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Generation of Prolactin-like Neurons in the Dorsal Strand of Ascidians

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The adult ascidian neural complex forms from a thin tube called the neurohypophyseal duct and from the primordium of the cerebral ganglion from the sensory vesicle in metamorphosing larvae. Neurohypophyseal duct cells, located in the anterior left side of the sensory vesicle of swimming larvae, are derived from the anterior embryonic neural plate, which expresses common transcription factors in vertebrates and urochordates. The cerebral ganglion primordium is probably derived from the posterior sensory vesicle during metamorphosis. After metamorphosis begins, the duct elongates anteriorly and fuses with the stomodeal ectoderm, where the dorsal tubercle, a large ciliated structure that opens into the upper part of the pharynx, later develops. The rudiment of the cerebral ganglion and the duct elongate posteriorly. The duct also differentiates into the neural gland. The dorsal wall of the neural gland in adult ascidians has a thick epithelium (placode), the central part of which forms the dorsal strand by repeated invaginations along the visceral nerve. Both gonadotropin-releasing hormone (GnRH) neurons and prolactin-like (non-GnRH) neurons are generated in the dorsal strand and migrate to the cerebral ganglion along the visceral nerve throughout adulthood. Thus, the epithelium derived from the neurohypophyseal duct possesses neurogenic potential to generate neural stem cells of the central (cerebral ganglion) and peripheral (dorsal strand) nervous systems. The generation of prolactin-like neurons and their migration into the brain with GnRH neurons suggest that the ascidian dorsal strand is homologous to the craniate olfactory placode, and provide unequivocal support for the existence of the clade Olfactores.

Key words: ascidian, PRL-like neuron, neurogenesis, olfactory placode, dorsal strand, chordate, Olfactores

INTRODUCTION

The evolutionary origin of neurogenic placodes remains controversial due to morphological divergence in the chordate subphyla Urochordata, Cephalochordata, and Vertebrata. Despite the importance of neurogenic placodes for understanding phylogenetic relationships among chordates, morphological and developmental data regarding their evolutionary origin remain scarce. In craniates, peripheral gonadotropin-releasing hormone (GnRH) neurons arise from the olfactory placode, whose cells are derived from the anterior region of the embryonic neural plate (Okubo et al., 2006; Cariboni et al., 2007; Schwarting et al., 2007; Bhattacharyya and Bronner-Fraser, 2008; Chen et al., 2009; Kanaho et al., 2009). Generation of peripheral neurons is a unique phenomenon that does not conform to the central origin of most neurons. The possible presence of a neurogenic placodal structure in invertebrate chordates has long been debated (Manni et al., 2004, 2005, 2006; Mackie and Burighel, 2005; Mazet et al., 2005, 2006; Schlosser, 2005). Recently, it was directly shown that GnRH neurons are generated in the dorsal strand and migrate into the cerebral ganglion (Terakado, 2009). It was hypothesized that the dorsal strand is homologous to the craniate olfactory placode, an idea that is based on the generation of peripheral GnRH neurons, which are commonly derived from the anterior region of the embryonic neural plate (Satoh, 1994; Cole and Meinertzhagen, 2001, 2004). It has been suggested that the anterior region of the embryonic neural plate is the territory of the olfactory/adenohypophyseal placodes of vertebrates, and that it expresses certain transcription factors common in craniates. Similarly, prolactin (PRL)-like neurons are generated in the dorsal strand, which is formed from the dorsal epithelium of the neural gland by repeated invaginations (Fig. 1A).

Molecular phylogenetic analyses have suggested that urochordates are the closest living relatives of vertebrates (Blair and Hedges, 2005; Delsuc et al., 2006, 2008; Putnum et al., 2008). It is therefore likely that developmental novelties of chordates (neural crest and placode) arose during evolution of the common ancestor of urochordates and vertebrates.

In cephalochordates (amphioxus), the similarity to vertebrates during embryonic development and in the expression of certain common transcription factors in the anterior region suggests the possible presence of a placode(s) that is homologous with those in craniates (Gorbman, 1995; Yasui

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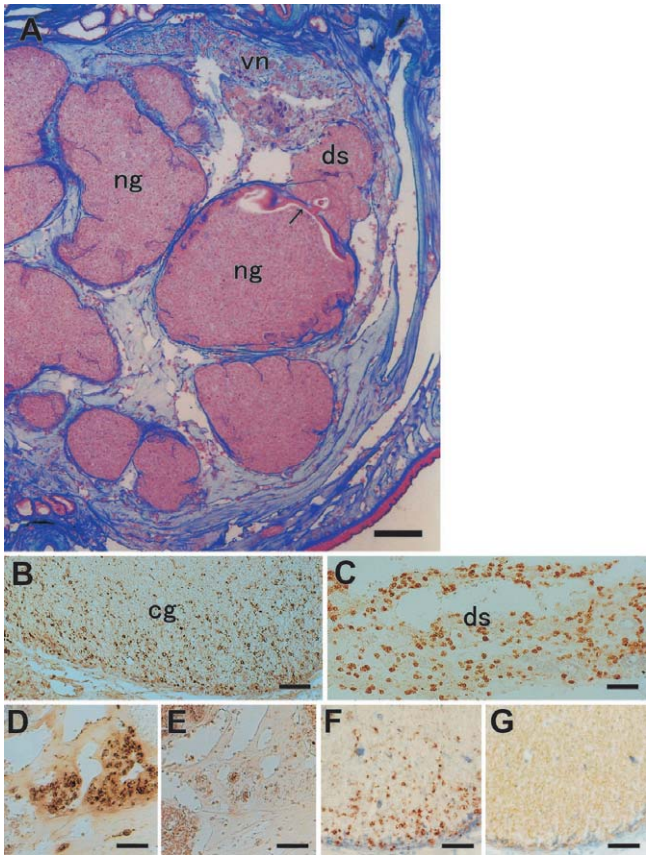

Fig. 1. Formation of the dorsal strand, localization of PRL-like neurons and controls. **A.** Formation of the dorsal strand (ds) from the placode (arrow) in the dorsal epithelium of the neural gland (ng) by invagination facing the visceral nerve (vn). Aldehyde fuchsin stain. **B** and **C**. Occurrence of PRL-like neurons in the cerebral ganglion (cg) and the dorsal strand (ds). PRL-like neurons in the cerebral ganglion are located mostly in the cortical region and possess long neurites, while neurons in the dorsal strand possess very short or lack neurites. Anti–bullfrog PRL stain. **D** and **E**. Specificity of GnRH immunoreactivity. Anti-human GnRH reactivity (D) is completely abolished by treatment with antiserum (E) that had been preabsorbed with the same antigen (2 μg/ml). **F** and **G**. Specificity of PRL-like immunoreactivity. Anti–bullfrog PRL reactivity (F) is completely abolished by treatment with antiserum (G) that had been preabsorbed with the same antigen (10 μg/ml). Bars: (A) 100 μm; (B–G) 50 μm.

et al., 2000; Boorman and Shimeld, 2002). However, the proposed placode generates neither neuroendocrine cells that give rise to GnRH neurons nor endocrine cells that give rise to adenohypophyseal endocrine cells. Thus, the first chordate may have lacked placodes (Meulemans and Bronner-Fraser, 2007). These observations provide unequivocal support for the existence of the clade Olfactores (tunicates + vertebrates).

Several neuronal populations are generated from the olfactory placode in vertebrates. Recent results have demonstrated certain morphological, developmental (Burighel et al., 1998; Manni et al., 2004; Terakado, 2009), and molecular (such as *Six*, *Pitx*, *Eya*, *Pax*, *Coe*, *Dach*, *POUIV* gene families) commonalities between urochordates and vertebrates (Bassham and Postlethwait, 2005; Boorman and Shimeld, 2002; Christiaen et al., 2002; Mazet et al., 2005; Mazet and Shimeld, 2005; Schlosser, 2005), but information on the localization on these transcription factors and on the sites of emergence of neuroendocrine/endocrine cells remains controversial. The aim of this study was to describe the morphological features of PRL-like non-GnRH neurons that are generated in the dorsal strand, and to provide the morphological bases for further cellular and molecular studies.

MATERIAL AND METHODS

Animals

Halocynthia roretzi larvae (3 days after hatching) and metamorphosed larvae (2 weeks after hatching) were reared in plastic Petri dishes in a refrigerator in which the temperature was nearly the same as natural seawater (10–11°C). Some of the swimming and metamorphosing larvae were supplied by Dr. Hiroki Nishida (Osaka University). Adult specimens (approximately 2- to 5-month-old juveniles and 3-year-old mature individuals) were cultured in natural seawater in Mutsu Bay in Aomori City, Japan, and purchased from a fisherman. They were stored in a water tank before use.

Histochemical staining

Swimming and metamorphosed larvae that were kept in filtered seawater were fixed in seawater Bouin's fluid for 12 hrs, and then stored in 70% ethanol. Neural complexes from juveniles and mature individuals were processed similarly. Tissue was embedded in Paraplast (Fisher Scientific Company, USA) and sectioned at 6 μm thick. Rehydrated sections were stained with Mayer's hematoxylin (Wako) and eosin (Merck). Adult neural complexes were also stained with aldehyde fuchsin.

Immunocytochemistry

Immunocytochemistry was performed as reported (Terakado et al., 1997). Briefly, adult neural complexes were fixed for 12 hrs in a 1:1 mixture of 0.2 M cacodylate buffer, pH 7.2, containing 8% paraformaldehyde (Merck) and saturated picric acid aqueous solution. Sections (6 μm thick) were mounted on gelatin-coated slides (Matsunami, Japan) and dewaxed, and endogenous peroxidase activity was blocked by treatment with 0.5% H_2O_2 in 70% methanol for 40 min. Sections were then treated with 2% normal swine serum (Dako, Kopenhagen, Denmark) to reduce nonspecific staining. The primary antiserum (rabbit polyclonal against bullfrog PRL; Yamamoto and Kikuyama, 1982) was diluted 1:2000 in 0.01 M phosphate-buffered saline, pH 7.5 (Sigma, Missouri, USA) containing 0.1% bovine serum albumin (Sigma), and applied to the sections overnight at 4°C. Detection was carried out using the ABC (streptavidin-biotin-peroxidase) method. Biotinylated swine anti–rabbit immunoglobulin (Dako) and peroxidase-conjugated streptavidin (Dako) were applied sequentially for 1 hr each. Immunoreactivity was visualized by exposing the sections to 3, 3'-diaminobenzidine (WAKO) containing 0.006% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.5, for 3–10 min. Control staining was carried out by incubating sections overnight at 4°C in primary antiserum (1:1000, 1 ml) that had been preabsorbed with the same antigen (10 μg). As an additional control, anti-human GnRH (SAN) antiserum (1:1000) was preabsorbed with the same antigen (2 μg/ml). Additional sections were immunostained with swine ACTH primary antiserum (Tanaka and Kurosumi, 1986) to illustrate the intimate topological relationships between the neural gland, the dorsal strand, and the visceral nerve.

Conventional electron microscopy

The neural complexes (from mostly 3-year-old individuals) were dissected into small pieces and fixed for 4 hrs with 3% glutaraldehyde (TAAB Laboratories, Equipment, UK) in 0.2 M cacodylate buffer, pH 7.2, containing 2% tannic acid (WAKO) and 5 mM calcium chloride. Tissue sections were washed overnight at 4°C in 0.1 M cacodylate buffer, pH 7.2, post-fixed for 1 hr with 2% osmium

tetroxide (Merck) in the same buffer, dehydrated, and embedded in Epon-Araldite (TAAB). Ultrathin sections (6–7 nm thick) were collected, mounted on copper grids, double-stained with aqueous uranyl acetate (TAAB) and lead citrate (Sato and Shamoto, 1973), and examined with a Hitachi H700H electron microscope operated at 100 kV.

Immunoelectron microscopy

Neural complexes were cut into small pieces and fixed for 4 hrs at 4°C in 0.2 M sodium phosphate buffer, pH 7.4, containing 4% paraformaldehyde (Merck) and 0.5% glutaraldehyde (TAAB). Tissue was washed overnight at 4°C in phosphate-buffered saline, post-fixed for 1 hr at 4°C with 1% osmium tetroxide in 0.15 M phosphate buffer, dehydrated, and embedded in Epon-Araldite. Ultrathin sections (approximately 8 nm thick) were collected and mounted on nickel grids (Nisshin EM, Tokyo) coated with Formvar (TAAB) that had been stored in a refrigerator at –20°C in order to ensure the adhesion of ultrathin sections, and treated with 1% meta-periodic acid for 10–30 min. An antiserum to bullfrog PRL (1:120) was used as the primary antibody. The sections were then treated with gold-labeled (10 nm) secondary antibody (1:20; British BioCell International, Cardiff, UK) for 1 hr, washed, double-stained lightly with aqueous uranyl acetate and lead citrate, and examined with a Hitachi H700H electron microscope operated at 100 kV.

RESULTS

Specificity of immunoreactivity for PRL-like or GnRH neurons

Immunocytochemistry using anti–bullfrog PRL revealed numerous PRL-like neurons in the dorsal strand and the cerebral ganglion (Terakado et al., 1997; Figs. 1B, C). In control sections using preabsorbed antibody, immunoreactivity was undetectable (compare Figs. 1F and G). Using immunoelectron microscopy in control sections with the same preabsorbed antibody, no gold particles were observed in any neurons or endocrine cells (data not shown). Because GnRH and PRL-like neurons are often localized side by side, the specificity of the GnRH antiserum for the dorsal strand or the cerebral ganglion was also examined. We observed no GnRH immunoreactivity when sections were stained with preabsorbed GnRH antiserum (compare Figs. 1D and 1E).

Development of the neurohypophyseal duct

The neurohypophyseal duct is a thin duct located on the anterior left side of the sensory vesicle of larvae, and remains a duct-like structure until the onset of metamorphosis (Figs. 2A, B, C; Satoh, 1994; Cole and Meinertzhagen, 2001, 2004; Manni et al., 2005). In metamorphosed juveniles, the rudiment of the cerebral ganglion appears as a small mass of cells on the dorsal side of the preexisting neurohypophyseal duct (Figs. 2D, E, F). Brain development proceeds slowly in *H. roretzi*, and thickening of the epidermis (placode formation) and delamination/migration of pioneer cells into the brain are not discernible with light microscopy (Figs. 2D, E, F). The neurohypophyseal duct elongates anteriorly and fuses with the stomodeal ectoderm (Fig. 2E), later forming the dorsal tubercle. The cerebral ganglion elongates posteriorly to form a thin, long ganglion in young juveniles (Fig. 2G).

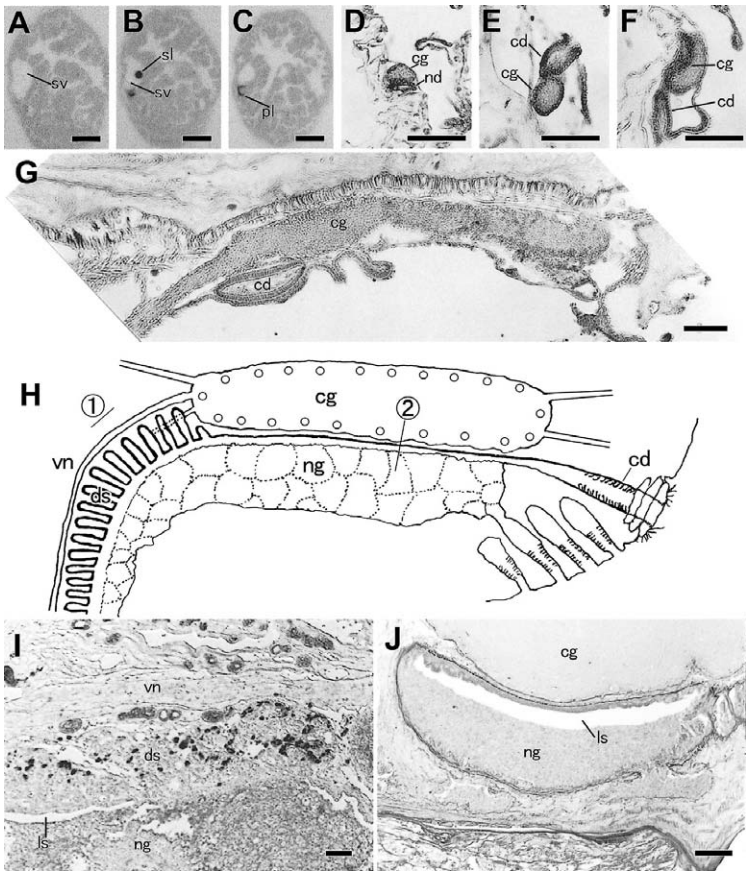

Fig. 2. Development of the neural complex and the topological relationships of the dorsal strand. **A, B, C**. Consecutive sections through the sensory vesicle (sv) of a late swimming larva. The statolith (sl) and photolith (pl) are visible within the sensory vesicle (B). Hematoxylin/eosin stain. **D, E, F**. Early stages of neural complex formation. The rudiment of the cerebral ganglion (cg) appears on the dorsal side of the neurohypophyseal duct (nd), attaching closely to it (D, anterior is bottom). The neurohypophyseal duct elongates to fuse with the stomodial epithelium (E, anterior is top. cd; ciliated duct). The cerebral ganglion begins to elongate posteriorly (F, anterior is bottom). Hematoxylin/eosin stain. **G**. The cerebral ganglion in a 5-mm juvenile. The cerebral ganglion (cg) elongates further posteriorly to form a thin, long structure. The nerve fibers run along the long axis. The ciliated duct (cd) is seen in the anterior ventral side (anterior to the left). Hematoxylin stain. **H**. Schematic representation of the adult neural complex. The epithelium of the dorsal strand (ds) is continuous to the ciliated duct (cd) through the dorsal region of the neural gland (ng, anterior to the right. vn; visceral nerve). **I**. Longitudinal section along the neural gland (ng)–dorsal strand (ds)–visceral nerve (vn) axis at the position indicated by ① in panel H. The dorsal strand (ds) is closely associated with the visceral nerve (vn). The luminal space (ls) of the neural gland lies just under the dorsal epithelium. Dense cells in the dorsal strand are ACTH-positive cells. Anti–swine ACTH stain. **J**. Transverse section at the position indicated by ② in panel **H**. The cerebral ganglion (cg) is separated by the dorsal epithelium of the neural gland (ng). The luminal space (ls) lies in the most dorsal region of the neural gland. Aldehyde fuchsin stain. Bars: 50 μm.

Neural complex

The adult neural complex of *H. roretzi* is schematically described, with special reference to the continuation of the epithelium of dorsal strand (ds, Fig. 2H). The most anterior

part is the dorsal tubercle, which opens into the upper part of the pharyngeal cavity, where it presents as a screw-like structure in which cilia on the outer surface move towards the pharyngeal cavity. In contrast, cilia on the inner surface move towards the interior of the body. Next to the dorsal tubercle towards the inside is the ciliated duct, in which cells are elongated. The ciliated duct contains both young cells, as judged by their small size and high nucleus/cytoplasm ratio, and degenerating cells, as judged by disintegrating organelles. Cilia are embedded in prominent microvilli, and are arranged obliquely to point towards the interior. The region between the ciliated duct and the anterior part of the neural gland body is non-ciliated and has an exocrine function; some large granules (0.3–0.8 μm in diameter) lie on the apical side and are often exposed to the lumen (Terakado et al., 1997). In the neural gland region, the epithelial structure is apparent in the dorsal side that faces the cerebral ganglion or the visceral nerve, but is indistinct in other regions. The luminal spaces that are continuous through the ciliated duct, the neural gland, and the dorsal strand terminate at the tips of the tubular structures of the dorsal strand (Figs. 2H, I, J).

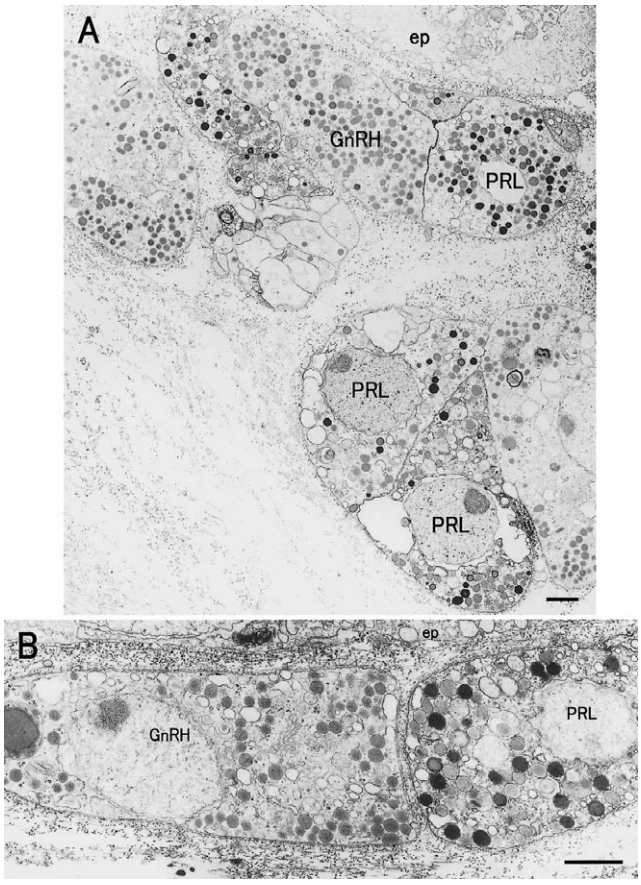

Fig. 3. Electron micrographs of parts of the dorsal strand. (**A**). Many young and developing GnRH (GnRH) and PRL-like (PRL) neurons are localized beside the epithelium (ep) of the dorsal strand. (**B**). Two types of neurons in the dorsal strand. PRL-like neurons possess dense and moderately dense secretory granules, while GnRH neurons contain similar, moderately dense granules. Uranyl acetate-lead citrate double stain. Bars: 1 μm.

Other than the dorsal epithelial cells, the cells of the neural gland are mostly, if not entirely, binucleate and loosely connected, and have no secretory granules. The neural gland elongates posteriorly along the visceral nerve and forms the dorsal strand from the dorsal epithelium by invagination towards the visceral nerve (Fig. 1A). There is an intimate topological relationship between the neural gland, the dorsal strand, and the visceral nerve (Figs. 2H, I). Neuroendocrine cells containing GnRH and non-GnRH (PRL-like) neurons are localized in the dorsal strand and the cerebral ganglion.

Neurogenesis in the neural complex

Although ascidian neural complex contains several organs and distinct regions (cerebral ganglion, neural gland, dorsal strand, ciliated duct, and non-ciliated duct), neurogenesis occurs in the cerebral ganglion and the dorsal strand. The latter is probably the exclusive site of peripheral neurogenesis under normal conditions. Using immunostain-

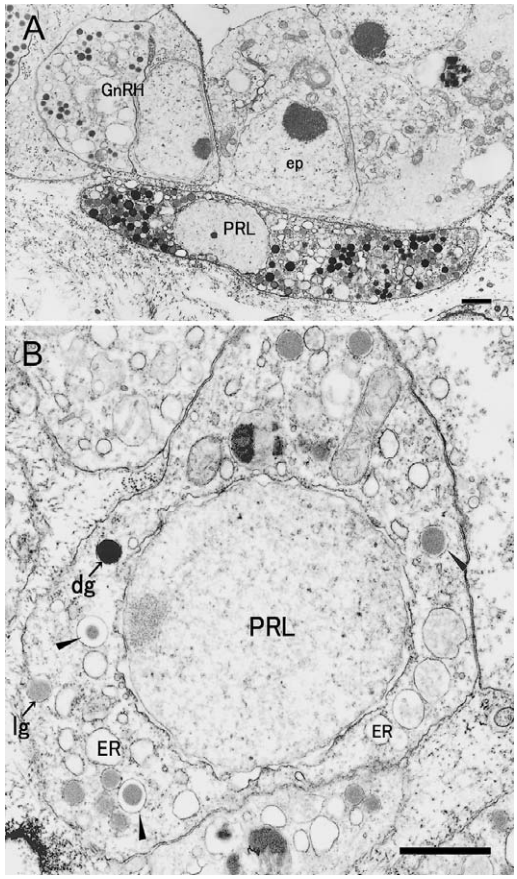

Fig. 4. Localization and ultrastructural features of young PRL-like neurons. (**A**). PRL-like neurons (PRL) with dense and moderately dense granules are often located beside the epithelium (ep). Growing GnRH neurons (GnRH) are seen in the epithelium of the dorsal strand. (**B**). A young PRL-like neuron is shown possessing a few dense (dg) and moderately dense (lg) granules (arrows) that are membrane-bound. Developing granules (arrowheads), which are centrally condensed, were often observed. Rough endoplasmic reticulum (ER) is distended in several places, which is indicative of extensive protein synthesis. Uranyl acetate-lead citrate double stain. Bars: 1 μm.

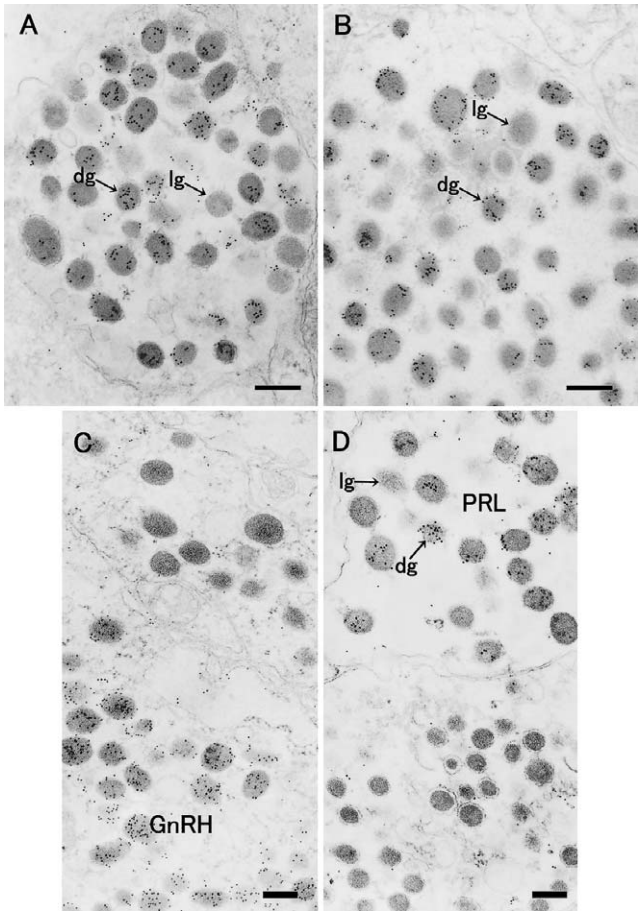

Fig. 5. Distinction of PRL-like neurons from GnRH neurons. **A** and **B**. Immunoelectron micrographs of PRL-like neurons in the cerebral ganglion (A) and the dorsal strand (**B**). Gold particles are localized on the dense granules (dg) in both neurons. Gold particles are not localized in the moderately dense granules (lg). Anti–bullfrog PRL labeling. **C** and **D**. Comparison of anti-GnRH and anti-PRL immunoreactivity between GnRH neuron and probable PRL-like neuron, and between PRL-like neuron and probable GnRH neuron. **(C)**. GnRH immunoreactivity in the cerebral ganglion reveals that granules in one cell type are GnRH immunopositive (bottom), whereas those in the other cell type are GnRH immunonegative (top). **(D)**. PRL-like immunoreactivity in the cerebral ganglion reveals that granules in the cell on the top are PRL-like immunopositive, whereas those of the cell on the bottom are immunonegative. Bars: (**A, B**) 250 nm; (**C, D**) 500 nm.

ing with anti–bullfrog PRL, we observed PRL-like cells along the dorsal strand and in the cerebral ganglion (Terakado et al., 1997). Using electron microscopy, two types of morphologically distinct neurons that occur side by side were discernible (Fig. 3A). In contrast to the generation of GnRH neurons, which occurs both within and adjacent to the epithelium (Terakado, 2009), PRL-like neurons were primarily generated adjacent to the epithelium (Figs. 3A, B, 4A). GnRH neurons contained a single kind of moderately dense secretory granules, while PRL-like neurons contained both very dense and moderately dense granules of similar diameter (Figs. 3A, B). Young PRL-like neurons, as judged by a high nucleus/cytoplasm ratio and the presence of a few secretory granules, were frequently found within cell masses

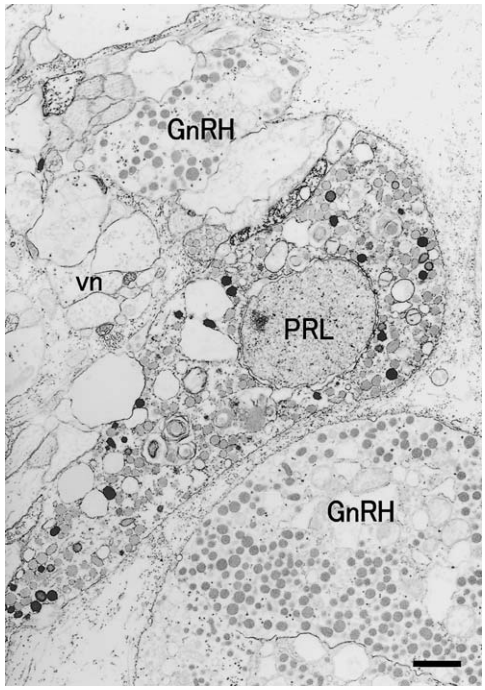

Fig. 6. Contact region between the dorsal strand and the visceral nerve (vn). GnRH neurons and PRL-like neurons are often attached to the nerve, elongated along the nerve fibers, and distributed continuously towards the cerebral ganglion. Uranyl acetate-lead citrate double stain. Bar: 1 μm.

lying beside the epithelium (Fig. 4B). Using immunoelectron microscopy, PRL-immunoreactive material was detected in dense granules, but not in moderately dense ones (compare Figs. 5A, B). Granules of one cell were often immunopositive for GnRH, whereas those of a neighboring cell were immunonegative (Fig. 5C). Similarly, granules of one cell were frequently immunopositive for PRL, whereas those of a neighboring cell were immunonegative (Fig. 5D). Most notably, GnRH and PRL immunoreactivities were mutually exclusive, suggesting that these neurons are distinct.

Migration along the visceral nerve

Abundant PRL-like neurons are distributed along the dorsal strand (Fig. 1C), and it is evident that they are generated in this tissue. PRL-like neurons were also observed among the fibers of the visceral nerve (Fig. 6), in addition to GnRH neurons. Unattached PRL-like neurons were numerous and were located also near the visceral nerve (Fig. 6. bottom), suggesting their invasion of the bundle of visceral nerve fibers and migration into the brain.

DISCUSSION

Occurrence of PRL-like (non-GnRH) neurons in the dorsal strand

The dorsal strand of *H. roretzi* is generated by repeated invaginations of the dorsal strand placode, and it produces GnRH neurons, some of which migrate into the cerebral ganglion through the visceral nerve (Terakado, 2009). Cells derived from the anterior region of the embryonic neural plate and their topological relationships suggest striking similarities between the dorsal strand and the olfactory placode,

which suggests that the dorsal strand of urochordate ascidians is homologous to the olfactory placode of vertebrates (Terakado, 2009). Because the ascidian dorsal strand is a single organ, this notion is compatible with the formation of a single olfactory placode in agnathans (Uchida et al., 2003). The dorsal strand also generates many PRL-like (non-GnRH) neurons (Figs. 1C, 3A, B), a finding that has been reported in other species. Anti–salmon PRL also stains some cells of the dorsal strand in *H. roretzi* (unpublished observation). The presence of PRL-like cells has been reported in the cerebral ganglion of *Ciona* (Fritsch et al., 1982) and *Styela* (Pestalino, 1983), and in the brain of vertebrates (Fuxe et al., 1977; Krieger and Liotta, 1979; Toubeau et al., 1979; Hansen and Hansen, 1982). We previously suggested that these PRL-immunoreactive neurons in the vertebrate brain may be homologous to those in the cerebral ganglion of ascidians (Terakado et al., 1997). It is well known that molecular features of prolactin in the adenohypophysis resemble those of vertebrate growth hormones (Kawauchi and Sower, 2006). Immunoreactivity of some neurons of the cerebral ganglion and some cells of the dorsal strand to anti-PRL and anti–teleost growth hormone antisera (unpublished observation) suggests the presence of ancestral molecule(s) of the growth hormone family in ascidians. Even the presence of a single molecule in ascidians raises the possibility that multiple antisera raised against molecules belonging to the growth hormone family react to ascidian prolactin due to the presence of common epitopes. Additional information is needed.

Most neurons are generated in the central nervous system in vertebrates and invertebrates. However, the vertebrate olfactory placode (peripheral organ) commonly generates GnRH neurons as well as other non-GnRH neurons (Murakami and Arai, 1994; Hilal et al., 1996; Yamamoto et al., 1996). The reason(s) why the GnRH neurons originate peripherally in vertebrates and ascidians is unknown; however, it is evident that GnRH neurons generated in peripheral organs are crucial for reproduction in vertebrates and ascidians, making this event a characteristic feature of the clade Olfactores (tunicates + vertebrates).

PRL-like neurons in both the dorsal strand and the brain contain very dense and moderately dense secretory granules that are similar in size to those in GnRH neurons (Figs. 3A, B). It will be interesting to determine whether PRL-like neurons also contain GnRH peptides in addition to PRL-like material. Our immunological data suggest that PRL-like neurons do not contain GnRH peptides and vice versa; that is, they may be mutually distinct neurons. Our data also suggest that PRL-like material is contained in dense granules. The material in the moderately dense granules of the PRL-like neurons remains to be identified.

Migration of PRL-like neurons to the brain

The observation that granulated PRL-like neurons are often found among the fibers of the visceral nerve (Fig. 6) suggests that they migrate towards the cerebral ganglion, similar to GnRH neurons (Terakado, 2009). The PRL-like neurons in the dorsal strand are mostly oval, whereas those in the cerebral ganglion have long neurites (compare Figs. 1B and C). This suggests that PRL-like neurons elongate after entering the brain. The intimate morphological relationship between the dorsal strand and the visceral nerve (Figs. 2H, I) has long been emphasized (see review by Goodbody, 1974; Chiba et al., 2004). In *H. roretzi*, this relationship may now be explained by the possibility that GnRH and PRL-like neurons generated in the dorsal strand invade the bundles of visceral nerve fibers and migrate towards the brain. This close topological relationship may be very important for invasion and migration from the site of generation (the dorsal strand) to the cerebral ganglion. This morphological relationship may partly explain the observation that the brain of *H. roretzi* easily reverts from a thin, cord-like structure (after breeding season, in winter) to the normal brain shape via migration of neurons from the dorsal strand. Similar phenomena are observed during regeneration of other brain structures; for example, after extirpation of the brain in *C. intestinalis*, the regenerating brain contains GnRH neurons, suggesting that GnRH neurons originate in the dorsal strand and subsequently migrate into the regenerating brain (Bollner et al., 1997). Together with the previous demonstration that GnRH neurons are generated in the dorsal strand and migrate into the brain (Terakado, 2009), the current results reveal that PRL-like neurons are also generated in the dorsal strand and migrate into the brain during normal development and maintenance of brain function throughout the life of the organism. Even in colonial ascidians, dorsal strand cells divide frequently and become incorporated directly into the cerebral ganglion (Koyama, 2002).

Formation of the neural complex

The neurohypophyseal duct is thought to be a rudiment of the adult neural complex (Willey, 1893). It is clear that the neurohypophyseal duct is derived from the anterior region of the embryonic neural plate (Nishida, 1987; Satoh, 1994; Cole and Meinertzhagen, 2001, 2004). However, Takamura (2002) used a monoclonal antibody against to the ovary homogenate of *C. intestinalis* and proposed that a posterior sensory vesicle remained throughout metamorphosis and became an adult cerebral ganglion. The neurohypophyseal duct, by contrast, becomes a ciliated funnel of the neural gland (Takamura, 2002). The origin of the neural gland is unclear because of the absence of a specific marker. Our observations clearly revealed that the epithelium of the ciliated duct is continuous with the dorsal epithelium of the neural gland and further with that of the dorsal strand. Therefore, it is evident that the ciliated funnel (ciliated duct)–neural gland–dorsal strand system is a single entity that is derived from the neurohypophyseal duct. The rudiment of the cerebral ganglion in *H. roretzi* appears immediately on the dorsal side of the neurohypophyseal duct of metamorphosed larvae. Delamination/migration of cells from the neurohypophyseal duct to the rudiment of the cerebral ganglion was not ascertained in the present study. The above results suggest that the adult neural complex may be formed from multiple origins and that its components may develop separately at an early phase (before migration of neurons from the dorsal strand), although the rudiments of the cerebral ganglion and the neural gland are closely associated. This hypothesis is compatible with Takamura's proposition, rather than the hypothesis that the entire adult neural complex is generated from the neurohypophyseal duct (Willey, 1893). Peripheral GnRH and PRL-like neurons are generated in the

dorsal strand, the epithelium of which is derived from the dorsal wall of the neurohypophyseal duct. In colonial ascidians, neurogenesis may also occur in the dorsal strand (if present), which remains fused with the cerebral ganglion for a long time, supplying neural cells (Manni et al., 1999; Koyama, 2002). The vertebrate olfactory epithelium (derived from the olfactory placode) is capable of prolonged neurogenesis that continues throughout adulthood (Beites et al., 2005; Murdoch and Roskams, 2007). This phenomenon also occurs during regeneration of neurons in the olfactory epithelium (Beites et al., 2005). This observation shows that the neurohypophyseal duct (and its derivatives) is homologous to the olfactory placode of vertebrates and can generate neural cells (neural stem cells) throughout the life of the organism, and that this phenomenon has been maintained throughout evolution from urochordates to mammals. Urochordate species that lack a dorsal strand, such as thaliaceans, appendicularians, and some colonial ascidians, seem to not generate peripheral neurons such as GnRH and PRL-like neurons. These species may have regressed peripheral neurogenesis as an adaptation to asexual reproduction and/or perhaps due to a gross downsizing of body size.

The neurohypophyseal duct and its derived tissues and cells generate a number of cell types including the ciliated duct cells, epithelial cells of the neural gland, luminal cells of the neural gland, epithelial cells of the dorsal strand, and neuroendocrine/endocrine cells in the dorsal strand. Similarly, the rudiment of the cerebral ganglion that is probably derived from the posterior sensory vesicle may generate various kinds of neural cells in the brain, according to an observation in *Ciona* by Takamura (2002). Of these cell types, those that are generated in the dorsal strand and migrate into the brain via the visceral nerve may be exclusively GnRH or PRL-like neurons. Although the function of PRL-like neurons is unknown, their abundance in the cerebral ganglion and the dorsal strand may correspond to the gigantism seen in this species and the corresponding necessity for a large neural network to adjust to external/internal changes in the environment.

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