with immunohistochemistry for urotensins (U) and neuropeptide Y (NPY). The precursors of the Dahlgren cells first appeared as agranular ovoid cells in the caudal region of the neural tube of 40-day-old embryos about one week before hatching. The occurrence of cytoplasmic granules in the immature Dahlgren cells became evident by the 14th day after hatching. At this moment, the U-positive reaction was merely demonstrated in some of the granules. Close association of NPY-positive fibers with the caudal neurosecretory structures was recognizable in 1-month-old larvae. Thus, it is apparent that the salmon Dahlgren cells start their secretory activity (production of the secretory granules) in early larval stages and that, thereafter, NPYergic afferent innervation of the caudal neurosecretory system becomes evident.

INTRODUCTION

The caudal neurosecretory system in fishes designated by Enami (1955) consists of the caudal neurosecretory cells (Dahlgren cells), fibers, and the neurohemal terminal complex, known as the urophysis. The roles of this system in osmo- (or electrolyte-) regulation and vasopressor activities have been suggested (Bern 1985; Kobayashi *et al*., 1986), but not established conclusively. The caudal neurosecretory neurons secrete at least two major peptide hormones, urotensins I (UI) and II (UII). UI has an amino acid sequence homologous with that of 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).

The ontogenetic development of this system has been studied in several fishes by means of routine histological techniques (Sano and Kawamoto, 1959; Sano *et al*., 1962; Fridberg 1962, Belsare, 1974). However, the origin or differentiational process of the Dahlgren cells has not been definitively determined (Kobayashi *et al*., 1986). As to the development of the urophysis, the exact time of its appearance differs among different species of fishes (Sano and Kawamoto, 1959; Fridberg, 1962; Sano *et al*., 1962; Belsare, 1974). Our immunohistochemical study of the chum salmon showed that UI and UII-positive structures first appeared in the embryos immediately before hatching and that the first sign of the formation of the urophysis was recognized in the 3-month-old larvae (Oka *et al*., 1993). However, information is still limited or scanty about the early development of the Dahlgren cells and the time of the commencement of their neurosecretory activities. So far, no ultrastructural data have been provided for this subject.

Concerning the control of the caudal neurosecretory system, previous studies have demonstrated the afferent innervation of the neurosecretory cells (Kobayashi *et al*., 1986). The chemical nature of the neuronal inputs to the caudal neurosecretory system has been analyzed by various techniques including 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; Oka *et al*., 1997). With respect to the peptidergic innervation, we reported that NPY-positive fibers made occasional contact with UI- and/or UII-positive cells and fibers in several fishes and cyclostomes, strongly suggesting NPYergic afferent innervation of the Dahlgren cells (Oka *et al*., 1997). However, almost nothing is known about the development of the peptidergic (NPYergic) nerve elements in the caudal neurosecretory system.

* Corresponding author: Tel. +81-25-267-1500;
FAX. +81-25-267-1134
E-mail. okashun@ngt.ndu.ac.jp

The aim of the present study is to offer ultrastructural and immunocytochemical evidences for early development of the Dahlgren cells and for developmental aspects of the NPYergic innervation of the caudal neurosecretory system in the chum salmon. For these purposes, we adopted conventional electron microscopy and immunocytochemistry for UI, UII and NPY.

MATERIALS AND METHODS

Embryos and larvae of the chum salmon, *Oncorhynchus keta*, were reared in a glass tank at the Johetsu Municipal Aquarium. Specimens from the 16-day stage after artificial insemination to 5 months post-hatching were fixed at 1-week to 1-month intervals. Two to four specimens were examined at each stage. After anesthesia with 0.1% *m*-aminobenzoate-methanesulfonate (MS-222), the caudal spinal cords were removed and immersed overnight in acid-free Bouin's fluid for light microscopy or in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1M phosphate buffer for electron microscopy.

For light-microscopic immunohistochemistry, the caudal spinal cords were dehydrated and embedded in paraffin. Serial sections were cut at a 10 μm thickness, mounted on the glass slides coated with Biobond (British BioCell, UK), and immunostained by the streptavidin-biotin method with a commercial kit (Nichirei, Tokyo). The following antisera at the specified dilutions were used as the primary antibodies: anti-*Catostomus* UI (1:4000; Suess *et al*. 1986), anti-*Gillichthys* UII (1:3000; Bern *et al*. 1985), and anti-porcine NPY (1:3000; UCB Bioproducts, Belgium). For simultaneous demonstration of NPY and UI or NPY and UII immunoreactivities in the same section, double immunohistochemical labeling was applied according to the recommendations of Nakane (1968) and Hsu and Soban (1982). Specificity of immunostaining was controlled 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 according to the control procedures 1 and 2, but was not abolished in those treated by procedure 3.

For conventional electron microscopy and electron microscopic immunocytochemistry, the fixed caudal spinal cords were rinsed in phosphate buffer overnight, dehydrated through a graded ethanol, and embedded in resin (Quetol 653, Nissin EM, Tokyo).

Semithin sections were cut at a 1–2 μm thickness, mounted on the glass slides coated with Biobond (British BioCell, UK). After removing resin by potassium hydroxyde in ethanol and deosmicating by 0.5% sodium metaperiodate, selected semithin sections were immunostained for the UI or UII by the same procedures used for the paraffin sections. Other semithin sections were stained with 0.5% toluidine blue in borax.

Ultrathin sections, cut with a diamond knife, were mounted on nickel grids. Some sections were stained with uranyl acetate and Reynold's lead citrate for conventional electron microscopy, whereas others were treated with protein A-gold (PAG) method for the immunocytochemistry. In the PAG method, the sections were treated with the 0.01M phosphate-buffered saline containing 0.5% bovine serum albumin (BSAPBS), and then incubated for 8–12 hr with the primary antibody (anti-UI, 1:4000). After a wash with phosphate buffered saline (PBS), the sections were placed on a drop of protein A-gold (15-nm, British BioCell, UK) diluted 1:20 with BSAPBS and incubated for 1 hr. After a rinse with PBS and distilled water, the sections were stained with uranyl acetate and viewed with an electron microscope.

RESULTS

Electron microscopy and immunocytochemistry for both urotensins

Results obtained by conventional electron microscopy and immunocytochemistry are summarized in Table 1.

In semithin sections of 16-, 23- and 30-day-old embryos, mitotic figures of neuroepithelial cells were occasionally seen in the caudal region of the neural tube, i.e., the future caudal neurosecretory system. The Dahlgren cells were not evident until 30 days after insemination. In the 40-day-old embryo about one week before hatching (about 18 mm in total length), the large ovoid cells stained faintly with the dyes were discriminated in the ventrolateral region of the caudal neural tube (Fig. 1a). At the electron microscopic level, their cytoplasm displayed many polysomes and rough endoplasmic reticula (ER) (Fig. 1b), but neither secretory granules nor Golgi body could be observed. Considering the location and neuroblast-like features of these cells, they may be the precursors of the Dahlgren cells. In the cells of the 47-day-old embryo immediately before hatching (about 22 mm in total length), Golgi bodies appeared occasionally in the cytoplasm together with abundant polysomes and some profiles of rough ER (Fig. 2a), but no secretory granules could be demonstrated in the cell body. Nevertheless, in semithin sections adjacent to this

Table 1. Summary of results in conventional electron microscopy and immunocytochemistry

		Conventional Electron Microscopy Appearance or presence			Immunocytochemistry UI or UII mmunoreactivity in the secretory granules		
	Age (days)	Dahlgren cells	granules in the cell	granules in the tract	axo-somatic contacts	Dahlgren cells	Nerve tract
Embryo	16	–	–	–	–	–	–
	23	–	–	–	–	–	–
	30	–	–	–	–	–	–
	40	P	–	–	–	–	–
	47	+	–	+	–	–	±
Larva	14	+	+	+	–	±	+
	30	++	+	+	+	+	+
	150	++	++	++	+	+	+

Score: ++, frequent; +, few; ±, very weak or questionable; –, not demonstrated; P, precursor cell

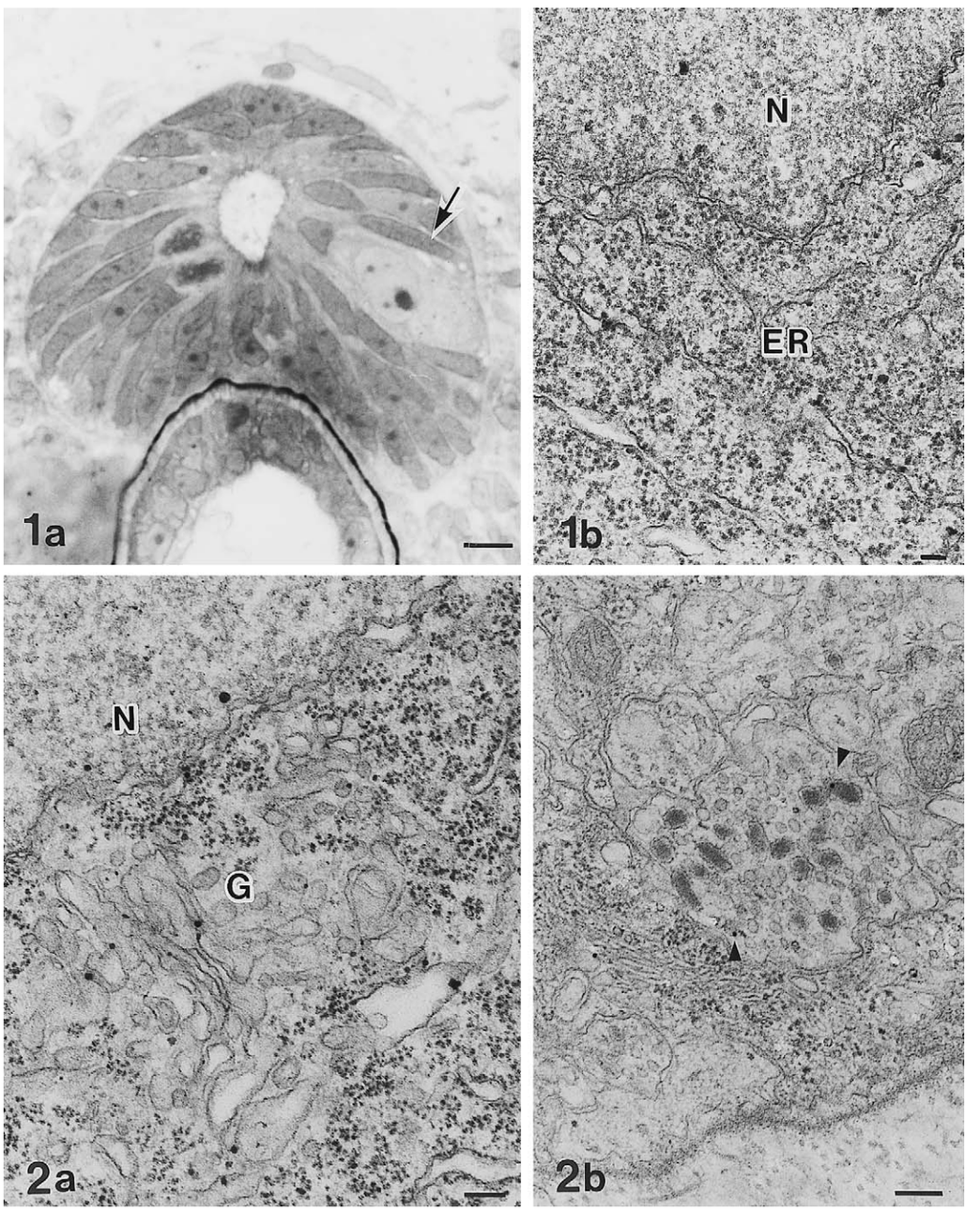

Fig. 1. Caudal part of the neural tube in the 40-day-old embryo of the chum salmon, *Oncorhynchus keta*. (a) Semithin sections cut at 1 μm thickness and stained by toluidine blue. In the ventrolateral region of the mantle layer, large pale cells are seen (arrow). (b) Ultrastructural features of the cells in Fig. 1a. Many polysomes and rough endoplasmic reticula (ER) are seen in the cytoplasm. These cells are regarded as the precursor cells of the Dahlgren cells. N, nucleus of the precursor cell. Scale bar: 1a; 5 μm, 1b; 200 nm

Fig. 2. Ultrastructural features of the cytoplasm of the precursor cells and neurosecretory tracts of the 47-day-old embryo just before hatching. (a) In the cytoplasm, many polysomes, rough ER and Golgi apparatus (G) are seen. However, neurosecretory granules were undetectable. (b) In the ventral part of the marginal layer of the caudal spinal cord, neurosecretory tracts contain a few granules about 100 nm in diameter. These granules are very weakly labeled by immunogold particles for urotensin I (arrowheads). N, nucleus of precursor cell. Scale bar: 200 μm

region, weak immunoreactivities for UI and/or UII were seen in the fibers running through the ventrolateral area of the caudal spinal cord. Ultrastructuraly, a few granules, about 100 nm in diameter, were observed in the neurosecretory fibers in the ventral area of the neural tube. Some of these granules were faintly labeled by immunogold particles for UI (Fig. 2b).

In the 14-day-old larvae (about 28 mm in total length), the developing Dahlgren cells contained secretory granules about 100 nm in diameter in the vicinity of the Golgi body. The morphological feature of the granules was nearly the same as those found in the preceding stage (Fig. 3a), and very weak immunoreactivity for UI was detected in the granules (Fig. 3b).

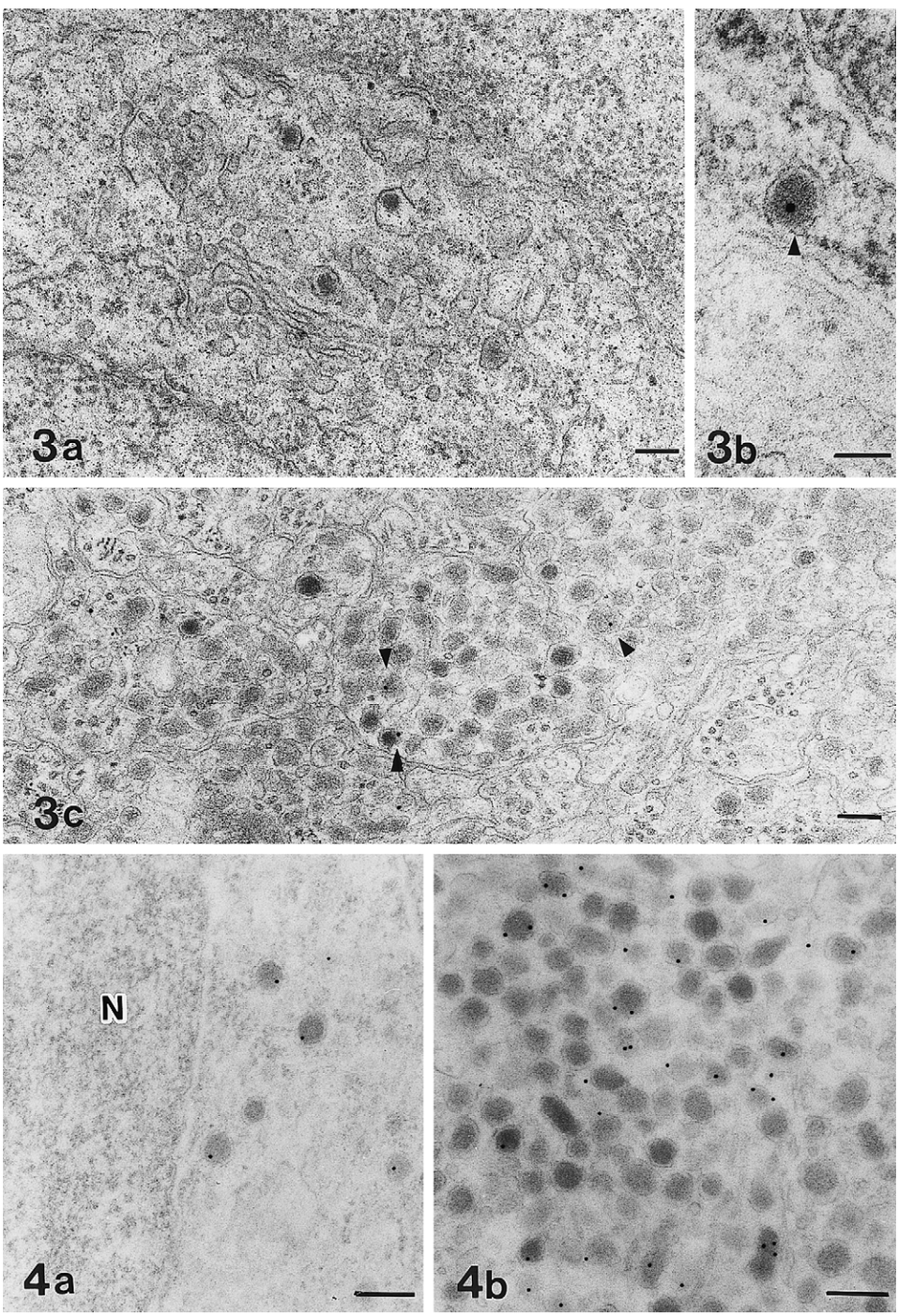

Fig. 3. Caudal spinal cord of a 14-day-old larva about 28 mm in total length. Conventional electron micrograph (a) and immunocytochemistry (b–c). (a) In the cytoplasm of developing Dahlgren cells, secretory granules are sporadically encountered in the vicinity of the Golgi body. (b) Some of these granules are very weakly labeled by immunogold particles for urotensin I (arrowhead). (c) In the ventrolateral part of the spinal cord, a number of granules are seen. Some granules show UI-immunoreactivity (arrowheads). Scale bar: 3a, 3c; 200 nm, 3b; 100 nm

Fig. 4. Caudal spinal cord of a 5-month-old larva at the smolt stage. (a) The Dahlgren cells contain numerous secretory granules, and many of them are positive for UI-immunocytochemistry. (b) In the urophyseal region, axonal tracts and terminals contain numerous UI-positive granules. N, nucleus of Dahlgren cell. Scale bar: 200 nm

The secretory granules were abundant in the neurosecretory tract of the same animal. Some of them showed UI-immunoreactivity (Fig. 3c). In 1-month-old larvae (about 30 mm in total length), feeding, displacement floating, and swimming began just after completion of the yolk sac absorption. Their Dahlgren cells were increased in number and size, and cyto-

plasmic granules showed weak immunoreactivity for UI. The ventrolateral portion of the caudal spinal cord, the presumptive urophysial area, also contained numerous axons and their terminals, in which distinct UI-positive granules were demonstrated. However, a histologically distinct neurohemal organ had not yet differentiated at this stage. With development, the Dahlgren cells and their processes gradually increased in number, and an elaborate urophysis comparable to that of adults was found in the 5-month-old larvae (about 75 mm in total length, at the smolt stage). A slightly bow-shaped swelling (the urophysis) of the ventral spinal cord was visible by the naked eye. The Dahlgren cells at this stage contained many granules (around 100 nm in diameter), most of which were positive for UI (Fig. 4a). In the urophysial region, neurosecretory fibers and terminals contained numerous UI-positive granules (Fig. 4b).

In the developing Dahlgren cells of the 14-day-old larva, no synaptic contacts, axo-somatic or axo-dendritic ones, were demonstrated by conventional electron microscopy. Only non-synaptic contacts of non-myelinated fibers with Dahlgren cells were merely observed in 1-month-old juveniles (Fig. 5a). Such contacts became more frequent with the development of this system until they became the 5-month-old juveniles. However, we failed to detect electron microscopic immunocytochemically any NPY-positive reaction.

Double immunohistochemistry

Double immunostaining for NPY and UI or NPY and UII revealed that NPY-positive fibers first appeared in the dorsal part of the caudal spinal cord of the 47-day-old embryo, although they were not in contact with UI and/or UII-positive structures. Later, closer association of the NPY-positive fibers

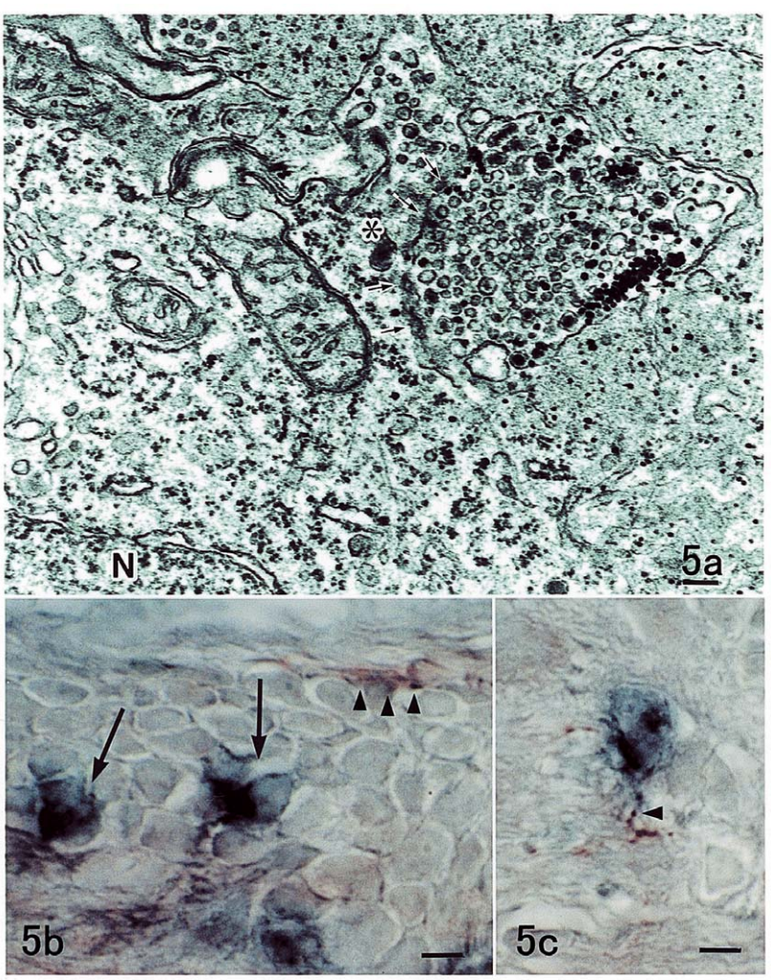

Fig. 5. Close relationships between the afferent nerve fibers and the Dahlgren cells in a 1-month-old larva. Conventional electron micrograph (a) and light microscopic double-immunohistochemistry (b–c).
(a) Non-synaptic contacts (arrows) of the non-myelinated fibers with Dahlgren cells. Asterisk, cytoplasmic granule of the Dahlgren cell; N, nucleus of the Dahlgren cell. (b)-(c) Double immunostaining applied to the caudal spinal cord of the same stage. (b) NPY-positive fibers (arrowheads: brown elements) are often observed in the vicinity of the Dahlgren cells showing UI-immunoreactivity (allows: dark blue elements). (c) Some NPY-positive fibers (arrowhead: brown element) are in contact with UI-positive cells (dark blue element) and fibers. Such contacts are first demonstrable in this stage. Scale bar: 5a; 150nm, 5b, 5c; 5 μm

with the UI and/or UII-positive cells and fibers were first demonstrated in the 1-month-old larvae (Fig. 5b, c). Such association of the NPY-positive and the UI and/or UII-positive structures became clearer with the development of the caudal neurosecretory system.

DISCUSSION

The present study on the chum salmon caudal neurosecretory system has demonstrated that 1) precursors of the Dahlgren cells first appeared as agranular ovoid cells in the 40-day-old embryo, about 1-week before hatching, 2) the cytoplasmic granules of the cells were first recognized in the 14-day-old larva, and 3) close association of NPY-positive fibers with caudal neurosecretory structures became evident later in the 1-month-old larva.

Studies on the ontogenetic development of the caudal neurosecretory system have been carried out on only a few species (Sano and Kawamoto, 1959; Fridberg, 1962; Sano *et al*., 1962; Belsare, 1974) by means of routine light microscopic techniques. Subsequently, immunohistochemical analysis of the development of the caudal neurosecretory system in the chum salmon demonstrated that UI and/or UII-immunoreactivity was first detectable in the embryos immediately before hatching (Oka *et al*., 1993). The present data may provide some insight into the origin or very early development of the Dahlgren cells, which has hitherto been obscure.

Origin of the Dahlgren cells

The origin or differentiating process of the Dahlgren cells has been the subject of much discussion among investigators (Kobayashi *et al*., 1986). Sano and Kawamoto (1959) supposed that the Dahlgren cells of the guppy originate from special hypochromatic ependymal cells in the region dorsal to the developing urophysis. On the other hand, Sano *et al*. (1962) mentioned that the Dahlgren cells of the rainbow trout might differentiate from neuroepithelial cells. Later, Belsare (1974) insisted that the Dahlgren cells of the catfish were definitely formed from the ependymal cells. On the contrary, Fridberg (1962) insisted that the Dahlgren cells could be traced from neuroblasts having the same appearance as those that give rise to motor neurons in the roach. All of these reports were based on only light microscopic observations and did not refer to the precursor cells in embryos. In the present study, hypochromatic neuroblast-like cells were demonstrated in the caudal part of the neural tube in the 40-day-old embryo of the chum salmon. In an earlier stage, i.e., in the 30-day-old embryo, mitotic figures were often in the germinal layer of the neuroepithelium; but neuroblast-like cells were not evident there. Moreover, no neuroblast-like cells could be observed among the cells in the differentiated ependymal layer in any specimens examined. Thus, we postulate that the Dahlgren cells originate from neuroblasts that become differentiated in the lateral plate of the caudal neural tube.

Previously, we reported that UI and UII-immunoreactivities were first demonstrable in the ventral area of the spinal cord just before hatching in the 47-day-old chum salmon embryo (Oka *et al*., 1993). In the present study, however, we failed to demonstrate the neurosecretory granules in the developing Dahlgren cells at the same stage, in spite of weak but evident immunoreactivity for UI in the neurosecretory tract. This disagreement may be partly ascribed to physiological differences among the individuals and/or to differences in the densities and process of maturation in the secretory granules between the cell body and the fiber tract.

Development of the NPYergic innervation

An involvement of NPY or NPY-related substance in control of the caudal neurosecretory system of fishes has been suggested based on light microscopic double immunostaining for NPY and UI and/or UII (Oka *et al*., 1997). Evidences were obtained for the neuronal input to the caudal neurosecretory system (Kobayashi *et al*., 1986). However, almost no information is available for ontogenetic aspects of the innervation of the caudal neurosecretory system (Kobayashi *et al*., 1986). In this connection, the present findings may contribute to a discussion of the neuronal control of the caudal neurosecretory system during ontogenesis. The present data suggest that the possible influence of NPY as a regulatory substance (Colmers and Whalestedt, 1993) on the caudal neurosecretory system begins in the post-hatching stage, being later than the onset of the secretory activity of the Dahlgren cells. However, we failed to provide valid immunohistochemical evidence for synaptic or non-synaptic contact between NPY-positive fibers and UI and/or UII-positive structures in the embryos or early juveniles. Nevertheless, non-synaptic contact between the Dahlgren cells and unmyelinated fibers containing dense granules and small clear vesicles was demonstrated ultrastructurally in the 1-month-old juveniles of the chum salmon. Thus, it is highly probable that the neuronal input to the Dahlgren cells begins in the post-hatching stage.

The present study has provided some information as to the origin of the Dahlgren cells and the afferent innervation of the caudal neurosecretory system during ontogenesis. Concerning the afferent peptidergic innervation of the caudal neurosecretory system, Miller and Kriebel (1986a, b) reported that descending peptidergic innervation in the caudal neurosecretory system of the molly may arise from the dorsal tegmental magnocellular nucleus, which includes GnRH immunoreactive neurons. More recently, four types of synaptic inputs to the Dahlgren cells have been identified, and at least two subpopulations of the Dahlgren cells were differentiated in the goldfish based on the ultrastructural features (Cioni *et al*., 1998). Further studies are needed to confirm the differentiation and development of the Dahlgren cells and their innervation in the chum salmon.

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