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論文題目	Development of serotonergic nervous system in sea urchin larvae （ウニ幼生セロトニン神経系の発生）
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論文内容の要旨

Acquisition of nervous system is one of the most characteristic events during evolution in both vertebrates and invertebrates. Despite still unsettled disputes of the precise phylogenetic position of echinodermata (Holland et al., 1991; Jefferies, 1991; Jefferies et al., 1996), it has been thought that the sea urchin embryo is an informative material to study how the nervous system of chordates has evolved (Lacalli et al., 1990; Hay-Schmidt, 2000; Zimmer, 2000), and previous molecular biological studies conducted based on the developmental regulatory genes supported that phylogenetically echinoderm locates at close position to chordates (Lowe and Wrey, 1997).

Sea urchin larvae develop several nervous systems, such as serotonergic, dopaminergic, and GABAergic ones (Bisgrove and Burke, 1986; 1987). Among them, serotonin cells firstly appear during embryogenesis and form a serotonergic apical ganglion (SAG) in the epithelium at the oral lobe (Bisgrove and Burke, 1986). However, some fundamental properties of SAG are still not well understood, such as; (1) where precisely it is present at the oral lobe epithelium, (2) where SAG-forming cells differentiate from, and (3) how different they are from the rest of the

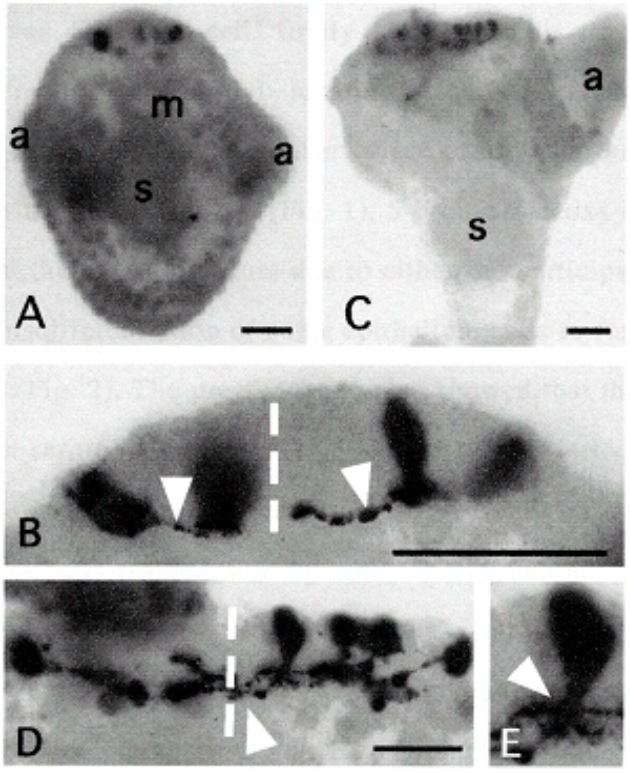

Fig. 1. Whole-mount immunohistochemistry of serotonergic apical ganglion (SAG) formation. Specimens were whole embryo-stained with anti-serotonin antibodies, and the image was reversed to show the serotonin cells better. A few serotonin cells were detected in the epithelium at the oral lobe in early prism larvae (A). These cells had a round cell contour with no visible cell processes. Early 2-arm plutei SAG cells had extended long axons with granular features on the basal side (B, arrowheads) toward the embryonic midline (white broken line). The number of SAG cells increased in 48-hour PF plutei (C). These serotonin cells extended longer axons toward the midline of the embryo (D, white broken line) on the basal side, and formed a neuro-plexus (D, arrowhead). Highly magnified photo of (D) shows several branched axons extended from a serotonin cell (E, arrowhead). a , m , and s in (A) and (C) show arm, mouth, and stomach, respectively. Bars, 20 μm (A-C); 10 μm (D); 5 μm (E).

ectodermal cells. Thus, initial half of the present study was designed to answer these questions by elucidating the cell surface property and the cell lineage of SAG, and the detailed developmental process whereby the ganglion is formed using immunochemical techniques. They are, however, based on immunohistochemistry of serotonin, a metabolic end product, and thus have little inner view of the gene activity that is expected to provide more accurate SAG differentiation process. The later half of this study was, thus, conducted by focusing on the molecular biological aspect of the differentiation of SAG and the serotonergic nervous system. It has been suggested that zinc finger proteins, such as Eagle and Huckbein contribute to the differentiation of serotonergic neurons in the ventral nerve cord of *Drosophila melanogaster* (Dittrich et al., 1997), and in mammals, Nkx family, a transcription factor, fibroblast growth factors (FGFs), and serotonin itself are needed for the development of serotonergic neurons in the central nervous system (CNS, e.g. Rubenstein, 1998). In sea urchin, involvement of the genes that have been known for CNS formation in other animals, such as *hedgehog*, *bone morphogenetic proteins* (*BMPs*), and *notch*, have been reported during embryogenesis (e.g. Sherwood and McClay, 1999; Angerer et al., 2000). However, they have never been considered in relation to the neurogenesis.

In the initial half of this study, development of serotonergic nervous system in sea urchin, *Hemicentrotus pulcherrimus*, was investigated with immunohistochemical techniques. Serotonin cells were initially detected by anti-serotonin antibodies in 36-hour post-fertilization (PF) prism larvae. Serotonin cells firstly detected at this stage were located randomly around the apical tuft, indicating the initial location of SAG cells was not precisely determined during early embryogenesis. The number of serotonin cells increased, and each of these cells extended axons during embryogenesis (Fig. 1). 5-bromo-2-deoxyuridine (BrdU) incorporation experiment showed that the increase occurs due to either the participation of presumptive serotonin cell group, or the transdifferentiation of some epithelium cells to form SAG from 36-hour PF until, at least, 72-hour PF (Fig. 2). The present study also showed that the SAG was comprised of the serotonin cells and non-serotonin cells that characteristically inserted between the serotonin cells (Fig. 1B). Usually one or two non-serotonin cells resided between two serotonin cells, and it was quite rare for the SAG cells resided side by side. The immunohistochemistry demonstrated that the occurrence of axon extension toward the embryonic midline, as was typically shown in Fig. 1B. This suggested that some axon guidance cues navigated the direction where SAG axons extend to during the early

period of the neuro-plexus formation. This process has been reported in axon fasciculation in the vertebrates (e.g. Marg et al., 1999). According to the observation using confocal microscopy, the three-dimensional image of SAG in 72-hour PF pluteus larvae was linear and forked into two branches at both ends. Double detection of serotonin and Epith-2, a lateral cell-surface specific marker of epithelium, showed that the serotonin cells had the protein on their cell surface, indicating SAG cells shared the same cell surface property as non-serotonin epithelial cells. However, unlike non-serotonin ectodermal cells Epith-2 was present also on the surface of axons extended from the basal surface of serotonin cells at pluteus stage, suggesting that serotonin cells had distinctive basal cell surface property from the other epithelial cells. Despite most of the cell-lineages in sea urchin embryos and larvae have been well characterized (Davidson et al., 1986), the neural cell-lineage including SAG cells has not been investigated yet. To answer the question where serotonin cells come from, the location of SAG was examined using anti-serotonin antibodies with oral (EctoV) and aboral (arylsulfatase; Ars) ectoderm- specific molecular markers. According to the double-labeled image with anti-serotonin and anti-Ars antibodies, the serotonin cell was apparently present at the anti-Ars antibody- positive ectodermal region, whereas the SAG cell was right on the edge of the anti-EctoV antibody-positive ectoderm, suggesting SAG was present at the aboral region adjacent to the ciliary band. Thus, these results strongly indicated that the origin of serotonin cells was aboral ectoderm, which

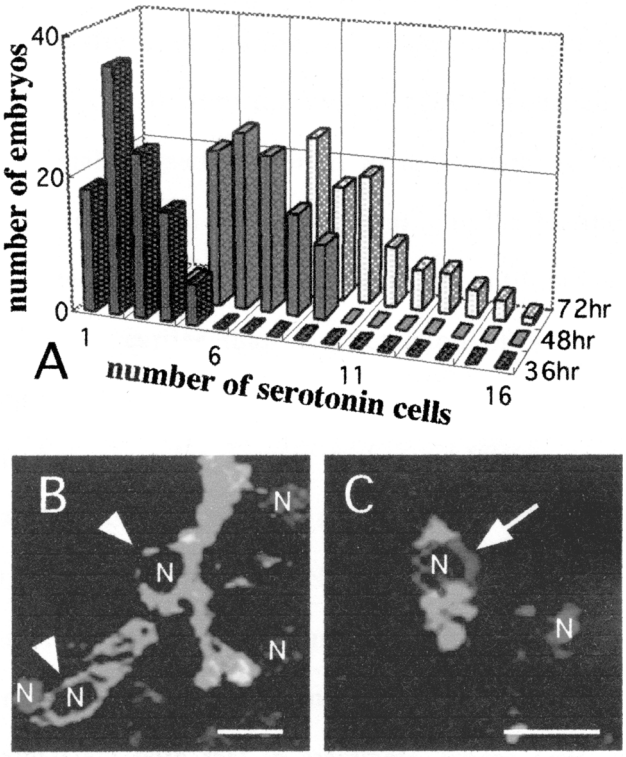

Fig. 2. **(A)** Histogram shows increasing number of serotonin cells examined in whole-mount immunohistochemistry samples. The abscissa shows the number of serotonin cells and the ordinate shows the number of larvae. Red, green, and yellow columns show 36, 48, and 72-hour PF larvae, respectively. (B) False-colored immunohistochemistry of 5-bromo-2-deoxyuridine (BrdU; red) in serotonin cells (green) incorporated for 12 hours from 36-hour PF to 48-hour PF. No positive signal was seen in nuclei (N) of serotonin cells (arrowheads). Non-serotonin cells, however, incorporated BrdU, as was shown with red nuclei (N). (C) A serotonin cell (green) incorporated BrdU (red) at the nucleus (arrow) in a larva incubated with BrdU soon after fertilization along with the nucleus (N) of non-serotonin cells (background red). Bars, 5 μm.

was contradictory to the observations previously reported. To elucidate the exact location of axons extended from the basal side of SAG cells, the observation around the oral lobe ectoderm was conducted using scanning electron microscope. According to the sample broken open at the oral lobe, the axons formed a plexus-like structure apparently on the ectodermal side of the basal lamina, which were consistent with previous report by transmission electron microscopy (Nakajima et al., 1993).

Next, molecular biological techniques were adopted to investigate the spatio-temporal expression of serotonin synthetase gene, *tryptophan 5-hydroxylase* (*TPH*; *HpTPH* in *Hemicentrotus pulcherrimus*), as a first neural-specific gene during sea urchin neurogenesis. BLAST search based on the amino acid sequence deduced from the cloned cDNA sequence of *HpTPH* showed high similarity to that in the animals already reported to date, such as 76% positive to that of chick, and 75% to that of human, rabbit, and mouse. According to the Northern hybridization, *HpTPH* expression begins to be detected in 34-hour PF late-gastrulae until, at least, through 72-hour PF pluteus larvae, which is a little earlier than the detection of serotonin accumulation. Whole-mount *in situ* hybridization combined with Tyramide Signal Amplification (TSA) system (T-WISH) of the gene showed that the expression pattern at the oral lobe region is quite similar to that of immunohistochemical localization of serotonin cells. *HpTPH* mRNA was detected exclusively in the cell body (Fig. 3B), implicating these mRNA were bound to the cell organelle, such as endoplasmic reticulum, as has been reported in mammal by Kim et al. (2002). Serotonin, on the contrary, spread in the entire cytoplasm of serotonin cell, including axons (Nakajima, 1986; Fig. 1). This observation is consistent with that in mammals, where *TPH* and TPH were expressed in a cell body (Kim et al., 2002), while TPH and serotonin were detected in the entire neuron (Chang et al., 2001). This indicates that the transcription and translation of TPH occur at the cell body, and yet only serotonin is transported into the axons. The location of TPH protein was not investigated in sea urchin larvae. According to the double-staining with serotonin immunohistochemistry and T-WISH of *HpTPH* conducted to examine whether all *HpTPH*- transcribed cells produce serotonin, a lower lip ganglion (LLG) contained serotonin-positive cells, but these cells did not express *HpTPH*. This observation indicated that serotonin is not produced at the LLG cells but only at the SAG cells (Fig. 3). This suggests that the LLG cells mainly accumulate the neurotransmitter by up-taking it secreted by SAG as the serotonin storage cells in mammals (Lebrand et al., 1996;

Galter et al., 1999) and the stomatogastric system of lobster (Richards et al., 2003).

By inhibiting serotonin synthesis with *p*-chlorophenylalanine (CPA), an irreversible inhibitor of TPH, or serotonin binding to the receptor with mianserin, a specific antagonist of serotonin receptor type-1D/2C, the larval swimming behavior was severely perturbed without any detectible deprivation of the developmental timing. However, the disturbed swimming behavior was not associated with the ciliary activity itself. In fact, the ciliary beating activity analysis conducted by visualizing the water current generated by the cilia on the larval surface indicated no significant difference between normal and serotonin-reduced larvae. Thus, serotonin is not important to maintain the ciliary beating activity, but is vital to regulate the normal swimming behavior that includes upward and downward swimming. This implicates the presence of the controlling system for serotonin-regulated swimming behavior. The embryos did not respond serotonin until the prism stage, whereas pluteus larvae did, implicating that the regulation system of swimming behavior switched from non-serotonergic to serotonergic at around the prism stage.

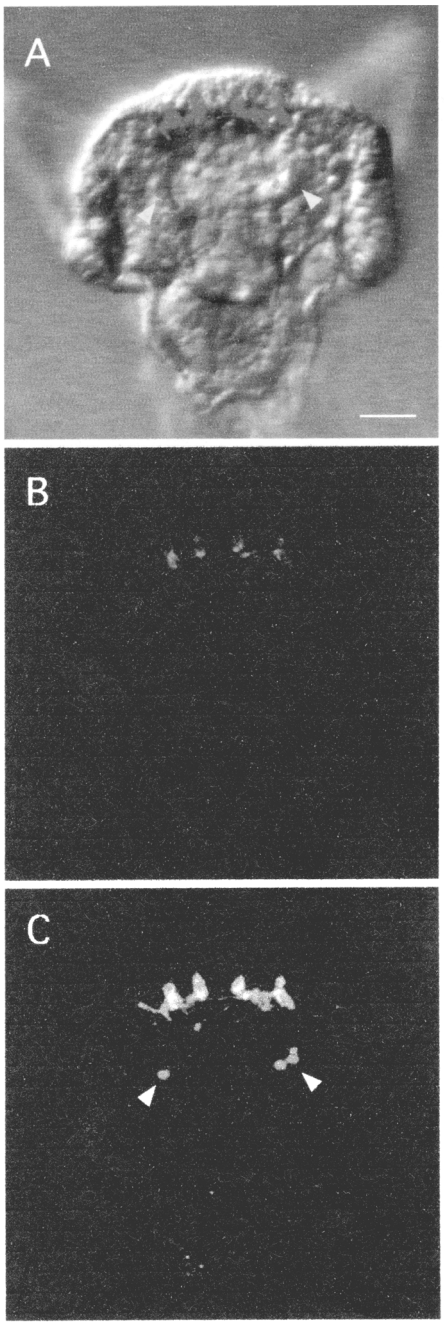

Fig. 3. Double detection of *HpTPH* and serotonin in a 72-h PF larva. (A) Immunofluoresence image of serotonin cells was combined with a phase-contrast image. (B) *HpTPH*-positive cells detected by T-WISH. (C) Merged images of (A) and (B). *HpTPH*-positive signals were detected only at the cell body of serotonergic apical ganglion (SAG) cells, but not in lower lip ganglion cells (yellow arrowheads). Bar shows 20 μm.

Serotonin receptor gene (*5-HThpr* in *H. pulcherrimus*) was cloned and sequenced to understand the signal transduction pathway of serotonergic nervous system. According to the deduced amino acid sequence, 5-HThpr is a seven-transmembrane protein that contains several glycosylation sites and G-protein coupling signature, and it phylogenetically locates at the close position to serotonin receptor 1A cluster. T-WISH of *5-HThpr* showed that the coelomic sacs, the gut, and the blastocoelic cells expressed the gene in the pluteus larvae. These tissues, however, were not directly involved in the swimming behavior, implicating that the serotonergic nervous system was indirectly contributing to the regulation of swimming behavior through the serotonin receptor-cells, such as blastocoelic cells, which are directly connected to the ectoderm. Genome project of sea urchin, *Strongylocentrotus purpuratus*, revealed other types of serotonin receptors, and strongly implicated that the serotonergic nervous system has several functions depending on the different types of the receptor as has been reported in vertebrate.

According to these immunohistochemical and molecular biological studies about the neurogenesis of sea urchin larvae, the SAG cells, which begin to transcribe *HpTPH* gene at late gastrula stage, comprise the linear-shaped ganglion around the apical tuft ectoderm. Serotonin from the SAG cells functions in the larval swimming behavior, but not in the ciliary beating itself. T-WISH of *5 HThpr* implicates that serotonin in sea urchin larvae has diverse functions.

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論文審査結果の要旨

1. 研究の目的:　神経系形成の分子的機構を系統進化的視点を交えながら棘皮動物のウニ幼生をモデルとして解明することを目的とした研究である。

2. 新しい知見:

(1) ウニ幼生において発生過程の最初に出現する神経系として知られるセロトニン神経系を構成する細胞はプルテウス幼生の反口側上皮組織由来であることを明らかにした。これは、従来詳細な解析なくして口側上皮由来としてきた見方を改めるものとなった。

(2) 海産無脊椎動物では初めてセロトニン合成酵素遺伝子を分離同定し、その全遺伝子構造を解明した。さらに、この構造解明により、ウニ幼生のセロトニン合成酵素は脊椎動物と無脊椎動物の中間に位置し、しかも脊椎動物の進化系統上にあることが推測された。

(3) 従来セロトニンの免疫組織化学観察からセロトニン神経節には頂部神経節と食道下神経節の２種類があると考えられていたが、今回セロトニン合成酵素遺伝子の *in situ* hybridization による解析から、食道下神経節はセロトニンの貯蔵組織である可能性が強く指摘された。これはウニ幼生においても脊椎動物に見られるセロトニン貯蔵機構があることを示している。

(4) セロトニンを受容するタンパクの存在を、セロトニン受容体遺伝子の分離・同定によって確定した。セロトニン受容体はラットのタイプ 1A に高い相同性を持ち、活性部位の分子構造は脊椎動物のそれと高い相同性を持っていた。

(5) セロトニン神経系の機能を幼生の遊泳運動によって解析した。その結果、幼生の遊泳器官である繊毛の運動は従来の知見とは異なり、セロトニンによって直接制御されているのではなく、繊毛運動の遊泳運動へと統合される介在神経系による機構が存在していることが明かとなった。これは、幼生の遊泳運動には複雑な制御機構が存在していることを示し、従来考えられてきた以上に高度に発達した神経系が棘皮動物幼生で出現していることを解明した。

(6) 以上のように、本研究は棘皮動物幼生神経系の機能機構に全く新しい認識を持たせるものともなった。

本論文の審査委員は全員一致で本研究の成果を高く評価し、谷口俊介君が自立して研究活動を行うに必要な高度の研究能力と学識を有することを示していることを認めた。

したがって, 谷口俊介提出の論文は, 博士（生命科学）の博士論文として合格と認める。