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Changes in the Nervous Systems from Larva to Juvenile in *Ciona intestinalis*

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Changes in the nervous systems during metamorphosis in the ascidian *Ciona intestinalis* were observed using the monoclonal antibody UA301, which specifically reacts with its nervous system. Although almost all larval nervous systems degraded during metamorphosis, a posterior sensory vesicle (PSV) remained and became an adult cerebral ganglion. On the other hand, an anterior part of the sensory vesicle, the so called "neurohypophisis", became a ciliated funnel of the neural gland rather than part of the cerebral ganglion. However, the origin of other part of the neural gland was still unclear in this study.

Key words: ascidian, adult nervous systems, cerebral ganglion, neural gland, metamorphosis, monoclonal antibody

Ascidians (Urochordata) are primitive members of the phylum chordates, including vertebrates, and occupy a unique phylogenetic position[1]. *Ciona intestinalis* used in this study is a solitary ascidian and its embryonic development is very rapid. Embryos start to hatch about 16 hours after fertilization (20°C) and become typical tadpole larvae. Tadpole larvae can swim by ocillating their tail. Their behavior depends on light and gravity and their responses change as they become older. Although they can express such complex behavior, the larval nervous system is simple compared with other higher chordates. The number of cells comprising their central nervous system is estimated to be about 300 cells, of which neurons are less than 100[2]. For this reason, studies on ascidian neurobiology have increased rapidly overe the last few years[3-5]. Our study particularly focused on attempting to describe the neural map in larvae and a systematic search for useful neural markers [6-8].

On the other hand, there are few studies on the adult nervous system. Larvae are allowed to settle in a plastic dish filled with sea water, and metamorphose within one or two days, making it easy to study the process of metamorphosis. In an other ascidian species, *Halocynthia roretzi*, the development of adult tissues after

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metamorphosis has been reported[9, 10]. However, adult gonad and nervous system development is not fully described in these papers. Recently, we traced the primordial germ cells and described gonad development from embryo to young adult of *Ciona intestinalis*[11, 12]. In this paper, the changes in the nervous systems during metamorphosis are described and the origin of the adult neural complex discussed.

Materials and Methods

Materials

The adult ascidian, *Ciona intestinalis* was collected near the Education and Research Center of Marine Bio-Resources of Tohoku University, Onagawa Bay, Japan and Mukaishima Marine Biological Station of Hiroshima University, the Inland Sea of Japan. Eggs and sperm were obtained by dissection of the gonoducts. After artificial insemination, they were raised until hatching at 18-20°C in an incubator. Larvae hatched at about 16 hours and they were allowed to settle and metamorphose in plastic dishes at 20°C.

Preparation of monoclonal antibody

As described in a previous report[13], the UA series monoclonal antibodies were prepared by immunizing the ovary homogenate of *C. intestinalis* as an immunogen. The antibody (UA301) used in this study is specific to the nervous systems of *C. intestinalis* larvae[6].

Whole-mount immunohistology

Collected samples were fixed with 10% formalin in sea water at 4°C for between several hours to overnight. The fixed samples were washed water several with sea times and dehydrated with an ethanol series. These samples were washed in 80% ethanol, 60% ethanol and then phosphate-buffered saline (PBS) before immunohistochemistry. They were immersed in 5% fetal bovine serum (FBS) /PBS containing 0.01% thimerosal for several hours to block nonspecific reactions, and then in the supernatant of UA301 hybridoma culture medium overnight at 4°C. After being washed for a day in PBS containing 0.05% Tween-20 (PBST), they were incubated overnight at 4°C with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG1 serum (Zymed labolatory, Inc., CA, USA) diluted 1:100 in 5% FBS/PBS containing 0.01% thimerosal. After being washed as before, they were reacted with DAB substrate (0.06% w/v diaminobenzidine, 0.015% v/v hydrogen peroxide in PBS), and then observed with a differential interference microscope (Axiophot 2, Carl Zeiss).

Results and Discussion

Nervous systems of young larvae

In a previous paper[6], the nervous systems of young larva were reported. Fig. 1 shows a summary illustration of the larval nervous network. The larval central nervous system consists of three major parts, the sensory vesicle, visceral ganglion and caudal nerve cord[2]. The sensory vesicle is a sac structure in which two kinds of sensory organs, otolith and ocellus are located. Additionally, its posterior region (posterior sensory vesicle, PSV) contains many neuropiles and neurons, including sensory neurons specific to light and gravity.

The visceral ganglion is the integral center of larval locomotion rather than visceral innervation, because the visceral organs in larva are almost undifferentiated and the visceral ganglion is specific to the larval period (see later). So I propose that its name must be changed to "the motor ganglion". Recently, it was suggested that it contains several motor neurons[3], including colinergic neurons[8]. Their axons run along both sides of the caudal neural tube and form the caudal nerve cord[14]. It has been said that the caudal neural tube itself does not contain any neurons[2].

The peripheral nervous system (PNS) has been described by some researchers[14-16]. In a previous paper[6], several types of PNS were identified by immunostaining with UA301 monoclonal antibody (Fig. 1). ATEN (apical-trunk epidermal neurons), DCEN (dorso-caudal epidermal neurons) and VCEN (ventro-caudal epidermal neurons) are epidermal primary sensory neurons[15] and their axons connect with the posterior end of the visceral ganglion. As their soma extends a long cilia to the fin[14, 15], they may monitor larval locomotion. Although RTEN (rostral-trunk epidermal neurons) also have cilia, they exist on the line connecting papilar neurons and the sensory vesicle. They may function as interneurons. Recently, it was reported that gelsolin mRNA and its protein product are detectable in these epidermal neurons of the PNS in other ascidian, *Halocynthia roretzi*[17].

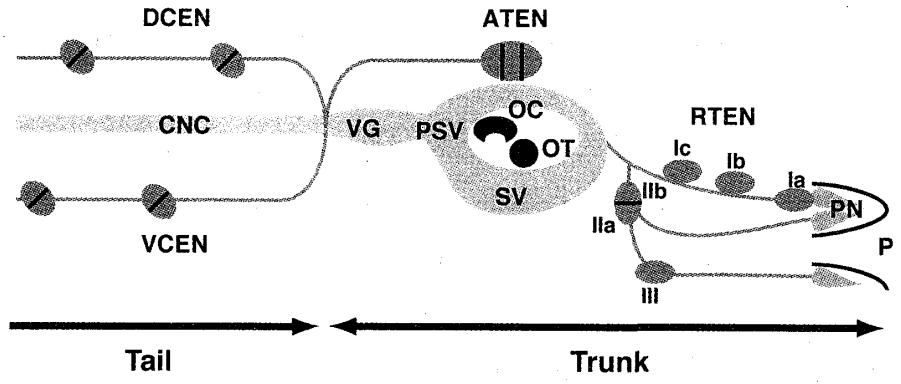

Fig. 1. Nervous networks in the larva seen from the right side.
Anterior is on the right. CNC: caudal nerve cord. DCEN: dorso-caudal epidermal neuron. OC: ocellus. OT: otolith. P: papillar. PN: papillar neuron. PSV: posterior sensory vesicle. ATEN: apical-trunk epidermal neuron. RTEN: rostral-trunk epidermal neuron. VCEN: ventro-caudal epidermal neuron. VG: visceral ganglion.

Nervous systems of larvae resorbing the tail

The changes in the larval nervous systems were observed during tail resorbtion with UA301 monoclonal antibody. In this period, adult organs arising from endoderm became gradually apparent (Fig. 2). The anterior part of the endoderm developed into the endostyle and gill sac, while the posterior part developed into the esophagus, stomach and intestine. The only endodermal region stained with this antibody was the heart primordium, located in the ventro-central part of the endoderm.

UA301 antibody reacted with most of the larval central nervous system, including the dorsal and posterior regions of the sensory vesicle and the visceral ganglion. The ventral and anterior regions of the sensory vesicle were not stained. The latter region has been called a neurohypophysis primordium (Fig. 2D, red arrowhead)[19]. As the adult cerebral ganglion was strongly stained with this antibody (data not shown), this region probably became the ciliated funnel which was located anterior to the neural gland[18] rather than the cerebral ganglion in adults. The origin of the neural gland was unclear because of no specific marker.

Additionally, the epidermal regions, which developed into the oral and atrial siphons, were stained with this antibody. One primordium of the oral siphon was located anterior to the apical region of the sensory vesicle. Two primordia of the atrial siphons were located in the right and left side of the epidermis. After metamorphosis, two atrial siphons moved to the dorsal side of the body and finally joined together as one siphon within a month. These stained structures in each siphon became the circular neurons around each siphon. They first appeared as a circular cell mass and then connected with the posterior sensory vesicle with axons. In adults, these axons form the major nerve fibers arising from the cerebral ganglion[18].

Changes in the nervous systems during metamorphosis

During metamorphosis, most of the peripheral nervous systems degraded (Fig. 3). Only a few neurons of RTEN (perhaps, type II neurons in Fig. 1) remained after metamorphosis (Fig. 2C, black arrowhead; Fig. 3D, white arrowhead). It is unclear whether these neurons still remain or not in adulthood. In this period, the caudal nerve cord as well as DCEN and VCEN resorbed into the posterior end of the trunk with tail debris. The staining of the visceral ganglion became weaker, while that of the dorsal and posterior regions of the sensory vesicle (Fig. 3A-C, white arrowheads) were still intense. These regions connected with the oral and atrial siphon primordium with axons (Fig. 3D). Although the visceral ganglion degraded, the thin nerve cord stained with UA301 antibody ran posteriorly from the posterior sensory vesicle. This nerve cord existed even after metamorphosis and ran toward the tail debris (Fig. 3F), and in older juveniles, was observed to connect with the gonad rudiment[12]. This probably becomes the nerve cord running along the dorsal strand in adults. Recently, it was reported that GnRH neurons exist in the dorsal strand as well as the cerebral ganglion and are

related to the induction of gamete release in adult *Ciona intestinalis*[20, 21]. This nerve cord may also be related to the development and maturation of the gonad.

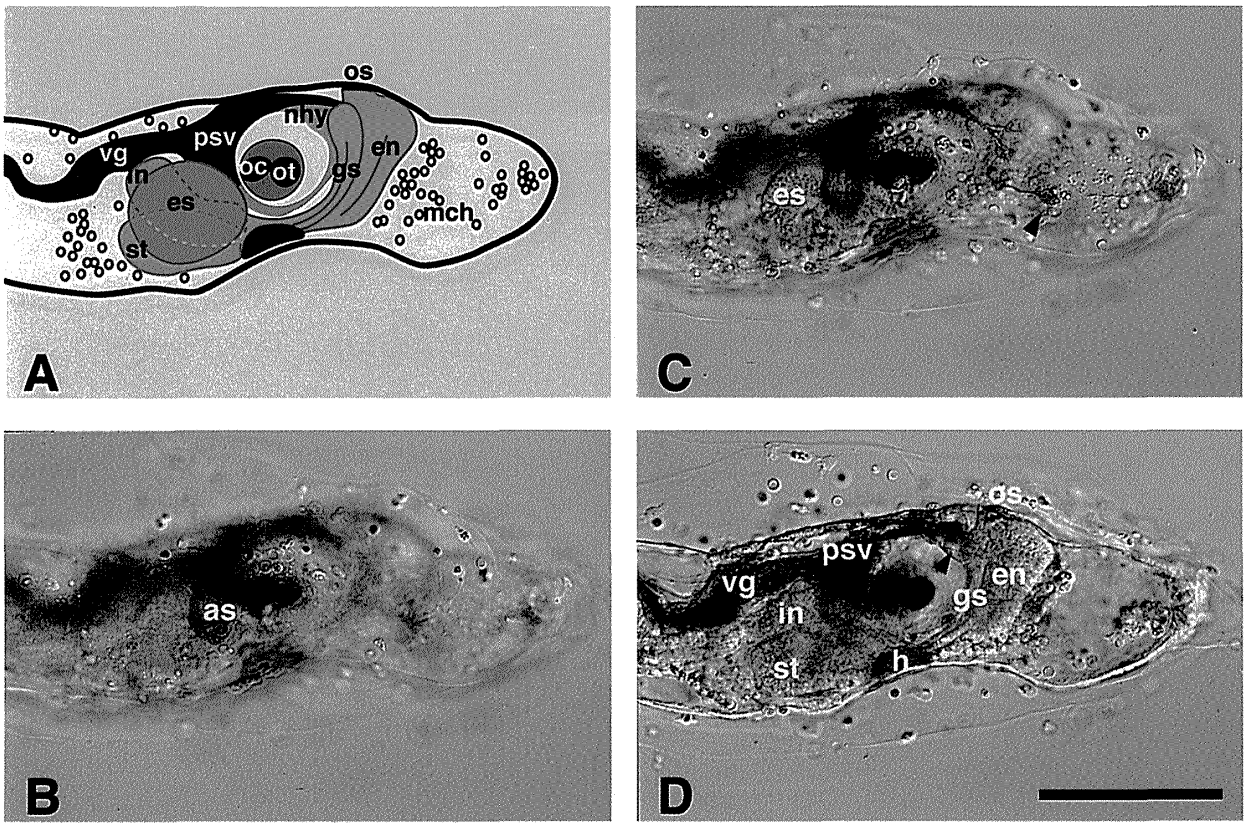

Fig. 2. Whole-mount immunostaining pattern of the larva resorbing the tail with UA301 monoclonal antibody. Scale bar is 100μm. Posterior is on the left. (A) Schematic diagram of larval organs and primordial juvenile organs. At this time, larval endoderm divide into several regions; endostyle (en), gill sac (gs), heart (h), intestine (in), esophagus (es) and stomach (st) primordia. Oral siphon primordium (os) originates from the dorso-apical region of the endoderm and the epidermis region connecting it. Larval sensory vesicle occupies the most central part of the trunk and is followed posteriorly by the posterior sensory vesicle (psv) and visceral ganglion (vg). Neurohypophysis (nhy) is located in the left-anterior part of the sensory vesicle. UA301-positive regions are indicated by red areas. oc; ocellus. ot; otolis. mch: mesenchymal cells. (B-D) The right view of UA301-immunostained larva. Focus plane was moved from the surface toward the inside. In (B), atrial siphon primordium (as) locating laterally posterior to the sensory vesicle was stained. In (C), several rostral-trunk epidermal neurons were stained. Black arrowhead indicates a pair cell of these neurons. Axons of these neuron connected with the dorso-apical region of the sensory vesicle posteriorly and papillae anteriorly. In (D), UA301 antibody reacted with most of the larval central nervous system, except for the ventral and a part of the dorso-apical region of the sensory vesicle. The latter region is a neurohypophysis primordium (red arrowhead). Additionally, UA301 staining was seen in the heart (h) and oral siphon (os) primordia.

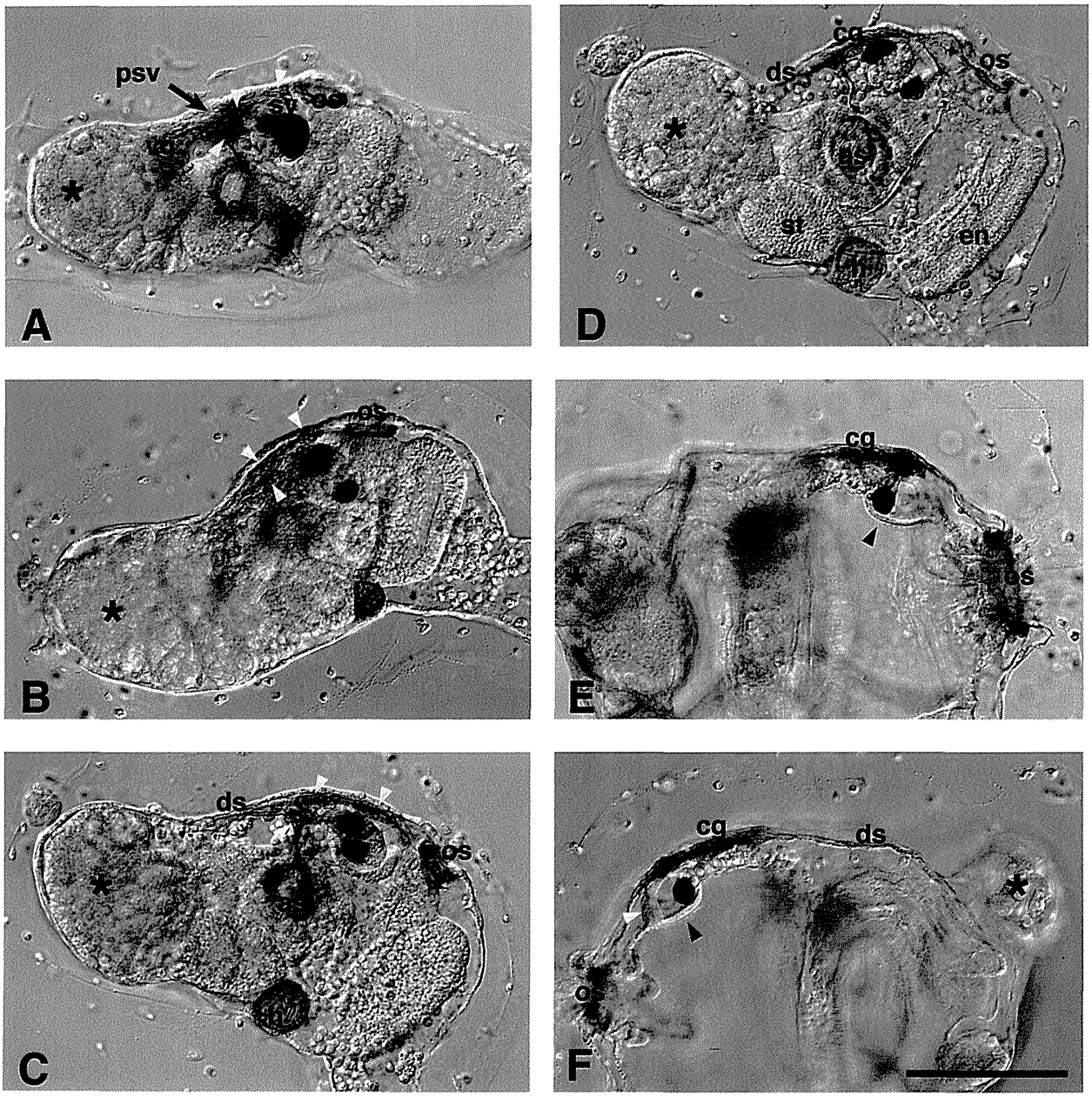

Fig. 3. Whole-mount immunostaining pattern during metamorphosis. Scale bar is 100μm. Posterior is on the left. In tail-resorbed individuals (A), the visceral ganglion (vg) was destroyed and decreased the reactivity against UA301 monoclonal antibody. On the other hand, the dorso-apical part of the sensory vesicle (sv), dorsal and ventral part of the posterior sensory vesicle (psv) still reacted intensely (white arrowheads). Their reactivity remained also during rotation of the body axis (B and C, white arrowheads). The axon from the oral siphon (os) and atrial siphon (as) primordium connected with the dorso-apical part of sv and the ventral part of psv, respectively. This indicates the anterior and posterior limit of juvenile neural ganglion, respectively (see text in detail). Three UA301-positive parts of CNS joined together, extended longitudinally and finally formed a juvenile cerebral ganglion (cg) (D). The posterior part of larval psv elongated antero-posteriorly during body axis rotation and formed a dorsal strand (ds) with connection to the tail debris in the just metamorphosed juveniles (E and F). In the left view of the juvenile (F), the funnel of the neural gland (white arrowhead) connected with the ciliated branchial epithelium (E and F, black arrowhead). * shows debris of the tail.

The heart primordium became more evident and separated from the other endodermal organs during metamorphosis. Even after metamorphosis, the sensory vesicle was distinguishable and two pigment cells remained in its lumen (fig. 3). However, because the connection between these cells and the sensory vesicle loosed, these cells floated in the lumen and, in some cases, drifted with coelomic cells throughout the body and finally disappeared. The anterior part of the sensory vesicle expanded (Fig. 3F, white arrowhead) and the ventral part became ciliated (Fig. 3E, F, black arrowheads). These structures probably become a ciliated funnel of the neural complex in adults (discussed later).

Origin of the adult neural complex

The adult neural complex consists of the cerebral (neural) ganglion and neural gland[18]. The cerebral ganglion is only one ganglion in the adult ascidian and has five main nerve fibers at each end, innervating the musculature in the surrounding mantle tissue. The neural gland lies ventrally to the ganglion in *Ciona intestinalis* and its anterior opening is called a "ciliated funnel". Some researchers[19, 22] have explained that the adult neural complex originates from the neurohypophisis. The neurohypophisis arises from the left side of the anterior end of the larval neural tube, while the right side gives rise to the sensory vesicle. During metamorphosis, the cerebral ganglion is formed by proliferation from the dorsal wall of the neurohypophisis. However, we propose another possibility for the origin of the neural complex. From the above observations, we suggest that the origin of the ciliated funnel is the left anterior wall of the sensory vesicle, the so called, "neurohypophisis" (Fig.3F, white arrowhead), although we have claimed that the adult cerebral ganglion arises from the posterior and dorsal region of the sensory vesicle, which remained and reacted intensely with UA301 antibody after metamorphosis (Fig. 3A-C, white arrowheads). As the UA301 antibody does not react to the adult neural gland, its origin is still unclear. However, it seems to be from a part of the PSV closely associated with the prospective cerebral ganglion. If true, there is very close link between the cerebral ganglion and neural gland in development.

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カタユウレイボヤ *Ciona intestinalis* の幼生から幼若体における神経系の変化

高村克美

神経系に特異的に反応するモノクローナル抗体 UA301 を用いて、カタユウレイボヤ *C. intestinalis* の変態に伴う神経系の変化を観察した。その結果、ほとんどの幼生神経系が変態中に崩壊するにもかかわらず、感覚胞後部の posterior sensory vesicle (PSV)は変態後もそのまま存続し、成体の脳神経節になることが明らかになった。一方で、今まで成体の脳神経節になるといわれてきたいわゆる"神経下垂体"は、むしろ神経腺前方に位置する繊毛を持った漏斗状器官になるものと思われる。神経腺自体の起源は今回は明らかにできなかった。