ZOOLOGICAL SCIENCE **26**: 398–405 (2009)

Placode Formation and Generation of Gonadotropin-Releasing Hormone (GnRH) Neurons in Ascidians

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Neurogenic placodes, a chordate innovation, generate several neuronal populations, including gonadotropin-releasing hormone (GnRH) neurons which are crucial for vertebrate and solitary ascidian urochordate reproduction. The dorsal strand placode of ascidians is derived from the anterior ridge of the embryonic neural plate and thus shares a common developmental origin and expression of various transcription factors with vertebrate placodes. Despite their importance for understanding vertebrate origins, the evolutionary and developmental origins of the neurogenic placode remain obscure. Here I demonstrate the formation of an elaborate neurogenic placode, which forms the dorsal strand, on part of the neural gland epithelium in a solitary ascidian urochordate, *Halocynthia roretzi*. Two modes of GnRH neurogenesis in the dorsal strand (a peripheral organ) and the migration of GnRH neurons into the brain along the visceral nerve are also described. Ontogenetically, GnRH neurons are first detected in the dorsal strand and cerebral ganglion of very young juveniles at almost the same time, demonstrating that ascidians possess morphological and developmental features in common with vertebrates. These results further indicate that the onset of peripheral GnRH neurogenesis and the ability of neurons to migrate into the brain predate the divergence of ascidians and vertebrates. Thus, based on the generation of GnRH neurons, the dorsal strand in ascidians may be homologous to the vertebrate olfactory placode. These organs are derived from the anterior region of the embryonic neural plate, which expresses several transcription factors that invertebrate chordates and vertebrates have in common. These results provide unequivocal support for the clade Olfactores (tunicates + vertebrates).

Key words: ascidian, GnRH neuron, neurogenesis, olfactory placode, dorsal strand, chordate, Olfactores

INTRODUCTION

The question of vertebrate origins is a highly interesting problem in biology that has been examined from various points of view (Gee, 1996). Recent molecular phylogenetic studies have shown that tunicates are the closest relatives of vertebrates (Blair and Hedges, 2005; Delsuc et al., 2006; Putnum et al., 2008), but the only supporting morphological evidence is the presence of neural crest-like pigment cells in ascidian embryos (Jeffery et al., 2004).

Neurogenic placodes are thought to have played a crucial role in the evolution of vertebrates from filter feeding ancestors (Northcutt and Gans, 1983). In invertebrate chordates such as ascidians, the neurohypophyseal duct (a rudiment of the adult neural complex) is considered homologous to the olfactory/adenohypophyseal/hypothalamic placode of vertebrates for several reasons: these structures share a common origin from the anterior region of the embryonic neural plate (which similarly expresses *Pituitary homeobox* [*Pitx*]; Boorman and Shimeld, 2002; Christiaen et al., 2002), the topological relationship between the pharynx and the brain is the same, and both structures have the capacity to produce neurons (Brighel et al., 1998; Manni et al., 1999; Manni et al., 2005).

Reproduction in colonial ascidians is very diverse, but they are largely ovoviviparous or viviparous and produce a small number of gametes. In contrast, solitary ascidians are oviparous and produce a large number of gametes. *Ciona intestinalis* (order Enterogona) and *Halocynthia roretzi* (order Pleurogona) are taxonomically very different, but both are solitary ascidians. Because numerous GnRH neurons are found around the dorsal strand of *C. intestinalis* (Mackie, 1995; Tsutsui et al., 1998; Terakado, 2001), it has been hypothesized that these neurons are derived from the dorsal strand epithelium by delamination (Mackie, 1995; Bollner et al., 1997). Direct proof of this has, however, been lacking. This paper aims to describe the morphological and developmental features that provide evidence that urochordate ascidians are the closest relatives of vertebrates.

MATERIALS AND METHODS

Animals

Adult specimens of the ascidian *H. roretzi* that were cultured in Mutsu Bay in Aomori City, Japan were purchased from a fisherman. Small individuals, about 3–5 mm in length, were obtained with tweezers from ropes submerged in Kesennuma Bay, Kesennuma City, Japan.

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doi:10.2108/zsj.26.398

Light microscopy

Tissues containing the neural complex were excised and fixed with Bouin's fixative for 24 h. The fixed tissues were dehydrated, embedded in Paraplast, sectioned at 6 μm, and stained with Mayer's hematoxylin-eosin or aldehyde fuchsin.

Immunohistochemistry

Excised neural complexes were fixed with Bouin's fixative without acetic acid, or with 1:1 fixative (equal parts of 0.2 M cacodylate buffer [pH 7.2] containing 8% paraformaldehyde and saturated picric acid aqueous solution) for 12–15 h. The latter generally gave good results and was primarily used for the present study. Sections were mounted on gelatin-coated slides and immunostained by using the ABC (streptavidin-biotin-peroxidase) Elite Kit (Vector Laboratories, USA) with a polyclonal anti-human (Monosan, Netherlands) or anti-salmon GnRH primary antibody (Bachem, Switzerland). Dewaxed and rehydrated sections were treated with 0.5% H_2O_2 in 70% methanol for 40 min to block endogenous peroxidase activity. The primary antiserum was diluted 1:800 in 0.01 M phosphate-buffered saline (PBS; pH 7.5) containing 0.1% bovine serum albumin and was incubated with the sections overnight at 4°C. Immunoreactivity was visualized by exposing the sections to 0.02% 3,3′-diaminobenzidine containing 0.06% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.5) at room temperature for 3–5 min and then lightly counterstaining the sections with Mayer's hematoxylin. As a negative control, sections were incubated in anti-GnRH antiserum (human or salmon) that had been pre-absorbed with the corresponding antigen (2 μg/ml).

Immunoelectron microscopy

To detect GnRH neurons among various neuroendocrine and endocrine cells, immunoelectron microscopy was carried out. Dissected neural complexes were cut into small pieces and fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 4 h at 4°C. Tissue pieces were washed overnight at 4°C in 0.1 M cacodylate buffer containing 7% sucrose, postfixed with 1% osmium tetroxide in 0.15 M phosphate buffer (pH 7.2) for 1 h at 4°C, dehydrated, embedded in LR white resin (London Kes & Company, Berkshire, UK), and then incubated at 55°C for 48 h. Ultrathin sections (~70 μm) were placed on Formvar-coated nickel grids kept in a refrigerator, treated with 1% meta-periodic acid for 30 min, and washed with 1% BSA in PBS for 1 h. A polyclonal antiserum against salmon GnRH (1:200) was used as the primary antibody. Sections were incubated overnight with this antiserum at 4°C, washed with PBS, treated with gold-labeled (10 nm) secondary antibody (1:20; British BioCell International, Cardiff, UK) for 3 h, washed in PBS and then in distilled water, and examined with a Hitachi H-700H electron microscope operated at 100 kV. Control staining was performed in parallel using adjacent sections, but the primary antiserum was pre-absorbed with antigen (2 μg/ml). These methods are essentially the same as those described by Terakado et al. (1997) and Kawahara et al. (2002).

RESULTS

Formation of the dorsal strand placode

Halocynthia roretzi (order Pleurogona) is one of the larg-

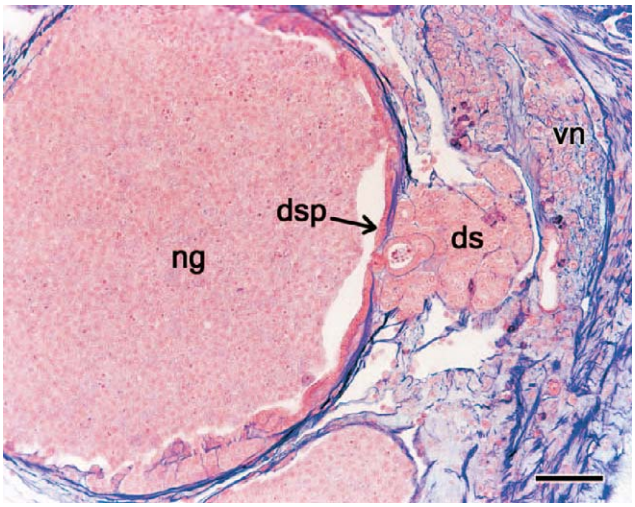

Fig. 2. Formation of the dorsal strand (ds) by invagination at the center of the thick part (dsp) of the neural gland epithelium facing the visceral nerve (vn). ng, neural gland. Aldehyde fuchsin stain. Scale bar: 90 μm.

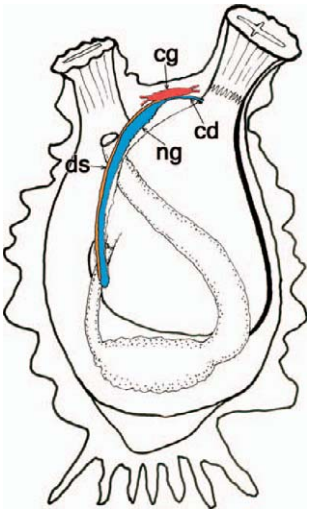

Fig. 1. Schematic drawing of the neural complex in a 3-year-old *Halocynthia roretzi*. The cerebral ganglion (cg) lies between the atrial and branchial siphons. The neural gland (ng) is located just beneath the ganglion, and its lumen opens anteriorly to the pharynx through the ciliated duct (cd). The neural gland extends posteriorly with the dorsal strand (ds).

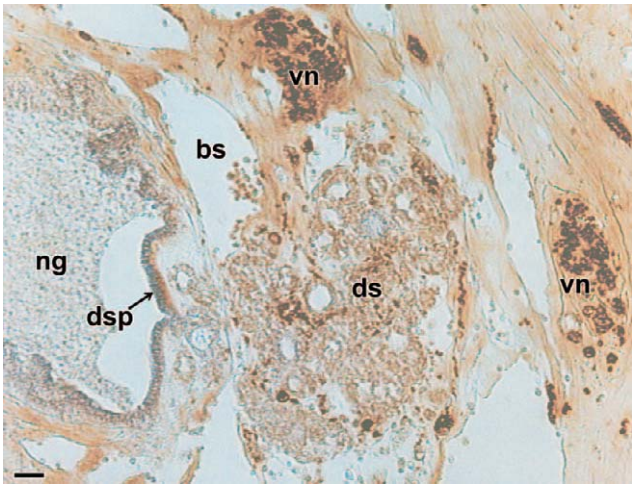

Fig. 3. Immunostaining with anti-human GnRH antibody shows the presence of GnRH neurons in the dorsal strand (ds). The oval structures in the dorsal strand are cross sections of the invaginated tube(s). The dorsal strand runs parallel with the visceral nerve (vn), which is also GnRH-immunoreactive, within the blood sinus (bs). dsp, dorsal strand placode; ng, neural gland. Scale bar: 20 μm.

est solitary ascidians; it survives for 4–5 years or more, and reaches a body weight of over 500 g. It is distantly related to *C. intestinalis* (order Enterogona), which is the model organism of Subphylum Urochordata. In *H. roretzi*, the adult neural complex is large and is comprised of the cerebral ganglion, the associated neural gland, and the dorsal strand (Fig. 1). The lobulated neural gland extends posteriorly and can be >5 cm long; from the midline of the thickened epithelium of the neural gland, the dorsal strand arises by repeated invaginations (Figs. 2, 3). Formation of the GnRH neurogenic placode occurs after metamorphosis, although GnRH mRNA is expressed as early as the 4-cell stage and continuously before metamorphosis in *Ciona* (Adams et al., 2003). The neurogenic placode and numerous GnRH neurons are maintained throughout adulthood in *H. roretzi*. After metamorphosis, the structure is termed the dorsal strand placode, as it possesses important characteristics of a placode in a strict sense, such as a thickened epithelium, an invagination at the center, production of different cell types, and delamination/migration of cells (Northcutt and Gans, 1983; Schlosser, 2005). The invagination is most frequently observed in the posterior region of the brain, where it forms curved tubes and derived cells in large quantities. The curved tubes and derived cells take a cord-like form that runs parallel with the neural gland (Fig. 1) and measures about 100–200 μm in diameter in the thick region. The largest morphological difference in the neural complex between *H. roretzi* and other ascidians is the long (over 5 cm), bunch-like neural gland associated with the dorsal strand. Sometimes, the neural gland/dorsal strand complex branches and follows a branch of the visceral nerve for up to 0.5 cm to the gill basket. The dorsal strand epithelium contains several morphologically distinct cell types, including intermediate filament-rich cells, ciliated cells, dark cells, ribosome-rich undifferentiated cells, and GnRH-containing cells. The epithelium is sometimes innervated with granule-containing nerve fibers. In the neural gland, however, no innervation was found. The luminal spaces of the dorsal strand are continuous with the pharyngeal cavity through luminal spaces of the neural gland and the ciliated duct. In contrast to the neural gland, cilia and microvilli project from the ciliated cells of the epithelium to the lumen, to a greater or lesser extent. Because the dorsal

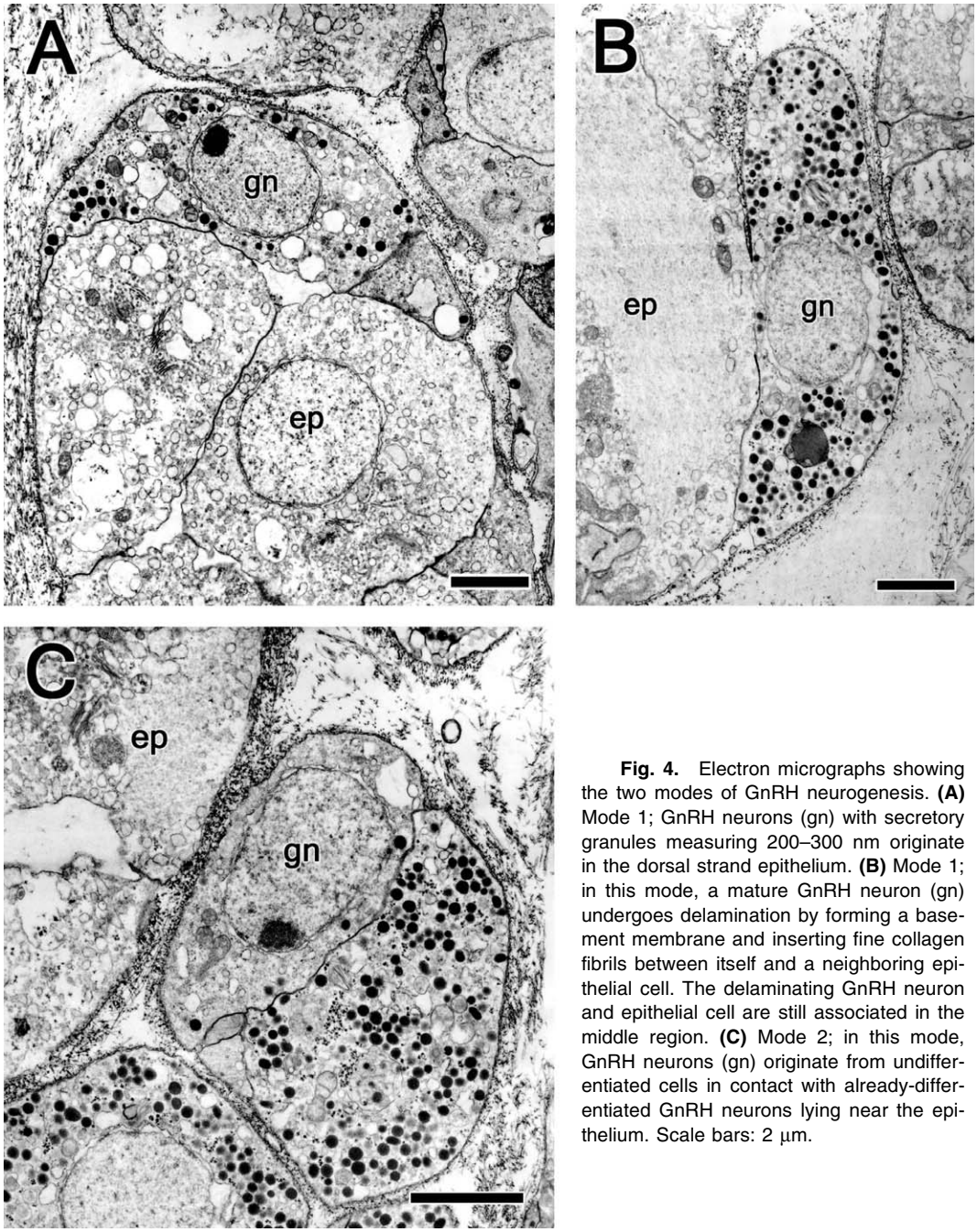

Fig. 4. Electron micrographs showing the two modes of GnRH neurogenesis. **(A)** Mode 1; GnRH neurons (gn) with secretory granules measuring 200–300 nm originate in the dorsal strand epithelium. **(B)** Mode 1; in this mode, a mature GnRH neuron (gn) undergoes delamination by forming a basement membrane and inserting fine collagen fibrils between itself and a neighboring epithelial cell. The delaminating GnRH neuron and epithelial cell are still associated in the middle region. **(C)** Mode 2; in this mode, GnRH neurons (gn) originate from undifferentiated cells in contact with already-differentiated GnRH neurons lying near the epithelium. Scale bars: 2 μm.

strand placode and numerous GnRH neurons are present in every season, GnRH neurons may be continuously produced throughout adult life. This contrasts with *C. intestinalis* (whose life span is about 3 months), in which the number of GnRH neurons is constant in mature individuals.

Modes of GnRH neurogenesis in the dorsal strand

GnRH neurogenesis in *H. roretzi* occurs in two modes. The first mode includes the early development of GnRH neurons from ribosome-rich undifferentiated cells within the dorsal strand epithelium (Fig. 4A). After delaminating from the epithelium, mature GnRH neurons with numerous secretory granules separate from neighboring epithelial cells by forming a basement membrane that has thin collagen fibers attached (Fig. 4B). During the second mode, undifferentiated cells near the epithelium, which probably delaminated earlier, develop into GnRH neurons in contact with earlier-differentiated GnRH neurons (Fig. 4C). As in the first mode, cells are separated by the formation of a basement membrane after the new neuron differentiates. A common feature in the two modes of GnRH neurogenesis may be a direct contact between neurons (without a basement membrane); in the first mode, single developing GnRH neurons were sometimes innervated from differentiated GnRH neurons lying outside of the epidermis, and in the second mode, neuron-to-neuron contact is common. The contact is significant, as it may be possible to prove an inductive role for the differentiated GnRH neurons. Some of the numerous GnRH neurons located outside the epithelium attach to neighboring visceral nerve fibers (Fig. 5A). GnRH neurons

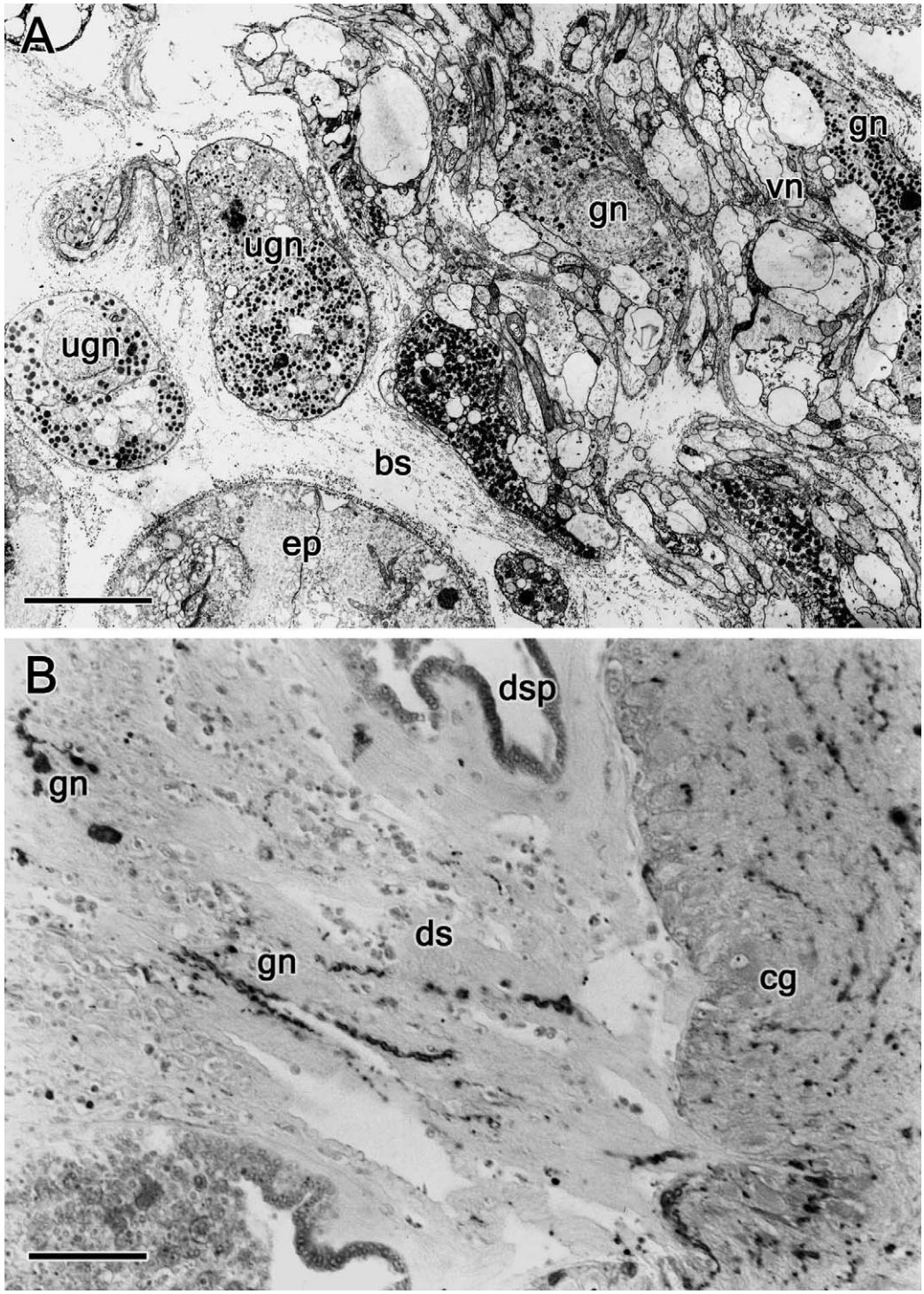

Fig. 5. Electron and light micrographs suggest that GnRH neurons migrate to the cerebral ganglion. **(A)** Electron micrograph; GnRH neurons (gn) are very often attached to visceral nerve fibers (vn). Unattached GnRH neurons (ugn) are found between the dorsal strand and the visceral nerve fibers, running within the blood sinus (bs). ep, dorsal strand epithelium. **(B)** Light micrograph; immunostaining with anti-human GnRH reveals that GnRH neurons (gn) in the dorsal strand (ds) elongate antero-posteriorly and are distributed continuously to the cerebral ganglion (cg). dsp, dorsal strand placode. Scale bars: 5 μm (A), 100 μm (B).

in the dorsal strand elongate antero-posteriorly and become continuously distributed toward the cerebral ganglion (Fig. 5B). In the cerebral ganglion of young *H. roretzi* (1 year old), there are numerous GnRH neurons in the vicinity of the visceral nerve base or at the site where the dorsal strand comes close to the cerebral ganglion. The dorsal strand always runs along the visceral nerve (Figs. 2, 3) within the blood sinus. There are only a few cell bodies of GnRH neurons in the distal regions of the dorsal strand. The GnRH neurons around the dorsal strand are bi- or multi-polar, and lack sensory cilia and processes.

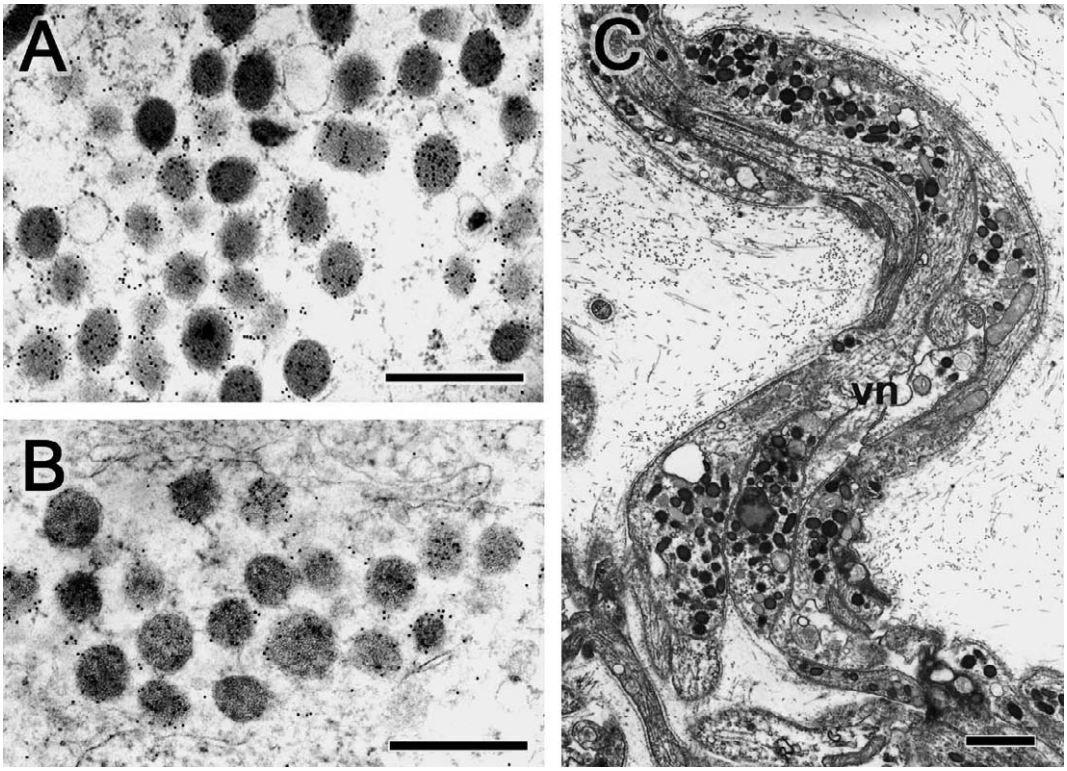

Fig. 6. Immunoelectron and electron micrographs of GnRH neurons. **(A)** Ultrathin section of part of a cell body, stained with anti-salmon GnRH antiserum. Immunogold particles are localized in secretory granules. **(B)** Neurite of a GnRH neuron, also containing immunoreactive granules, similar in size to those in (A). **(C)** The number of secretory (GnRH containing) granules in visceral nerve fibers (vn) increases greatly during the breeding season (compare vn with the visceral nerve fibers in Fig. 5A, which was from material collected in the non-breeding season). Scale bars: 500 nm (A, B), 1 μm (C).

Origin of dorsal strand epithelial cells

GnRH neurons and fibers contain granules that are about 200–300 nm in diameter. Immunostaining with anti-GnRH antibody heavily labeled these granules, revealing GnRH condensation in them (Fig. 6A, B). The central invagination of undifferentiated cells at the placode suggests that a decrease in epithelial cell number, due to delamination from the dorsal strand epithelium, induces cell proliferation at the placode. Bilateral evagination of the placode into the neural gland lumen also suggests that cell proliferation takes place at the placode (Figs. 2, 3). Here, the single columnar epithelium becomes loose and multilayered, mono-nucleate cells become bi-nucleate, and detached cells lacking a basement membrane may migrate into the lumen (Ogawa et al., 1985). Thus, the entire neural gland, except for the dorsal strand placode, consists of bi-nucleate cells (Ogawa et al., 1985), which may originate in the periphery of the dorsal strand placode where mono-nucleate cells become bi-nucleate. Cell division is thought to have ceased in these bi-nucleate cells (Ogawa et al., 1985). In contrast, cells of the dorsal strand epithelium are likely to originate from the center of the placode by continuous invagination (Figs. 2, 3).

DISCUSSION

Occurrence of GnRH neurons among ascidians

Obvious differences in the occurrence of GnRH neurons exist among ascidians. The occurrence of GnRH neurons only in solitary ascidians (Mackie, 1995; Tsutsui et al., 1998; Terakado, 2001) is apparently related to external fertilization (oviparous) in these ascidians. In *H. roretzi* (order Pleurogona), gonoducts are on the surface of gonads that lie far from the dorsal strand, but GnRH nerve fibers whose cell bodies probably lie in the cerebral ganglion run just beneath the epithelium of the gonoducts. These fibers either innervate the gonoducts or release GnRH into the blood sinus, where GnRH acts hormonally to cause spawning. The latter process occurs in mature *Ciona* during the breeding season (Terakado, 2001; Adams et al., 2003), when secretory granules accumulate in the GnRH neurons and fibers in the dorsal strand (Terakado, 2001). Some visceral nerve fibers of *Ciona* (Mackie, 1995) and macular sensory neurons of *Corella inflate* (Mackie and Singla, 2004) are also GnRH immunoreactive, and send their axons to the cerebral ganglion through the visceral nerve. However, the GnRH in the sensory neurons of cupular organ in *Corella inflate* differs from the GnRH-like molecule found in the dorsal strand plexus (Mackie and Singla, 2004). Similarly, the visceral nerve fibers of *H. roretzi* are GnRH-immunoreactive (Fig. 3). It is possibile that their initial GnRH neurons are derived from cells that have accumulated GnRH mRNA during early embryonic stages (see Adams et al., 2003). During the breeding season, the number of secretory granules in visceral nerve fibers greatly increases (Fig. 6C).

In most colonial ascidians, on the other hand, fertilization is internal (ovoviviparous or viviparous) and occurs with

a small number of eggs and embryos. No GnRH neurons are known to exist around the oviduct of colonial ascidians, in which the dorsal strand or its homologous structure is poorly differentiated (Burighel et al., 1998). This structure does, however, maintain the capacity for cell proliferation even into adulthood (Burighel et al., 1998; Koyama, 2002). *Polyandrocarpa misakiensis* is exceptionally oviparous among colonial ascidians and possesses a GnRH receptor, but no GnRH gene has yet been identified (Kobayashi et al., 2005). Formation of the dorsal strand and the neural gland in the large solitary ascidian *H. roretzi* is shown schematically in Fig. 7. Other chordates, such as salps (Lacalli and Holland, 1998) and larvaceans (Bassham and Postlethwait, 2005), have only a ciliated duct or funnel rather than a typical neural gland and a dorsal strand. Amphioxus lacks both an organ equivalent to the dorsal strand of ascidians and peripheral GnRH neurons (Nozaki and Gorbman, 1992), though the amphioxus genome contains GnRH receptors (Holland et al., 2008), and there are GnRH-immunoreactive cells in the central nervous system (Castro et al., 2006). Thus, it seems that among invertebrate chordates with an elaborate dorsal strand, peripheral GnRH neurons are conspicuous in solitary ascidians such as *C. intestinalis* and *H. roretzi*, presumably reflecting the superiority of sexual reproduction. On the other hand, the difficulty of detecting GnRH neurons associated with a peripheral origin may reflect the regression of the dorsal strand, or equivalent organ, in colonial ascidians, salps, larvaceans, and amphioxus.

Migration of GnRH neurons into the cerebral ganglion

Among invertebrate chordates, the large ascidian *H. roretzi* is unique in having GnRH neurons in the cerebral ganglion. The cell bodies of GnRH neurons localize around, but not within, the cerebral ganglion in *C. intestinalis*, suggesting their peripheral origin and migration. Accumulation of GnRH neurons in the posterior-right side of the cerebral ganglion (Tsutsui et al., 1998) also suggests that GnRH neurons migrate onto the surface of the cerebral ganglion along the dorsal strand and/or the visceral nerve, because the dorsal strand and the visceral nerve run down from the posterior-right side of the neural gland and cerebral ganglion, respectively, as viewed from the dorsal side (Mackie, 1995). In *Ciona*, GnRH axons within the cerebral ganglion and the visceral nerve apparently stem from GnRH cell bodies in the dorsal strand plexus (Mackie, 1995; Bollner et al., 1997). Collectively these results strongly suggest that all the GnRH neurons in the cerebral ganglion of *H. roretzi* may have migrated from the dorsal strand via the visceral nerve and connective tissue filaments surrounding the dorsal strand. The possibility that some GnRH neurons originate in the cerebral ganglion cannot, however, be excluded. Migration of GnRH neurons into the cerebral ganglion in *H. roretzi* is very similar to the migration of vertebrate GnRH-1 neurons, which originate in the olfactory placode and migrate along the vomeronasal nerves into the brain (Okubo et al., 2006; Schwarting et al., 2007). Common features between ascidian and vertebrate GnRH neurons include their generation in a neurogenic placode, delamination from the peripheral organ, migration into the brain along a nerve, and critical participation in reproduction. These similarities indicate that these functions were acquired before the divergence of urochordates and vertebrates. This is the first morphological evidence that the neurogenic placode is similar in urochordates and vertebrates, which is consistent with recent molecular phylogenetic analyses (Blair and Hedges, 2005; Delsuc et al., 2006; Putnam et al., 2008). Because cephalochordates, which are evolutionarily older than urochordates (Blair and Hedges, 2005; Delsuc et al., 2006; Putnam et al., 2008), do not have regions of thickened ectoderm homologous to the dorsal strand placode of ascidians or the cranial placodes of vertebrate (Schlosser, 2005), the first chordates may have lacked placodes (Moulemans and Bronner-Fraser, 2007). The present result in which the olfactory placode occurs solely in tunicates and vertebrates provides unequivocal support for the clade Olfactores (tunicates+vertebrates) (Jefferies, 1991). There are GnRH receptors in the amphioxus genome (Putnam et al., 2008), and GnRH-immunoreactive cells in the central nervous system of amphioxus (Castro et al., 2006) may be identical to those in lampreys (Tobet et al., 1996) and some invertebrates (Zhang et al., 2008), with regard to developmental origin.

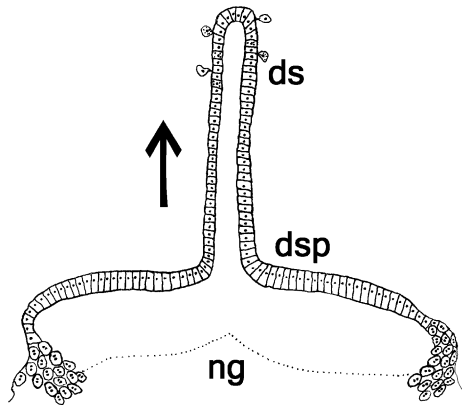

Fig. 7. Schematic drawing of dorsal strand formation and cell proliferation at the placode in the large solitary ascidian *Halocynthia roretzi*. The dorsal strand (ds) is formed by invaginations at the center of placode (dsp), due to cell proliferation in the placode. A decrease in cells by the delamination of pre-mature GnRH neurons and undifferentiated cells from the dorsal strand epithelium may induce subsequent cell proliferation in the placode. Alternatively, luminal cells of the neural gland (ng) may originate from both sides of the placode, where mono-nucleate cells become bi-nucleate and detach from the basement membrane of the epithelium. The arrow indicates the direction of growth during development of the dorsal strand after the delamination of cells from the epithelium.

Homology to the vertebrate pituitary

The idea that the ascidian neural gland may be homologous to the vertebrate pituitary gland was proposed more than a century ago (Julin, 1881). This idea has recently received renewed attention based on the expression of *Pitx,* a gene involved in the development of the adenohypophysis, and embryological evidence for a common origin of the neural crest and placodes (Boorman and Shimeld, 2002; Christiaen et al., 2002; Mackie and Burighel, 2005; Manni et al., 2005), which suggests direct homology between the adenohypophysis of vertebrates and the ciliated duct of

ascidians (Christiaen et al., 2002; Mackie and Burighel, 2005; Manni et al., 2005; Mazet and Shimeld, 2005). However, the presence of a hypophysis in invertebrate chordates has not been generally accepted (Holland et al., 2008), because direct evidence, such as common genetic or molecular similarities with the vertebrate adenohypophyseal hormones, has not yet been obtained, though endocrine cells in the dorsal strand of *H. roretzi* show prolactin-like immunoreactivity (Terakado et al., 1997), adrenocorticotropin-like immunoreactivity (Kawahara et al., 2002; Kawahara et al., 2003), and gonadotropin-like immunoreactivity (Terakado and Ogawa, 1995). Further investigations are required to gain insight into pituitary evolution.

Contradictions between the expression of transcription factors and the generation of endocrine cells/GnRH neurons

Among tunicates, ascidians (Boorman and Shimeld, 2002; Christiaen et al., 2002) and larvaceans (Bassham and Postlethwait, 2005) express common transcription factors in the preoral ectoderm, but they do not generate endocrine cells or GnRH neurons in the ectoderm and its derived organs. The absence of a correlation between regions that express transcription factors for the GnRH system and regions that generate endocrine cells and GnRH neurons argues against the presence of adenohypophyseal and olfactory placodes in ascidians (Schlosser, 2005). This contradiction may, however, be explained by the absence of the full genetic network required for generating these systems or by regression from an already complete endocrine/neuroendocrine system through gene loss. Although solitary and colonial ascidians belong to the same order, the regression of GnRH immunoreactivity in colonial ascidians may reflect degeneration of the dorsal strand, a consequence of the ascendance of asexual reproduction (colony formation). Regression of the GnRH neuronal system may also be the result of strong downsizing, or vice versa, and it may have occurred after the urochordates diverged from vertebrates by adapting to various environments and the accompanying lifestyle alterations. The expression of certain common, fundamental transcription factors is, however, conserved in the preoral ectoderm/ciliated funnel (Boorman and Shimeld, 2002; Christiaen et al., 2002), which is most compatible with the secondary regression of GnRH neurons in extant colonial ascidians. This would also indicate that colonial ascidians are derived from solitary ascidians, and not the reverse. In thaliaceans, another group of colonial tunicates, the GnRH-neuronal system may also have degenerated, and this group may have directly or indirectly originated from the exclusively sexually reproducing tunicates (solitary ascidians). This hypothesis is supported circumstantially by the fact that no deuterostomes except for the colonial tunicates reproduce asexually. In summary, *H. roretzi,* a large, solitary ascidian, possesses structural and functional features very similar to those of vertebrates, including the formation of a neurogenic placode and migration of GnRH neurons into the cerebral ganglion. In colonial tunicates, the dorsal strand and GnRH neuronal system have regressed to various degrees, along with the ascendance of asexual reproduction. Thus, colonial tunicates may have originated from sexually reproducing tunicates (solitary ascidians), which possess a dorsal strand and its derived GnRH neurons.

ACKNOWLEDGMENTS

I thank the members of the Laboratory of Endocrinology and Neuroethology, Saitama University, for the use of equipment and assistance with photography.

REFERENCES

Adams BA, Tello JA, Erchegyi J, Warby C, Hong DJ, Akinsanya KO (2003) Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, *Ciona intestinalis.* Endocrinology 144: 1907–1919

Bassham S, Postlethwait JH (2005) The evolutionary history of placodes: a molecular genetic investigation of the larvacean urochordate *Oikopleura dioica*. Development 132: 4259–4272

Blair JE, Hedges SB (2005) Molecular phylogeny and divergence times of deuterostome animals. Mol Biol Evol 22: 2275–2284

Bollner T, Beesley PW, Thorndyke MC (1997) Investigation of the contribution from peripheral GnRH-like immunoreactive 'neuroblast' to the regenerating central nervous system in the protochordate *Ciona intestinalis.* Proc Roy Soc Lond B 264: 1117–1123

Boorman CJ, Shimeld SM (2002) *Pitx* homeobox genes in *Ciona* and amphioxus show left-right asymmetry is a conserved chordate character and defines the ascidian adenohypophysis. Evol Dev 4: 354–365

Burighel P, Lane NJ, Zaniolo G, Manni L (1998) Neurogenic role of the neural gland in the development of the ascidian, *Botryllus schlosseri* (Tunicata, Urochordata). J Comp Neurol 394: 230–241

Castro A, Becerra M, Manso MJ, Sherwood NM, Anadon R (2006) Anatomy of the Hesse photoreceptor cell axonal system in the central nervous system of amphioxus. J Comp Neurol 494: 54–62

Christiaen L, Burighel P, Smith WC, Vernier P, Bourrat F, Joly JS (2002) *Pitx* genes in tunicates provide new molecular insight into the evolutional origin of pituitary. Gene 287: 107–113

Delsuc F, Brinkmann H, Chourrout D, Philippe H (2006) Tunicate and not cephalochordates are the closest living relatives of vertebrates. Nature 439: 965–968

Gee H (1996) Before the Backbone. Views on the Origin of the Vertebrates. Chapman & Hall, London

Holland LZ, Albalat R, Azumi K, Benito-Gutierrez E, Blow MJ, et al. (2008) The amphioxus genome illuminates vertebrate origins and cephalochordate biology. Genome Res 18: 1100–1111

Jefferies RPS (1991) Two types of bilateral symmetry in the Metazoa: Chordate and bilaterian. In "Biological Asymmetry and Handedness" Ed by GR Bock, J Marsh, Wiley, Chichester pp 94–127

Jeffery WR, Strickler AG, Yamamoto Y (2004) Migratory neural crest-like cells form body pigmentation in a urochordate embryo. Nature 431: 696–699

Julin C (1881) Etude sur l'hypophysis des ascidians et sur les organes qui l'avoisinent. Bull Acad R Med Belg 3 s 1: 151–170

Kawahara G, Terakado K, Sekiguchi T, Inoue K, Kikuyama S (2002) Adrenocorticotropin-like immunoreactivity in the granules of neural complex cells of the ascidian *Halocynthia roretzi.* Zool Sci 19: 1061–1065

Kawahara G, Terakado K, Tanaka S, Kikuyama S (2003) Occurrence of prohormone convertase-like substances in the neural complex cells of the ascidian *Halocynthia roretzi.* Gen Comp Endocrinol 131: 32–37

Kobayashi Y, Ohashi M, Kawamura K, Yubisui T, Fujiwara S (2005) An ascidian homologue of the gonadotropin-releasing hormone receptor is a retinoic acid target gene. Comp Biochem Physiol B

141: 274–280
Koyama H (2002) The dorsal strand of *Polyandrocarpa misakiensis* (Protochordata: Ascidiacea): a light and electron microscope study. Acta Zool (Stockh) 83: 231–243
Lacalli TC, Holland LZ (1998) The developing dorsal ganglion of the salp *Thalia democratica,* and the nature of the ancestral chordate brain. Phil Trans Roy Soc B 353: 1943–1967
Mackie GO (1995) On the 'visceral nervous system' of *Ciona*. J Mar Biol Assoc UK 75: 141–151
Mackie GO, Burighel P (2005) The nervous system in adult tunicates: current research directions. Can J Zool 83: 151–220
Mackie GO, Singla CL (2004) Cupular organs in two species of *Corella* (Tunicata: Ascidiacea). Invertebr Biol 123: 269–281
Manni L, Lane NJ, Sorrentino M, Zaniolo G, Burighel P (1999) Mechanism of neurogenesis during the embryonic development of a tunicate. J Comp Neurol 412: 527–541
Manni L, Agnelletto A, Zaniolo G, Burighel P (2005) Stomodial and neurohypophysial placodes in *Ciona intestinalis*: Insights into the origin of the pituitary gland. J Exp Zool B 300: 324–339
Mazet F, Shimeld SM (2005) Molecular evidence from ascidians for the evolutionary origin of vertebrate cranial sensory placodes. J Exp Zool B 304: 340–346
Moulemans D, Bronner-Fraser M (2007) The amphioxus Sox family: implications for the evolution of vertebrate placodes. Int J Biol Sci 3: 356–364
Northcutt RG, Gans C (1983) The genesis of neural crest and epidermal placode: a reinterpretation of vertebrate origins. Q Rev Biol 58: 1–28
Nozaki M, Gorbman A (1992) The question of functional homology of Hatschek's pit of amphioxus (*Branchiostoma belcheri)* and the vertebrate adenohypophysis. Zool Sci 9: 387–395
Ogawa M, Orikasa C, Terakado K (1985) Binucleate cells in the neural gland of the ascidian, *Halocynthia roretzi* (Drasche). Zool Sci 2: 213–218
Okubo K, Sakai F, Lau EL, Yoshizaki G, Takeuchi Y, Naruse K, Aida K, Nagahama Y (2006) Forebrain gonadotropin-releasing hormone neuronal development: insights from transgenic medaka and the relevance to X-linked Kallman syndrome. Endocrinology 147: 1076–1084
Putnam NH, Butts T, Ferrier DEK, Furlong RF, Hellsten U, et al. (2008) The amphioxus genome and the evolution of the chordate karyotype. Nature 453: 1064–1071
Schlosser G (2005) Evolutionary origins of vertebrate placodes: insights from developmental studies and from comparisons with other deuterostomes. J Exp Zool B 304: 347–399
Schwarting GA, Wierman ME, Tobet SA (2007) Gonadotropin-releasing hormone neuronal migration. Semin Reprod Med 25: 305–312
Terakado K (2001) Induction of gamete release by gonadotropin-releasing hormone in a protochordate, *Ciona intestinalis.* Gen Comp Endocrinol 124: 277–284
Terakado K, Ogawa M (1995) Secretory cells scattered along the dorsal strand of ascidians. 3. Gonadotropic hormone-positive cells. Proc Jpn Soc Comp Endocrinol 10: 30
Terakado K, Ogawa M, Inoue K, Yamamoto K, Kikuyama S (1997) Prolactin-like immunoreactivity in the granules of neural complex cells in the ascidian *Halocynthia roretzi.* Cell Tissue Res 289: 63–71
Tobet SA, Chickering TW, Sower SA (1996) Relationship of gonadotropin hormone (GnRH) neuron to the olfactory system in developing lamprey (*Petromyzon marinus*). J Comp Neurol 376: 97–111
Tsutsui H, Yamamoto N, Ito H, Oka Y (1998) GnRH-immunoreactive neuronal system in the presumptive ancestral chordate *Ciona intestinalis* (Ascidian). Gen Comp Endocrinol 112: 426–432
Zhang L, Tello JA, Zhang W, Tsai PS (2008) Molecular cloning, expression pattern, and immunocytochemical localization of a gonadotropin-releasing hormone-like molecule in the gastropod mollusk, *Aplysia californica*. Gen Comp Endocrinol 156: 201–209

(Received November 13, 2008 / Accepted April 9, 2009)