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Glutamatergic Networks in the *Ciona intestinalis* Larva

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ABSTRACT

Glutamate is a major neurotransmitter in the excitatory synapses of both vertebrate and invertebrate nervous systems and is involved in many neural processes including photo-, mechano-, and chemosensations, neural development, motor control, learning, and memory. We identified and characterized the gene (*Ci-VGLUT*) encoding a member of the vesicular glutamate transporter subfamily, a specific marker of glutamatergic neurons, in the ascidian *Ciona intestinalis*. The *Ci-VGLUT* gene is expressed in the adhesive organ, the epidermal neurons, and the brain vesicle, but not in the visceral ganglion. The *Ci-VGLUT* promoter and an anti-Ci-VGLUT antibody were used to analyze the distribution and axonal connections of prospective glutamatergic neurons in the *C. intestinalis* larva. The green fluorescent protein (GFP) reporter driven by the 4.6-kb upstream region of *Ci-VGLUT* recapitulated the endogenous gene expression patterns and visualized both the cell bodies and neurites of glutamatergic neurons. Papillar neurons of the adhesive organs, almost all epidermal neurons, the otolith cell, and ocellus photoreceptor cells were shown to be glutamatergic. Each papillar neuron connects with a rostral epidermal neuron. Axons from rostral epidermal neurons, ocellus photoreceptor cells, and neurons underlying the otolith terminate in the posterior brain vesicle. Some caudal epidermal neurons also send long axons toward the brain vesicle. The posterior brain vesicle contains a group of Ci-VGLUT-positive neurons that send axons posteriorly to the visceral ganglion. Our results suggest that glutamatergic neurotransmission plays a major role in sensory systems and in the integration of the sensory inputs of the ascidian larva. J. Comp. Neurol. 508:249–263, 2008.

Indexing terms: ascidian; vesicular glutamate transporter; otolith; photoreceptor cells; sensory systems; glutamatergic neurons

The ascidian larva has a simple central nervous system (CNS) derived from the dorsal neural tube and consisting of about 330 cells, of which about 100 cells are presumed to be neurons based on the cytological appearance of the cells (Nicol and Meinertzhagen, 1991; Cole and Meinertzhagen, 2004). The expression patterns of developmental regulatory genes along the anteroposterior axis are conserved in the developing CNS between ascidians and vertebrates, suggesting that the developmental mechanisms of the CNS are conserved among chordates (Wada et al., 1998; Imai et al., 2002). Recently, the draft sequence of the ascidian genome was generated in a widely studied species, *Ciona intestinalis* (Dehal et al., 2002). Thus, the larval CNS of ascidians can serve as a simple model system for studying the development and function of the chordate CNS (Meinertzhagen et al., 2004).

The anterior brain vesicle (also called the sensory vesicle) of the ascidian CNS contains two sensory organs, a photoreceptor organ (ocellus) and a gravity-sensing organ (otolith), which are responsible for the swimming behavior of the larva (Tsuda et al., 2003a; Sakurai et al., 2004). The visceral ganglion is located posterior to the brain vesicle and contains motor neurons that regulate the muscle con-

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traction of the tail (Okada et al., 2001; Yoshida et al., 2004). The ascidian larva also has a peripheral nervous system (PNS) consisting of an adhesive organ and epidermal neurons in the trunk and tail (Takamura, 1998). In spite of its simple organization, neuronal networks in the ascidian nervous system are poorly understood. This is mainly due to the lack of specific markers for neuronal subtypes. In an attempt to identify neuronal subtypes in the ascidian nervous system, we identified neuron-specific promoters of genes encoding vesicular transporters for GABA and glycine (Ci-VGAT) and for acetylcholine (Ci-VACHT) (Yoshida et al., 2004).

Although glutamatergic neurons have not been identified in ascidians yet, glutamate is expected to play an important role in the ascidian nervous system because it is a major neurotransmitter in the excitatory synapses of both vertebrate and invertebrate nervous systems. For example, glutamate is a neurotransmitter at neuromuscular junctions in arthropods and at specific peripheral and central synapses in arthropods and mollusks (Gerschenfeld, 1973; Walker and Roberts, 1982; Bicker et al., 1988; Horseman et al., 1988; Quinlan and Murphy, 1991; Dale and Kandel, 1993; Trudeau and Castellucci, 1993). In the vertebrate CNS, glutamate is also a major excitatory transmitter capable of exciting virtually all central neurons; its function is important for many neural processes including photo-, mechano-, and chemosensations, neural development, motor control, learning, and memory (Jahr and Lester, 1992).

Vesicular glutamate transporter (VGLUT), a glutamatergic neuron-specific protein, plays crucial roles in glutamatergic neurotransmission (Lee et al., 1999; Bellocchio et al., 2000; Takamori et al., 2000; Fremeau et al., 2004; Wojcik et al., 2004). VGLUT has been identified in mammals, *Caenorhabditis elegans, Drosophila, Xenopus*, and zebrafish and has been used as a marker to trace glutamatergic nerve networks (Lee et al., 1999; Bellocchio et al., 2000; Takamori et al., 2000; Fremeau et al., 2002; Gleason et al., 2003; Higashijima et al., 2004a,b; Daniels et al., 2004; Mahr and Aberle, 2006). In the present study we identified and characterized the *VGLUT* gene of the ascidian *C. intestinalis* and investigated the distribution and neuronal connections of glutamatergic neurons in the *C. intestinalis* larva. We attempted to visualize glutamatergic neurons by three different strategies: 1) whole-mount in situ hybridization detecting *VGLUT* transcripts; 2) immunohistochemical staining of VGLUT localization; and 3) visualizing cell bodies and neurites by GFP driven by the *VGLUT* promoter. Our results suggest that papillar neurons, epidermal neurons, the otolith cell, and ocellus photoreceptors are glutamatergic. We also found a group of prospective glutamatergic neurons in the posterior brain vesicle. Thus, glutamatergic neurotransmission seems to play a major role in both the PNS and CNS of the ascidian larva. Our results suggest that the posterior brain vesicle serves as a processing center that integrates sensory inputs and modulates motor outputs.

MATERIALS AND METHODS

Animals and embryos

Mature adults of *C. intestinalis* were collected from harbors in Murotsu and Aioi, Hyogo, Japan, and maintained in indoor tanks of artificial seawater (Marine Art, Senju Seiyaku, Osaka, Japan) at 18°C. Embryos and larvae were prepared as described previously (Nakagawa et al., 1999).

Isolation and characterization of *Ci-vGLUT* cDNA

The gene encoding vesicular glutamate transporter (*Ci-VGLUT*) was found in the *C. intestinalis* genome database (Dehal et al., 2002) by TBLASTN searches using amino acid sequences of mammalian vesicular glutamate transporter as queries. A 340-bp cDNA fragment encoding a central portion of Ci-VGLUT was amplified by polymerase chain reaction (PCR) with a pair of primers (5′-GTTGCCAACTTCTGCAGAAGCT-3′ and 5′-AGCCTGAAATGGCGAATCCACT-3′). To obtain the full-length coding sequence of *Ci-VGLUT*, cDNA fragments derived from 5′ and 3′ portions of *Ci-VGLUT* mRNA were amplified from a cDNA library of larvae using a gene-specific primer (5′-ATCACAGCACCAGCGTAGCTG-3′ for the 5′ portion and 5′-AGTGGATTCGCCATTTCAGGCT-3′ for the 3′ portion) and a vector-specific primer by the methods previously described (Yoshida et al., 2002). The 5′ end of *Ci-VGLUT* mRNA was further determined by the 5′ RACE method using a Gene Racer kit (Invitrogen, La Jolla, CA). The nucleotide sequences of the primers used for 5′ RACE were 5′-CGACCATTGCCACGCCCAGATTGC-3′ (for the primary PCR) and 5′-CGCCCAGATTGCATCGCATGCCAAAAG-3′ (for the nested PCR). The cDNA fragments were cloned into a pBluescript II SK(+) vector. The cDNA clones were sequenced on both strands with an automatic DNA sequencer (DSQ 1000L, Shimadzu, Kyoto, Japan).

Molecular phylogenetic analysis

The deduced amino acid sequence of Ci-VGLUT was aligned with amino acid sequences of solute carrier 17 (SLC17), SLC18, and SLC37 family proteins from various animals using the ClustalW program (Thompson et al., 1994). Sites with gaps were excluded from the analysis. Neighbor-joining (NJ) trees were constructed using the ClustalW program. Maximum-likelihood (ML) trees were reconstructed by the quartet puzzling algorithm using the TREE-PUZZLE program (Strimmer and von Haeseler, 1996). The reliability value for each internal branch indicates in percent how often the corresponding cluster was found among the 1,000 intermediate trees. Sequences used were: *Homo sapiens* VGLUT1 NP_064705, *H. sapiens* VGLUT2 NP_065079, *H. sapiens* VGLUT3 NP_647480, *Xenopus laevis* VGLUT1 AAQ12345, *Danio rerio* VGLUT2.1 BAD67437, *D. rerio* VGLUT2.2 BAD67438, *Drosophila melanogaster* VGLUT NP_608681, *C. elegans* EAT-4 AAC64972, *H. sapiens* SLC17A1 (NPT1) NP_005065, *H. sapiens* SLC17A2 (NPT3) NP_005826, *H. sapiens* SLC17A3 (NPT4) NP_006623, *H. sapiens* SLC17A4 NP_005486, *H. sapiens* SLC17A5 (sialin) NP_036566, *H. sapiens* SLC18A1 (VMAT1) NP_003044, *H. sapiens* SLC18A2 (VMAT2) NP_003045, *H. sapiens* SLC18A3 (VACHT) NP_003046, *H. sapiens* SLC37A1 NP_061837, *H. sapiens* SLC37A4 NP_001458.

In situ hybridization

We used a 1.0-kb cDNA encoding C-terminal portion of Ci-VGLUT as the template to synthesize a digoxigenin-

labeled antisense RNA probe using a DIG RNA labeling kit (Roche, Japan). In situ hybridization of whole-mount specimens was carried out as described in Kusakabe et al. (2001).

Antibody generation and immunohistochemistry

Complementary DNA fragments encoding the carboxyl-terminal region of Ci-VGLUT (Q535-E656) were amplified by PCR using a pair of primers, 5′-GAagaTctCCAGG-AAGAAGTTGGT-3′ and 5′-CTaAgCTTCCCTTACTCC-TTG-3′ (the lowercase letters in the primer sequences indicate mismatched nucleotides introduced to generate restriction sites), and cloned into the pQE40 vector (Qiagen, Germany). The construct was introduced into the *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA). The carboxyl terminal regions were expressed as a fusion protein with dihydrofolate reductase and a histidine tag and then were used to immunize mice. The preparation of the antiserum was performed as described previously (Tsuda et al., 2003b).

Ciona larvae were fixed with 10% formalin in artificial seawater for 3 hours at 4°C. Fixed specimens were subjected to whole-mount immunostaining as previously described (Tsuda et al., 2003b). The anti-Ci-VGLUT antiserum was diluted 1:1,000 in 10% goat serum in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (TPBS) and used as the primary antibody. For the negative control, the anti-Ci-VGLUT antiserum diluted 1:1,000 in TPBS was preabsorbed with 5 μg/mL of the antigen at 4°C for 12 hours before the primary antibody reaction. As the secondary antibody, an Alexa 488-conjugated antimouse IgG goat antibody (Molecular Probes, Eugene, OR) was used. The stained specimens were mounted in 50% glycerol and observed under a confocal microscope (LSM 510, Carl Zeiss, Germany).

Labeling prospective glutamatergic neurons with GFP

We determined the genomic organization of *Ci-VGLUT* by comparing the full-length cDNA sequences with whole-genome sequences in the NIG/Kyoto *C. intestinalis* Genome Project Database (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) and the JGI Ciona Genome Project Database (Dehal et al., 2002; http://www.jgi.doe.gov/ciona). The 5′ flanking sequences of *Ci-VGLUT* obtained from the Genome Databases were used to design gene-specific primers. Genomic DNA fragments containing a 4.7-kb upstream region of *Ci-VGLUT* were amplified from sperm DNA by PCR using a thermostable DNA polymerase (LA Taq, Takara BIO, Japan) and a pair of gene-specific oligonucleotide primers 5′-gatcgtcgacCCG-GTATGTCCACAGCATTC-3′ and 5′-gtcAggAtccCACTT-GTTGTTACTGCATAA-3 (the lowercase letters in the primer sequences indicate mismatched nucleotides introduced to generate restriction sites). The amplified upstream regions were inserted into the *Sal*I/*Bam*HI sites of pBluescript-EGFP (Yoshida et al., 2004).

The promoter-GFP fusion construct was electroporated into fertilized eggs of *C. intestinalis* as described by Corbo et al. (1997). Fluorescence was observed with the fluorescent microscopes SZX12-RFL3 (Olympus, Japan) and Axioplan 2 (Zeiss) or the confocal microscope LSM 510 (Zeiss). After the specimens were fixed with 10% formalin in artificial seawater, GFP localization was visualized by whole-mount immunofluorescent staining with a rat anti-GFP monoclonal antibody (GF090R, Nacalai Tesque, Kyoto, Japan), which had been raised against full-length GFP with a histidine-tag at the N-terminal end, and an Alexa 488-conjugated secondary antibody (Molecular Probes). The specificity of immunostaining with the anti-GFP has been demonstrated by the absence of GFP-immunoreactivity in larvae electroporated with *GFP* constructs containing inactivated promoters (Kusakabe et al., 2004). For double labeling, *C. intestinalis* arrestin Ci-Arr (Nakagawa et al., 2002) was detected with an anti-Ci-Arr primary antibody and an Alexa 546-conjugated secondary antibody (Molecular Probes) as described previously (Tsuda et al., 2003b). Preparation of the anti-Ci-Arr was describe previously (Tsuda et al., 2003b). The C-terminal peptide (D358-A418) of Ci-Arr conjugated with histidine-tagged dihydrofolate reductase was expressed from the pQE40 vector in *E. coli* and used as an antigen to immunize Balb/c mice. The specificity of anti-Ci-Arr antiserum was confirmed by Western blotting with a preabsorption negative control as described previously (Horie et al., 2005).

Photomicrograph production and processing

Confocal images of immunofluorescent staining were analyzed with the Zeiss LMS image software. Microphotographs of in situ hybridization specimens (Figs. 3, 8C) and control immunofluorescent staining (Fig. 4D,E) were obtained via analysis using a Zeiss Axioplan 2 microscope and a digital CCD camera VB-7010 (Keyence, Osaka, Japan). Image processing was performed using Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) as follows: microphotographs were assembled for Figures 4A–C, 5B, 7A,B, 8A,B and merged for Figure 6H; the contrast and brightness of photographs used in Figure 4D,E were so adjusted that the outline of larvae was easily recognized.

RESULTS

Identification and characterization of the *C. intestinalis VGLUT* Gene

We found a gene (*Ci-VGLUT*) encoding a protein similar to mammalian VGLUT in the *C. intestinalis* genome. The *Ci-VGLUT* gene corresponds to the annotated *Ciona* gene (Gene Model) ci0100152655. The prediction of the gene was partial in the genome database, and no EST/cDNA clones of *Ci-VGLUT* have been found in the cDNA project (Satou et al., 2002). We obtained *Ci-VGLUT* cDNA clones from a larval stage cDNA library and determined the full-length cDNA sequence. The genomic organization of the *Ci-VGLUT* gene was determined by comparing the genome sequence obtained from the JGI genome database with the full-length cDNA sequence. *Ci-VGLUT* consists of 13 exons (Fig. 5). The 5′ end of the *Ci-VGLUT* cDNA has a spliced leader sequence (5′-ATTCTATTTGAATAAG-3′), which is identical to that found in the *C. intestinalis* troponin I gene (Vandenberghe et al., 2001). The *Ci-VGLUT* cDNA encodes a protein of 663 amino acids (Fig. 1). The deduced amino acid sequence of Ci-VGLUT shows a high degree of amino acid similarity to mammalian VGLUT; the overall amino acid identity and similarity of human VGLUT to Ci-VGLUT are 54% and 71%, respectively (Fig. 1). All known vertebrate and invertebrate VGLUTs belong to the solute carrier 17 (SLC17) family

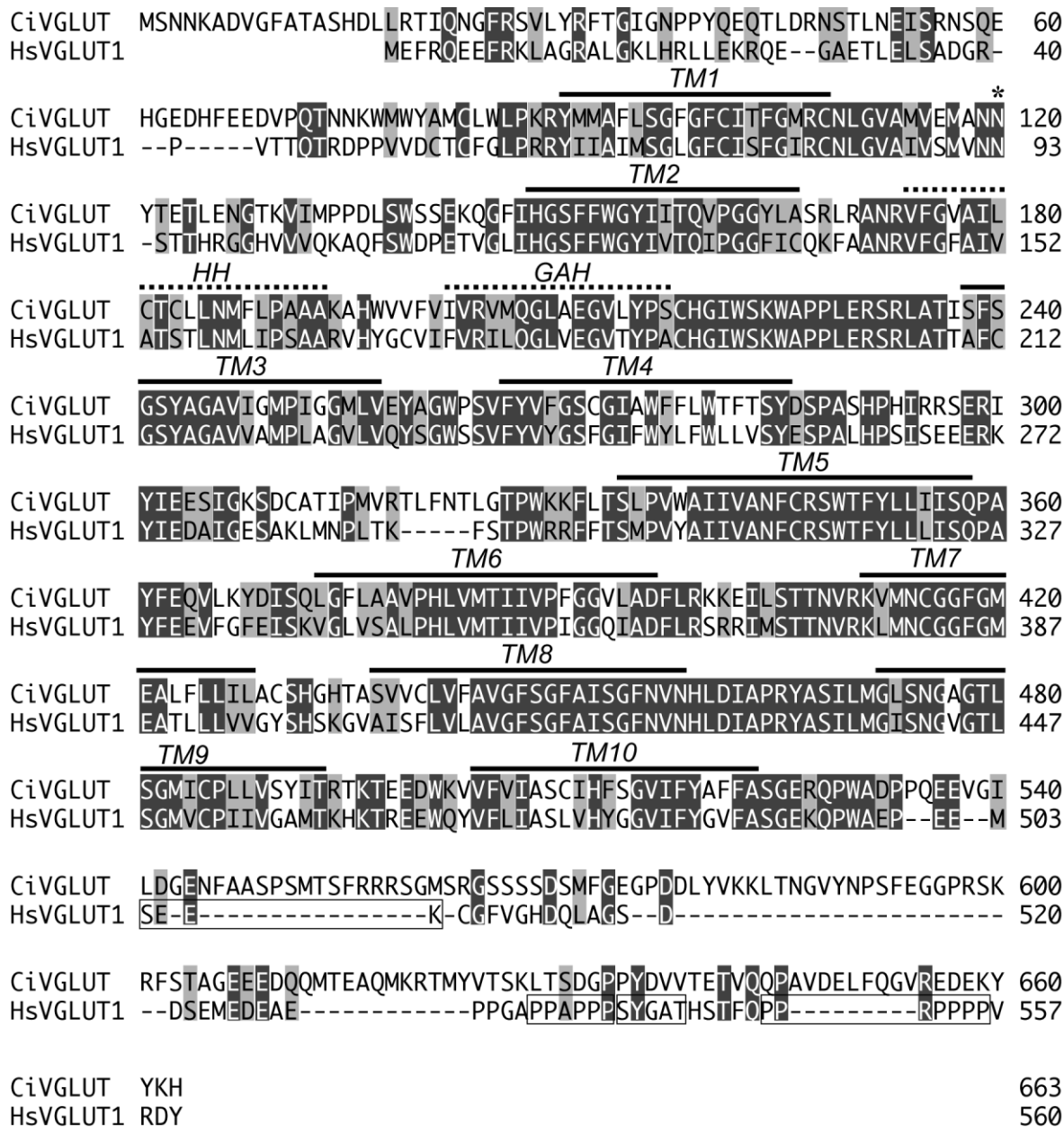

Fig. 1. Comparison of amino acid sequences of *Ciona intestinalis* VGLUT-like protein (Ci-VGLUT) and human VGLUT1. The predicted protein sequence of Ci-VGLUT was aligned with the amino acid sequence of human VGLUT1 using the ClustalW program. The numbers in the right column correspond to amino acid positions. White letters in a dark gray box indicate the positions where both proteins exhibit the same amino acid. Letters highlighted by a light gray box indicate the positions where *Ciona* and human proteins have similar amino acids. Dashes indicate gaps introduced in the sequence to optimize the alignment. Solid lines indicate predicted transmembrane domains (TM1–TM10). Dashed lines indicate a potential hydrophobic helix (HH) and a potential GxxxG motif and Amphipathic Helix (GAH). Transmembrane domains, HH, and GAH are predicted based on the membrane topology model of VGLUT proposed by Fei et al. (2007). An asterisk denotes a potential glycosylation site conserved among vertebrate and invertebrate VGLUTs. Two polyproline motifs (PPAPPP, PPRPPPP) and two conserved motifs (SEEK, SYGAT) in the cytoplasmic C-terminus of human VGLUT1 are indicated by open boxes.

and they are distantly related to other SLC families, such as SLC18 and SLC37 (Hediger et al., 2004; Eraly et al., 2004). Among these SLC proteins, Ci-VGLUT is the most closely related to vertebrate VGLUTs, as revealed by the molecular phylogenetic tree inferred by the maximum-likelihood method (Fig. 2). Molecular phylogenetic analysis by the neighbor-joining method gave essentially the same result (data not shown). Other SLC17 proteins and protostome VGLUTs are more distantly related. The position of Ci-VGLUT is basal to all members of the vertebrate VGLUT, and only one VGLUT-like gene was found in the *Ciona* genome. The position of Ci-VGLUT in the

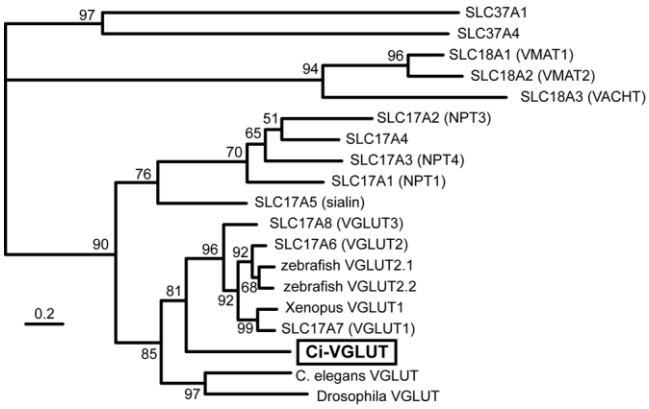

Fig. 2. Molecular phylogenetic trees of the VGLUT family and related transporters. Phylogenetic trees were inferred from amino acid sequences using the maximum likelihood method. Scale bar = 0.2 amino acid replacements per site. Numbers at nodes indicate the quartet puzzling reliability values.

molecular phylogenetic trees, as nested within a clade consisting solely of vertebrate and invertebrate VGLUTs, suggests that it has a physiological function similar to known VGLUT proteins. It should be noted, however, that the nature of the compounds transported by Ci-VGLUT has yet to be determined by biochemical studies. The nucleotide sequence of *Ci-VGLUT* cDNA has been deposited in the DDBJ, EMBL, and GenBank Nucleotide Databases under the accession number AB362939.

In mammalian VGLUTs several sequence motifs have been implicated as functionally important. For example, the cytoplasmic C-terminus of mammalian VGLUT1 contains two polyproline motifs (PPAPPP and PPRPPPP), the latter of which has been shown to be required for specific interaction with endophilins 1 and 3, synaptic vesicle proteins colocalized with VGLUT1 in glutamatergic synapses (De Gois et al., 2006). Two other motifs (SEE/DK, SYGAT) are conserved in the C-terminus of mammalian VGLUTs; SEE/DK is conserved across the three isoforms of vertebrate VGLUT (VGLUT1, VGLUT2, and VGLUT3), while SYGAT is found not only in vertebrate VGLUTs but also in *Drosophila* and *C. elegans* VGLUTs (Lee et al., 1999; Daniels et al., 2004). Interestingly, none of these motifs are present in the corresponding regions of Ci-VGLUT (Fig. 1). In mammalian VGLUTs, two possible N-glycosylation sites are predicted in the region between the transmembrane domains (TM) 1 and 2 (Fremeau et al., 2002; Schafer et al., 2002). One of the N-glycosylation sites is also conserved in Ci-VGLUT (Fig. 1).

Ci-VGLUT is specifically expressed in central and peripheral neurons of the larva

Spatial expression patterns of *Ci-VGLUT* were examined in embryos and larvae by whole-mount in situ hybridization (Fig. 3). At the mid-tailbud stage, *Ci-VGLUT* is expressed in particular cells in the anterior trunk epidermis (arrowheads in Fig. 3A) and in the central nervous system (white arrow in Fig. 3A); these cells are presumably precursors of anterior epidermal neurons and neurons in the brain vesicle, respectively. *Ci-VGLUT* expression was also found in a few cells on the dorsal and ventral

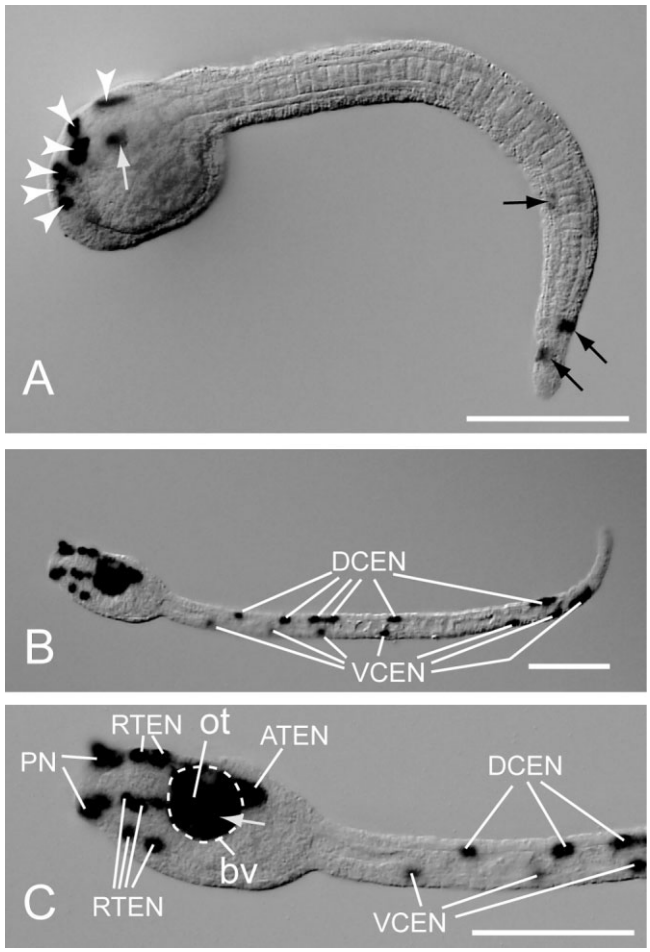

Fig. 3. Spatial expression patterns of *Ci-GLUT* visualized by whole-mount in situ hybridization. **A:** A mid-tailbud embryo. Arrowheads indicate cells expressing *Ci-VGLUT* in anterior trunk epidermis. A white arrow indicates *Ci-VGLUT* expression in the developing brain vesicle. Black arrows indicate prospective caudal epidermal neuron precursors. **B,C:** A tadpole larva. ATEN, apical trunk epidermal neuron; bv, brain vesicle; DCEN, dorsocaudal epidermal neuron; oc, ocellus; ot, otolith; PN, papillar neuron; RTEN, rostral epidermal neuron; VCEN, ventrocaudal epidermal neuron. Scale bar = 100 μm.

midlines of the posterior half of the tail (black arrows in Fig. 3A). In tadpole larvae, *Ci-VGLUT* is expressed in almost all peripheral epidermal neurons, including papillar neurons of the adhesive organ, rostral trunk epidermal neurons (RTEN), apical trunk epidermal neurons (ATEN), dorsocaudal epidermal neurons (DCEN), and ventrocaudal epidermal neurons (VCEN) (Fig. 3B,C). In the CNS of the larva, the *Ci-VGLUT* expression is restricted to cells in the brain vesicle; it is especially evident in a group of cells in the posterior half of the brain vesicle (white arrow in Fig 3C). At both the tailbud and larval stages, *Ci-VGLUT* expression was not observed in nonneural tissues.

Distribution of glutamatergic axons and nerve terminals in the larva

Axons and synaptic terminals of prospective glutamatergic neurons were visualized in *C. intestinalis* larvae by immunohistochemical staining of Ci-VGLUT (Fig. 4). A

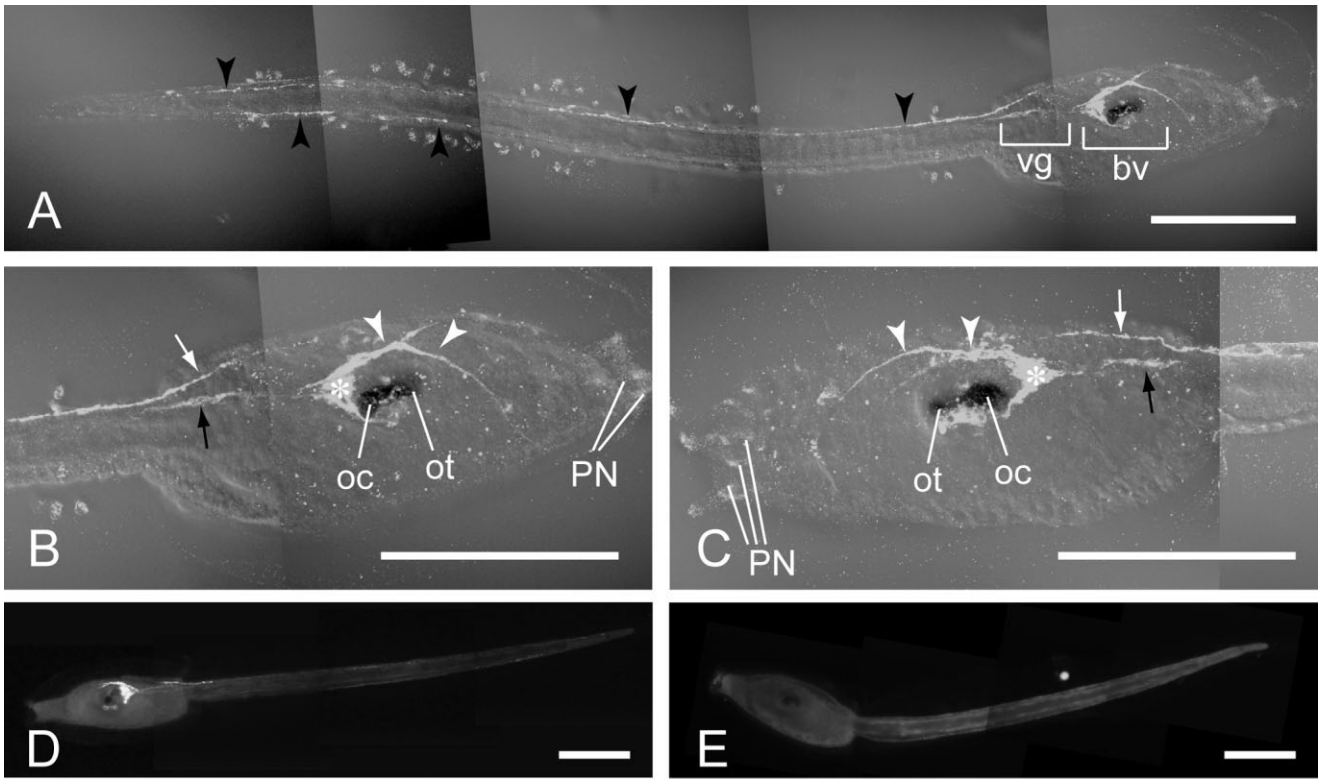

Fig. 4. Distribution of Ci-VGLUT-immunoreactive axons and nerve terminals in *C. intestinalis* larvae. Prospective glutamatergic axons and nerve terminals were visualized by immunofluorescent staining with an anti-Ci-VGLUT antibody. **A:** A whole tadpole larva. **B:** Right lateral view of the trunk of a larva. **C:** Left lateral view of the trunk of a larva. **D,E:** Control immunostaining showing specificity of the anti-Ci-VGLUT antibody. D: Left lateral view of a whole larva incubated with the anti-Ci-VGLUT antibody. E: Left lateral view of a whole larva incubated with preabsorbed antibody (negative control). White arrowheads indicate afferent axon bundles from rostral trunk epidermal neurons. Black arrowheads indicate axons of caudal epidermal neurons. White arrows indicate axons of posterior apical trunk epidermal neurons. Conspicuous staining of Ci-VGLUT-immunoreactive axons and nerve terminals were observed in the posterior brain vesicle (asterisks). Glutamatergic axons were also found along the anteroposterior axis of the visceral ganglion (black arrows). bv, brain vesicle; oc, ocellus; ot, otolith; PN, papillar neurons; vg, visceral ganglion. Scale bars = 100 μm.

polyclonal antiserum against a carboxy-terminal portion of Ci-VGLUT specifically recognized nerve tracts in both the PNS and CNS of the larva. Nerve tracts running beneath the tail epidermis of the dorsal and ventral midlines were stained (black arrowheads in Fig. 4A). Conspicuous staining was found in the posterior brain vesicle, suggesting that there are a large number of glutamatergic synapses at this site. Some of these nerve terminals seem to derive from axons coming from rostral epidermal neurons (white arrowheads in Fig. 4B,C). Others are likely to be axon terminals of neurons in the brain vesicle, including photoreceptor cells. Ci-VGLUT-positive nerve tracts were also found in the whole length of the visceral ganglion (black arrows in Fig. 4B,C). Another prospective glutamatergic nerve tract runs dorsal to the visceral ganglion along the midline of the posterior half of the trunk, and this extends to the anterodorsal part of the tail (white arrows in Fig. 4B,C). Papillar neurons were also stained with anti-Ci-VGLUT.

Identification of glutamatergic neurons by the *Ci-VGLUT* promoter-GFP fusion construct

Immunohistochemical staining of Ci-VGLUT failed to visualize the cell bodies of neurons. To visualize both cell bodies and neurites of glutamatergic neurons, we introduced a *GFP* reporter gene (*EGFP*) driven by the *Ci-VGLUT* promoter into *Ciona* fertilized eggs by electroporation, and GFP localization was detected in the larvae. The promoter-reporter fusion construct, pCi-VGLUT-EGFP, was made by connecting the 5′ flanking region, including 5′ exons and introns down to the 3′ end of intron II of *Ci-VGLUT* with a GFP coding sequence in a plasmid vector (Fig. 5A). The size of the upstream region included in pCi-VGLUT-EGFP was 4603 bp. *Ciona* larvae that were developed from fertilized eggs electroporated with pCi-VGLUT-EGFP showed the reporter gene expression in a pattern quite similar to that of the endogenous *Ci-VGLUT* gene (Fig. 5B). GFP expression in these cells was observed in 55% of the electroporated larvae (136 out of 245 larvae). No ectopic expression was observed. Thus, the 4.6-kb upstream region contained in pCi-VGLUT-EGFP is sufficient to recapitulate the expression of the endogenous *Ci-VGLUT* gene.

Because the expression of *GFP* introduced by electroporation in ascidian larvae is often ‘mosaic,’ we could stochastically label cell bodies and axons of different prospective glutamatergic neurons in pCi-VGLUT-EGFP-electroporated larvae. We shall describe below the

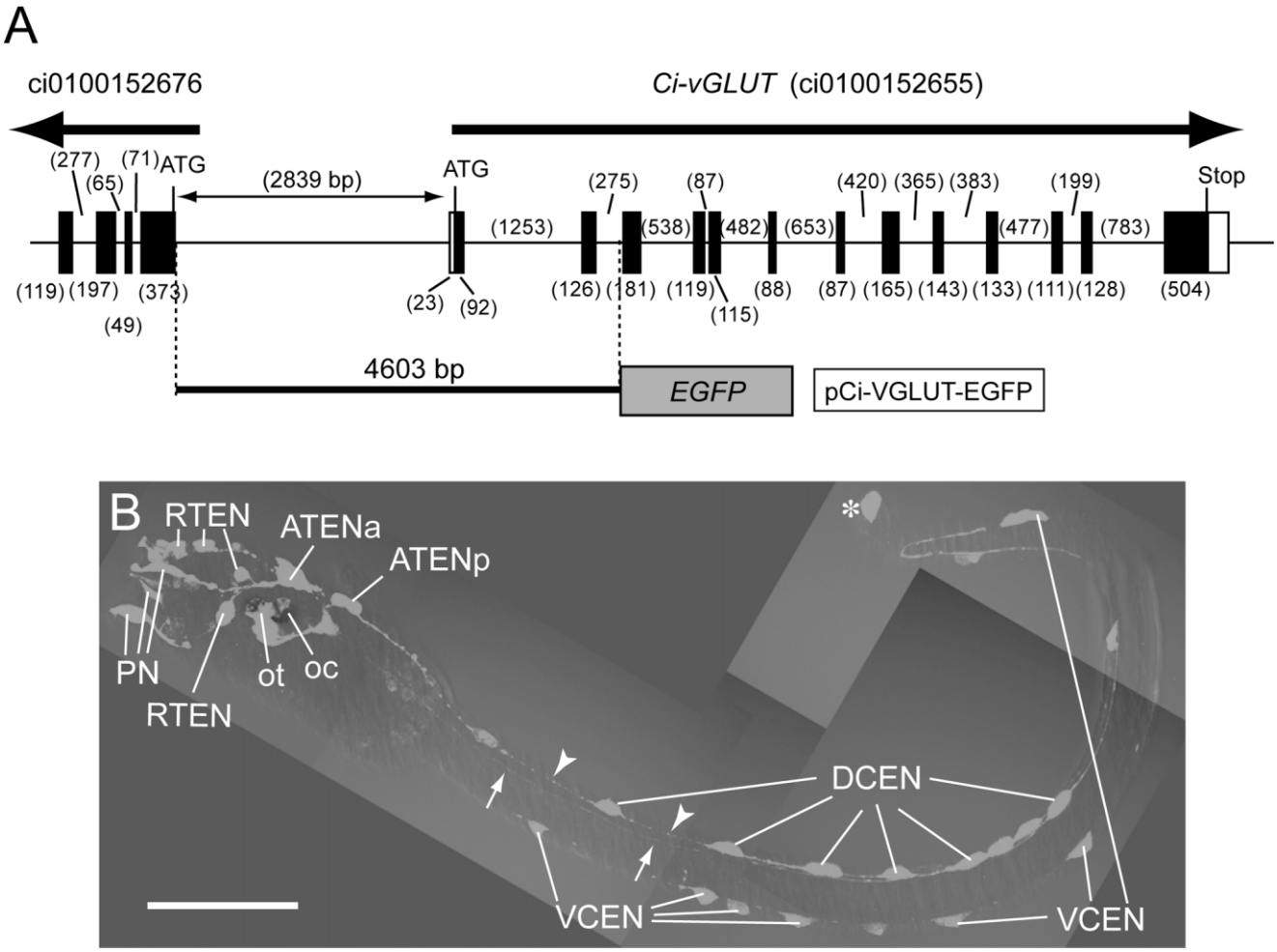

Fig. 5. Recapitulation of the endogenous gene expression patterns by the 4.6-kb upstream regulatory region of the *Ci-VGLUT* gene. **A:** Genomic organization of the *Ci-VGLUT* gene and structure of the promoter-*GFP* fusion construct pCi-VGLUT-EGFP. Arrows indicate the location and orientation of predicted genes. The exons are indicated by open boxes (noncoding regions) and solid boxes (coding regions). The numbers in parentheses below the diagram indicate the sizes of the exons and introns. **B:** An example of *GFP* expression in a *C. intestinalis* larva that developed from a fertilized egg electroporated with pCi-VGLUT-EGFP. Localization of GFP was visualized by immunofluorescent staining with an anti-GFP antibody. GFP localization was observed in the cell bodies of neurons located at the sites where the endogenous gene expression was observed by in situ hybridization. In neurons expressing *GFP*, axons were also labeled. An asterisk indicates a GFP-positive cell at the tip of the tail. ATENa, anterior apical trunk epidermal neuron; ATENp, posterior apical trunk epidermal neuron; DCEN, dorsocaudal epidermal neuron; oc, ocellus; ot, otolith; PN, papillar neuron; RTEN, rostoral trunk epidermal neuron; VCEN, ventrocaudal epidermal neuron. Scale bar = 100 μm.

positions and axonal trajectories for different populations of prospective glutamatergic neurons.

Papillar neurons. In *C. intestinalis* the adhesive organ contains six papillar neurons (Takamura, 1998). The papillar neurons are labeled with GFP and are therefore likely to be glutamatergic (Figs. 5, 6). Each of these neurons extends axons posteriorly and connects with one of the RTENs (Fig. 6A,B).

Trunk epidermal neurons. Both RTENs and ATENs are labeled with GFP (Figs. 5, 6). RTENs, which receive axonal input from papillar neurons, send a neurite into the brain vesicle (Fig. 6C–F). This neurite is presumed to be an axon because the corresponding region is clearly stained with the anti-Ci-VGLUT, as shown in Figure 4. The neurite terminates at the posterior brain vesicle, where glutamatergic inputs from the otolith and ocellus seem to occur (see below). In addition to the ATENs identified by Takamura (1998), a pair of two epidermal neurons is bilaterally located at the apical region of the trunk. These four neurons are located anterior to the previously identified ATEN and dorsal to the sensory pigment cells (Figs. 5, 6A). Therefore, we designated the newly identified neurons as anterior apical epidermal neurons (ATENa) and the previously identified ATENs as posterior apical epidermal neurons (ATENp). The neurites of ATENa extend posteriorly and end at the dorsolateral surface of the trunk (Fig. 7). The neurite of ATENp extends posteriorly and terminates at the anterior dorsal part of the tail; it seems to connect to the DCENs (Figs. 5B, 8B). Because the nerve tract between ATENp and DCENs were stained with anti-Ci-VGLUT (Fig. 4), it is likely to be axons of ATENp. The number and positions of RTENs and

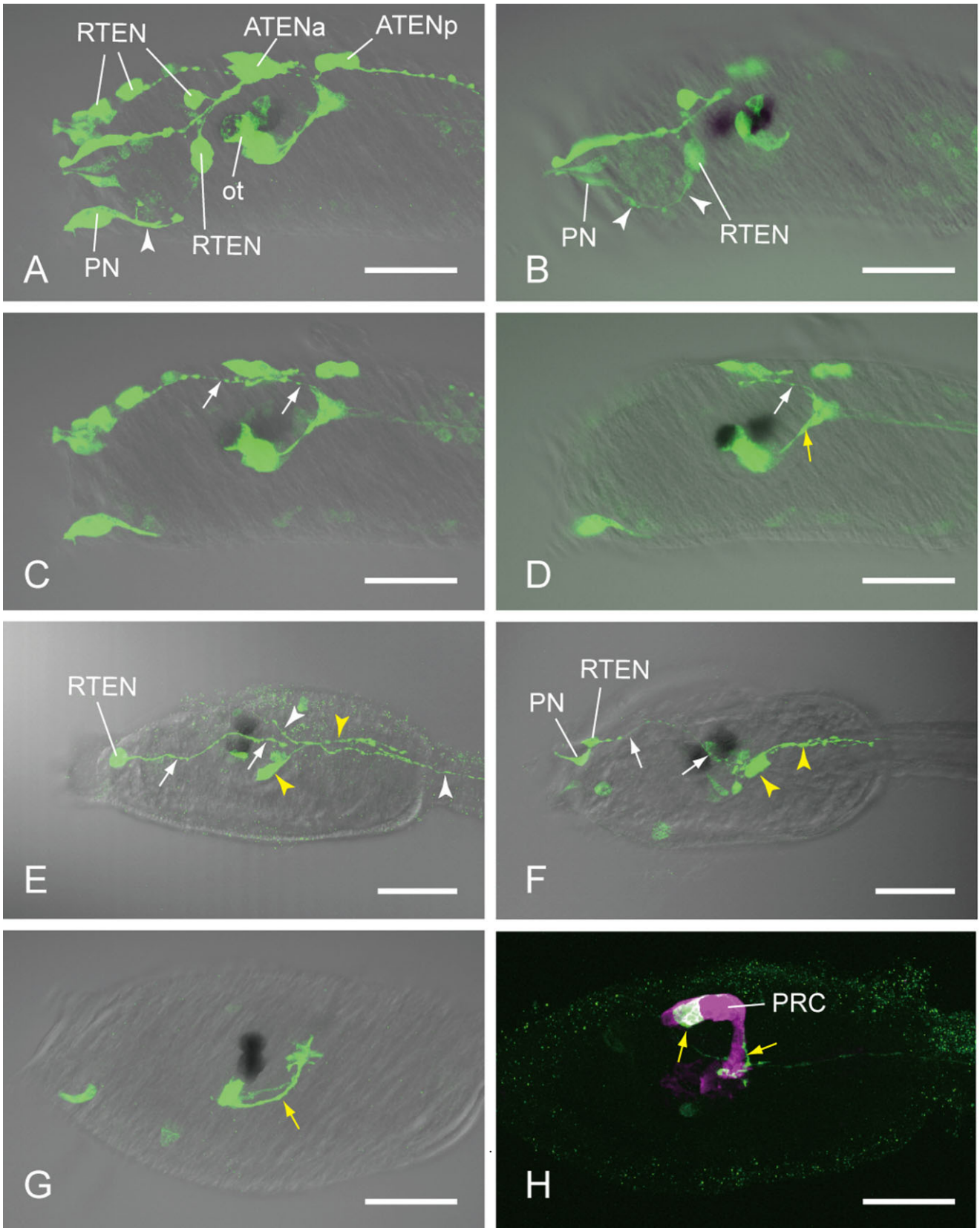

Figure 6

ATENs expressing *Ci-VGLUT* seemed to be consistent with those reported by Takamura (1998), except for ATENa, which were not reported in his article.

Caudal epidermal neurons. Caudal epidermal neurons seem to be glutamatergic (Fig. 5B). Their axons run along the dorsal and ventral midlines of the tail (Fig. 4). The axon of a DCEN located at the position about one-third of the tail length from the posterior tip runs a deeper path along the nerve cord and enters the posterior brain vesicle (Fig. 8). Another Ci-VGLUT-GFP-positive cell was often found at the tip of the tail (an asterisk in Figs. 5B, 8B). A *Ci-VGLUT* expressing cell was also found in this site by in situ hybridization, and an axon-like structure of this type of cell was observed in some specimens (Fig. 8C,D). Recently, Pasini et al. (2006) reported that caudal epidermal sensory neurons in *C. intestinalis* larvae are always found as pairs. Consistently, Ci-VGLUT-positive DCENs and VCENs are present as pairs; each pair consists of two cells adjacent to each other on dorsal or ventral midline of the tail (data not shown). Because two cells comprising a single pair are adjacent to each other, in situ hybridization signals of the two cells are not seen as two separate dots in the photograph shown in Figure 8C. The number of Ci-VGLUT-positive DCEN and VCEN pairs estimated by in situ hybridization varies among individuals: 7 to 20 pairs with an average of 15 pairs in total, n = 12 larvae analyzed. This is also consistent with the number of caudal epidermal sensory neurons reported by Pasini et al. (2006).

Ocellus. Photoreceptor cells were visualized with an antibody against *Ciona* arrestin Ci-arr (Nakagawa et al., 2002; Tsuda et al., 2003b). Photoreceptor cells are labeled with Ci-VGLUT-GFP, as clearly shown by double staining with Ci-arr (Fig. 6H). Axons of these cells project to the posterior brain vesicle. This suggests that some of the photoreceptor cells are glutamatergic like vertebrate retinal photoreceptor cells. As shown in Figure 6H, GFP signal was frequently observed in anterior population of photoreceptor cells while photoreceptor cells in posterior region of the ocellus were not labeled. Therefore, some photoreceptor cells might use neurotransmitters other than glutamate.

Otolith. The otolith cell is labeled with Ci-VGLUT-GFP (Figs. 5B, 6A). Its axon seems to connect to another prospective glutamatergic neuron(s) that resides ventral to the otolith. This secondary neuron(s) sends an axon to the posterior brain vesicle where photoreceptor nerve terminals are also located (Fig. 6D).

Other neurons in the brain vesicle. A group of cells located at the posterior part of the brain vesicle seem to be glutamatergic. These cells send neurites posteriorly to the visceral ganglion (Fig. 6E,F). These neurites seem to be axons because Ci-VGLUT-immunoreactivity was observed in a similar pattern (Fig. 4).

Visceral ganglion. We did not find any *Ci-VGLUT-GFP* expression in the visceral ganglion.

Fig. 6. Morphology of prospective glutamatergic neurons in the trunk of *C. intestinalis* larvae. Prospective glutamatergic neurons were visualized by immunofluorescent staining of GFP in pCi-VGLUT-EGFP-transfected larvae. **A:** A combined confocal microscope image of 46 optical sections (each 0.9 μm in depth) of the trunk of a larva. **B–D:** Confocal microscope images at different depths of the same series of optical sections as shown in A. In B (a combined image of optical sections #8–10) the axonal pathway from a papillar neuron connecting to one of the rostral trunk epidermal neurons is indicated by arrowheads. In C (a combined image of optical sections #23–46), afferent axons from rostral trunk epidermal neurons are indicated by white arrows. In D, nerve terminals from both rostral trunk epidermal neurons (white arrow) and otolith-associated neurons (yellow arrow) can be seen in one optical section (optical section #25). **E–G:** Examples of peripheral and central prospective glutamatergic neurons in the trunk region. White arrows indicate an axon of a rostral trunk epidermal neuron projecting to the posterior brain vesicle. A group of prospective glutamatergic neurons located in the posterior brain vesicle sends axons posteriorly to the visceral ganglion (yellow arrowheads). An axon from a caudal epidermal neuron runs along the visceral ganglion and projects onto the posterior brain vesicle (white arrowheads in E). A group of prospective glutamatergic neurons adjacent to the sensory pigment cells sends axons to the posterior brain vesicle (yellow arrow in G). **H:** Double immunofluorescent staining of GFP (green) with a photoreceptor-specific marker, arrestin (magenta). Some of the photoreceptor cells (PRC) express Ci-VGLUT-GFP (yellow arrows). Scale bars = 50 μm.

DISCUSSION

Ciona VGLUT ortholog and diversity of the VGLUT family in chordates

The present study identified *Ci-VGLUT*, an ortholog of the vertebrate *VGLUTs* in *C. intestinalis*. The whole genome analysis indicates that the *Ciona* genome contains only one *VGLUT* family gene. Molecular phylogenetic analysis strongly suggest that Ci-VGLUT is a vesicular glutamate transporter. In mammals, there are three VGLUT isoforms, VGLUT1, VGLUT2, and VGLUT3, each encoded by a different gene. Similarly, the zebrafish genome contains at least one *VGLUT1* ortholog and two *VGLUT2* orthologs (Higashijima et al., 2004a). The molecular phylogeny indicates that Ci-VGLUT is more closely related to vertebrate VGLUTs than *C. elegans* and *Drosophila* VGLUTs. The position of Ci-VGLUT is basal to all members of the vertebrate VGLUT family. Therefore, three subtypes of vertebrate VGLUTs are most likely to have appeared by means of gene duplications after the divergence between tunicates and vertebrates.

Sequence comparison with VGLUTs from other animals revealed both conserved and divergent features of Ci-VGLUT. The primary structure of the core region from TM1 to 10 is highly conserved not only in transmembrane regions but also in luminal and cytoplasmic loops, a potential hydrophobic helix (HH), and a potential GxxxG motif and Amphipathic Helix (GAH) (Fei et al., 2007). An N-glycosylation site between TM1 and 2 is conserved among all known VGLUTs, including those of *Ciona*, vertebrates, *Drosophila*, and *C. elegans*. These conserved features of the core region of Ci-VGLUT suggest conserved function of the protein, presumably as a vesicular glutamate transporter. Contrary to the highly conserved core region, the primary sequence of N-terminus is only moderately conserved and that of C-terminus is poorly conserved between Ci-VGLUT and VGLUTs of other organisms. The N- and C-terminal tails of VGLUTs are thought to face the cytoplasm and to contribute to functional differences and/or differential targeting to synaptic vesicles among isoforms via protein–protein interaction (Takamori, 2006; De Gois et al., 2006). One of the two polyproline motifs found in the C-terminus of mammalian VGLUT1 is required for binding specificity to endophilin 1 and endophilin 3,

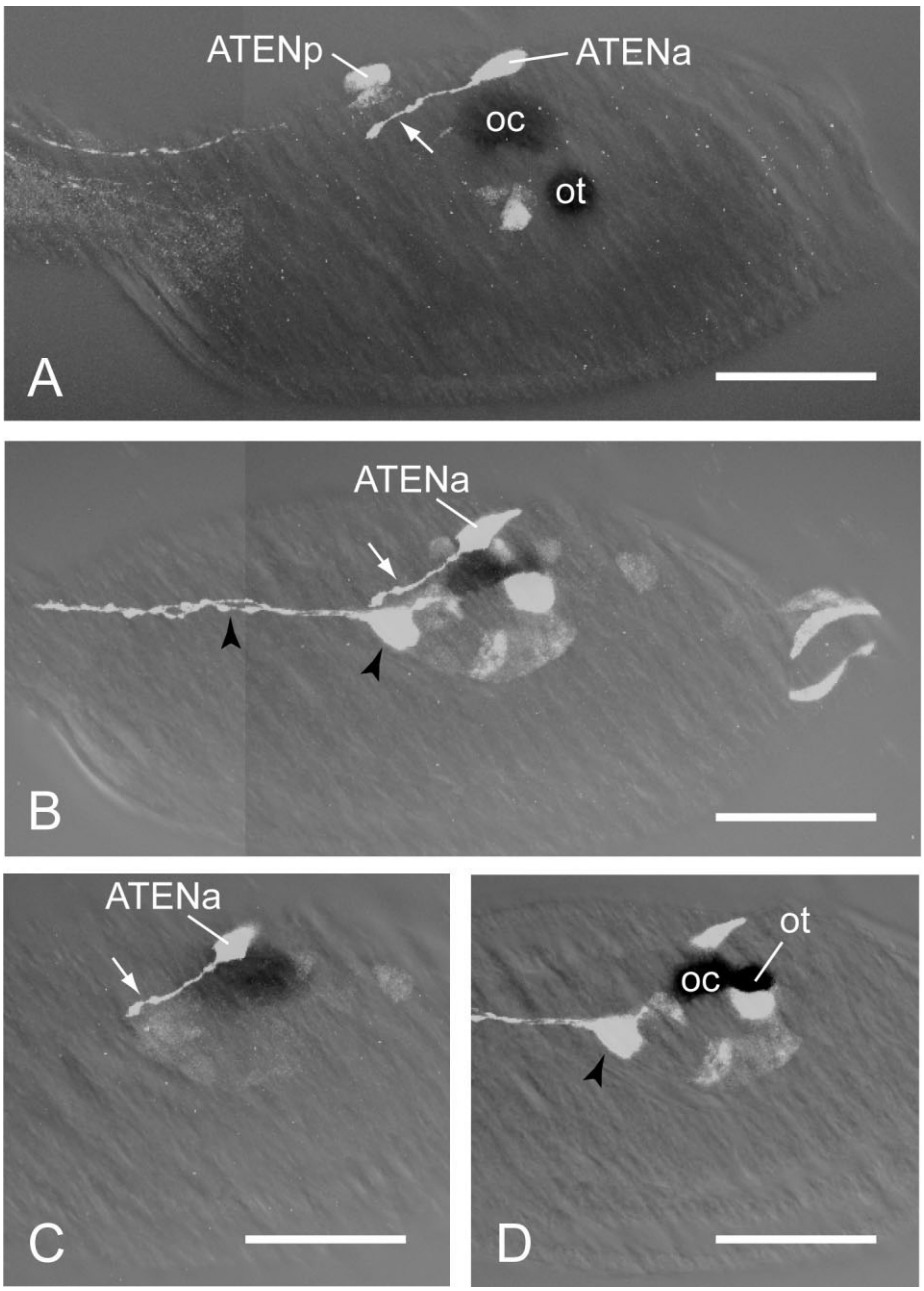

Fig. 7. Morphology of anterior apical trunk epidermal neurons (ATENa). A bilaterally aligned pair of two epidermal neurons is located dorsal to the sensory pigment cells. These cells are different from and located anterior to the previously identified apical trunk epidermal neurons (ATENp) (Takamura, 1998). **A,B:** Two examples of pCi-VGLUT-EGFP-transfected larvae that express GFP in ATENa. **C,D:** Confocal images at different depths of the same specimen as shown in B. Although the axon terminal of ATENa appears to occur close to or within the posterior sensory vesicle in the combined confocal image (B), the optical sections indicate that the axon actually ends at the dorsolateral surface of the trunk (C). Note that the axon terminal of ATENa is not seen in optical sections in which prospective glutamatergic neurons at the posterior brain vesicle are clearly seen (D). oc, ocellus; ot, otolith. Scale bars = 50 μm.

proteins considered to play an integral role in glutamatergic vesicle formation (De Gois et al., 2006). The C-terminus of Ci-VGLUT lacks polyproline motifs, suggesting functional regulation of Ci-VGLUT is different from that of mammalian VGLUT1. Because polyproline motifs are also not found in mammalian VGLUT2 and VGLUT3 as well as in nonmammalian VGLUTs, association with endophilins 1 and 3 may be a recently acquired regulatory mechanism for VGLUT1-mediated glutamatergic transmission in mammals. Lack of the highly conserved SEE/DK and SYGAT motifs in the C-terminus of Ci-VGLUT may indicate its unique function or regulation, which remains to be elucidated in future studies.

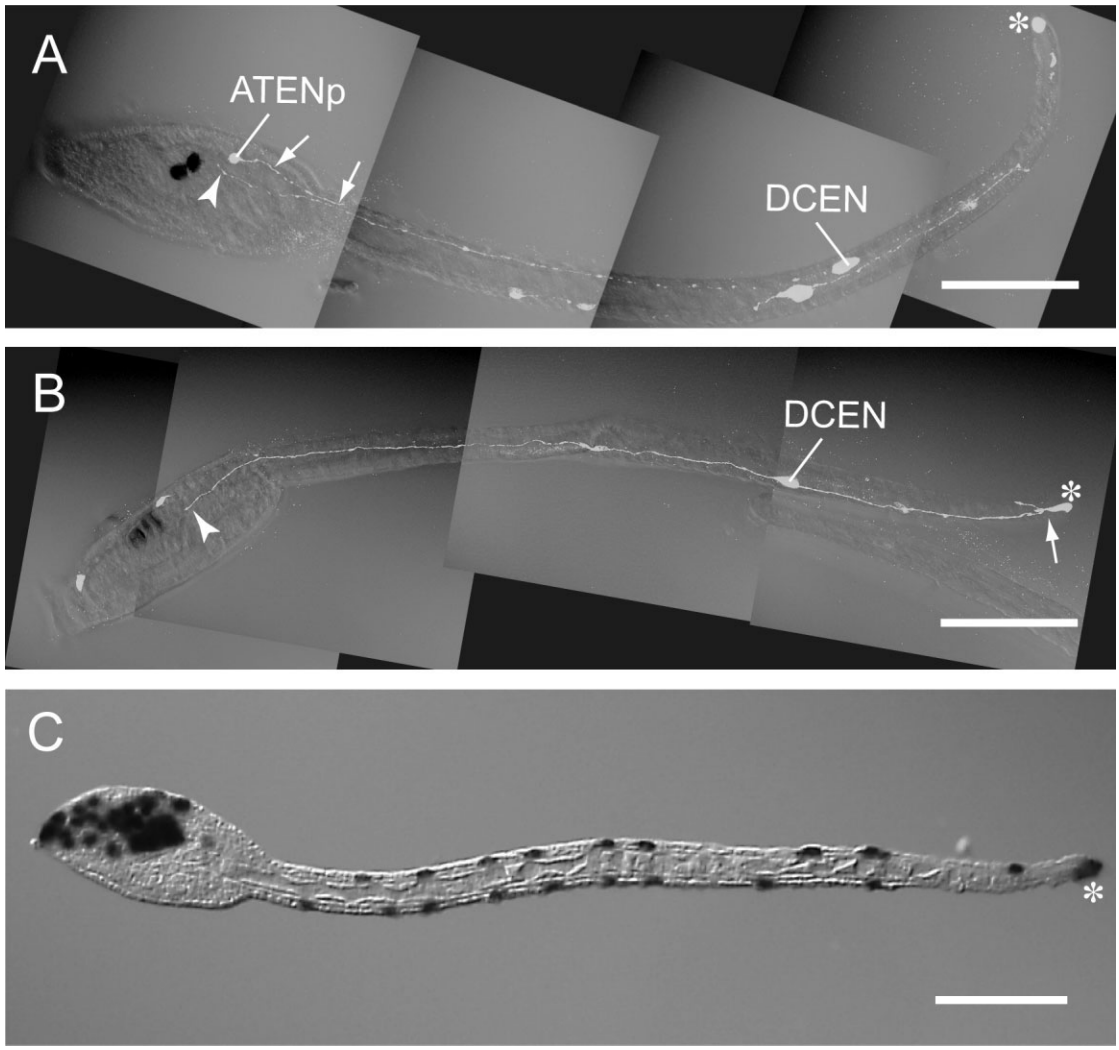

Fig. 8. Afferent axon of a dorsocaudal epidermal neuron. **A,B:** Two examples of pCi-VGLUT-EGFP-transfected larvae that express GFP in DCEN. The axon from a dorsocaudal epidermal neuron runs anteriorly along the nerve cord and visceral ganglion and ends at the posterior brain vesicle (arrowheads). Arrows in A indicate the axon of a posterior apical trunk epidermal neuron. Asterisks indicate a cell expressing *Ci-VGLUT-GFP* at the tip of the tail. An arrow in B indicates an axon-like protrusion of the GFP-positive cell at the tip of the tail. **C:** A larva showing *Ci-VGLUT* expression at the tip of the tail (asterisk) visualized by whole-mount in situ hybridization. ATENp, posterior apical trunk epidermal neuron; DCEN, dorsocaudal epidermal neuron. Scale bars = 100 μm.

Ci-VGLUT as a glutamatergic neuron marker

VGLUTs have been used as a reliable marker for glutamatergic neurons in *C. elegans* (Lee et al., 1999), *Drosophila* (Mahr and Aberle, 2006), zebrafish (Higashijima et al., 2004a,b), *Xenopus* (Gleason et al., 2003), and mammals (Fremeau et al., 2004; Hajszan et al., 2004; Moutsimilli et al., 2005; Nakamura et al., 2005). In the present study we used Ci-VGLUT as a specific marker for prospective glutamatergic neurons in three different ways: in situ hybridization, immunohistochemistry, and promoter-*GFP* transgenics. All the techniques consistently visualized particular populations of neurons in both the central and peripheral nervous systems. In situ hybridization of *Ci-VGLUT* visualizes cell bodies, while immunofluorescent staining with anti-Ci-VGLUT visualizes axons, especially their synaptic terminals. Expression of the *GFP* transgene driven by the 4.6-kb upstream region of *Ci-VGLUT* can recapitulate the endogenous *Ci-VGLUT* expression. The GFP localization labels both cell bodies and whole axons so that we can determine the axonal trajectory of each neuron. Thus, the combinatorial usage of the imaging protein, mRNA, or promoter activity of Ci-VGLUT provides conclusive clues to the distribution of glutamatergic neuronal elements in the *C. intestinalis* larva (Fig. 9).

Prospective glutamatergic neurons in the peripheral nervous system

Our results suggest that most of the peripheral neurons are glutamatergic in the ascidian larva. Although the functions of the PNS of ascidian larvae are poorly understood, morphological studies have suggested that most of the peripheral neurons are sensory (Torrence and Cloney,

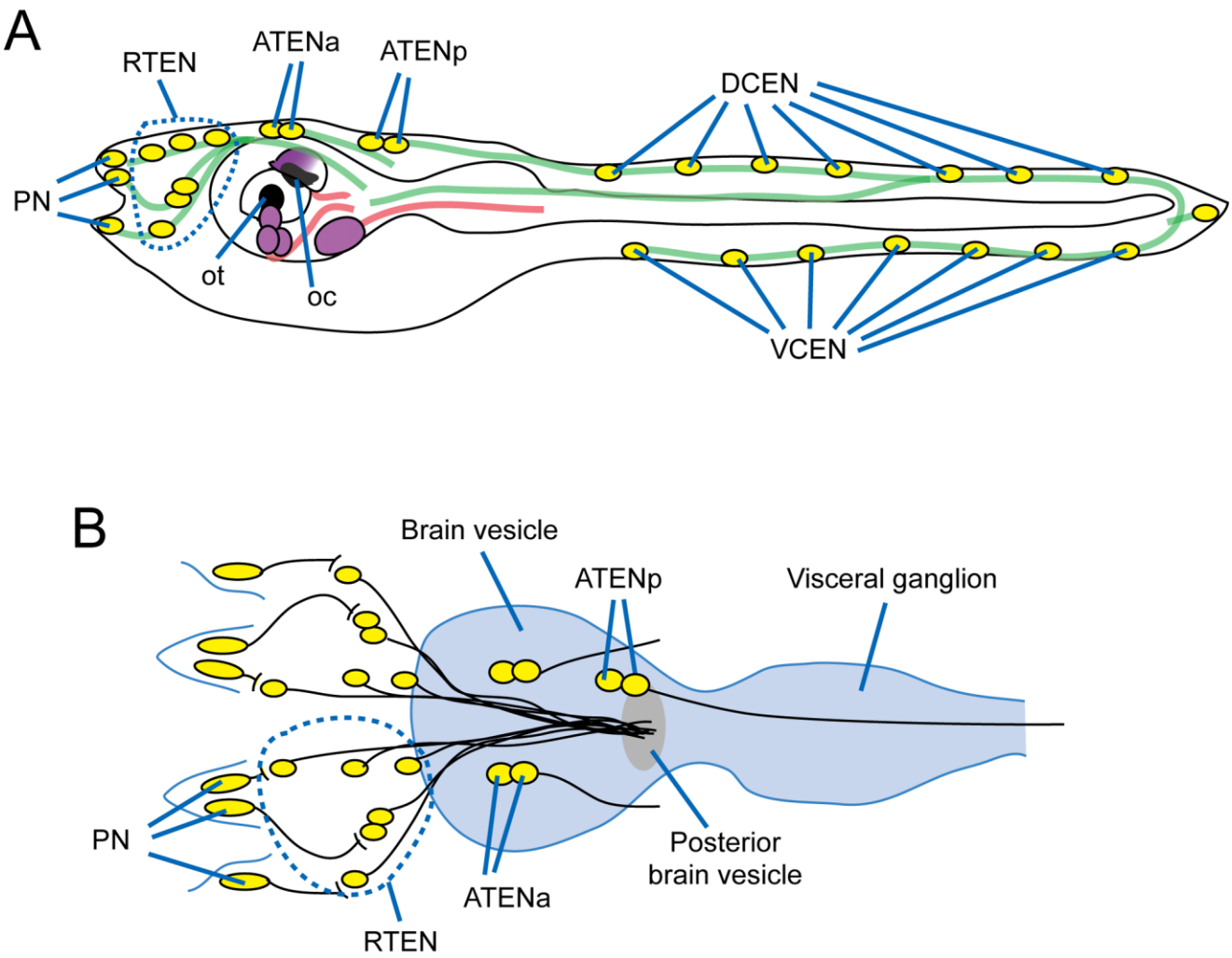

Fig. 9. Summary of Ci-VGLUT-positive neurons identified in this study. Cell bodies of Ci-VGLUT-positive peripheral neurons are indicated in yellow ellipses. Populations of Ci-VGLUT-positive neurons in the brain vesicle are indicated in purple. Purple gradient of the ocellus indicates the possibility that some of the photoreceptor cells are not glutamatergic (Horie et al., unpubl. data). **A:** Prospective glutamatergic networks in the *C. intestinalis* larva. Axon pathways from peripheral neurons are indicated by green lines and those from central neurons are indicated by orange-red lines. **B:** Axon pathways from papillar neurons and trunk epidermal neurons. ATENa, anterior apical trunk epidermal neuron; ATENp, posterior apical trunk epidermal neuron; DCEN, dorsocaudal epidermal neuron; oc, ocellus; ot, otolith; PN, papillar neuron; RTEN, rostral epidermal neuron; VCEN, ventrocaudal epidermal neuron.

1982, 1983; Crowther and Whittaker, 1994; Takamura, 1998).

The ciliated caudal epidermal neurons are thought to be mechanoreceptors (Torrence and Cloney, 1982; Crowther and Whittaker, 1994). Previous studies have reported that axons of the caudal epidermal neurons extend anteriorly to the CNS (Torrence and Cloney, 1982; Takamura, 1998; Ohtsuka et al., 2001). In the present analysis, we showed by using GFP reporter under the control of the *Ci-VGLUT* promoter that the caudal epidermal neurons send axons directly into the posterior brain vesicle, where they seem to form synapses. Thus, the posterior brain vesicle may receive sensory inputs from the caudal epidermal neurons. We also found that some of the caudal epidermal neurons send axons posteriorly; the neuronal connections of these axon are not clear but they run along the midline of the tail and may connect with other caudal epidermal neurons.

Based on behavioral and electron microscopic observations, Torrence and Cloney (1983) proposed that the adhesive organ of ascidian larvae has both mechanosensory and chemosensory functions. The papillae of the adhesive organ of the ascidian *Diplosoma macdonaldi* larva contain two types of sensory neurons: the anchor cells and basal cells (Torrence and Cloney, 1983). The basal sensory cells resemble the caudal sensory neurons; they are primary neurons whose presumptive sensory ending is a long cilium that arises from a deep apical pocket. Although the morphological and functional properties of papillar neurons are not fully elucidated in *Ciona*, the Ci-VGLUT-positive papillar neurons may have characteristics similar to those of the basal sensory cells of *Diplosoma*. Axons of

the papillar neurons extend posteriorly and seem to connect with the rostral trunk epidermal neurons, which are also Ci-VGLUT-positive. The rostral epidermal neurons then in turn send axons into the posterior brain vesicle. Therefore, sensory inputs from the adhesive papillae are also likely to be received by the posterior brain vesicle.

The apical epidermal neurons (ATENa and ATENp) are also Ci-VGLUT-positive, but their axons do not enter the brain vesicle. The putative axon of ATENp extends to the anterior dorsal region of the tail. It seems to constitute a continuous network with DCENs. The axons of ATENa terminate at the lateral surface of the trunk. There has been no report on the nervous or sensory structures in this region, and therefore the synaptic target of ATENa is unknown.

Very recently, while the earlier version of this article was under review, Imai and Meinertzhagen (2007) reported detailed morphology of neurons and axon-pathways in the peripheral nervous system of the *C. intestinalis* larva. There are some important differences between the present observations and their results. They reported that neurons in the adhesive papillae caudally project axons toward the brain vesicle, while our results suggest that Ci-VGLUT-positive papillar neurons do not directly project to the brain vesicle. Other important differences are the projection patterns of ATENs. Imai and Meinerthagen (2007) also identified two types of ATENs: anterior ATEN (aATEN) and posterior ATEN (pATEN), but locations of their axon terminals are different from those of ATENa and ATENp reported in this study. Because the two studies used a promoter of different genes to express GFP, different neurons of similar location might be visualized in each study. Future studies, including specific labeling of other neurotransmitter systems and double labeling using different promoters and reporters, would elucidate these seemingly contradictory observations.

Some features of the *Ciona* DCENs are reminiscent of the Rohon–Beard primary sensory neurons of anamniote vertebrates. The Rohon–Beard primary sensory neurons originate from cells at the neural plate border (Cornell and Eisen, 2002). The *Ciona* DCENs develop in the dorsal midline epidermis which shares with the vertebrate neural plate border its position within the embryos and the expression of orthologous genes (Pasini et al., 2006). The Rohon–Beard neurons are dorsocaudally located primary mechanoreceptors that innervate the skin and myotome, project rostrally to the hindbrain, and control early swimming behavior (Hughes, 1957; Roberts and Clarke, 1982; Nordlander, 1984). Similarly, DCENs of the *Ciona* larva are located in the dorsocaudal region and project rostrally to the posterior brain vesicle. Furthermore, the descending processes from these neurons extending into the dorsal fin tunic have been observed (Pasini et al., 2006; Imai and Meinertzhagen, 2007). Despite these similarities, important differences are also present. First, in vertebrate embryos development of sensory neurons, including the Rohon–Beard cells, requires Neurogenin function (Cornell and Eisen, 2002), whereas this may not be the case in epidermal sensory neurons of *Ciona* larvae according to the expression patterns of the *neurogenin* ortholog (Imai et al., 2004; Mazet et al., 2005). Second, the Rohon–Beard cells are located within the spinal cord, while ascidian DCENs are located in the epidermis. Certainly, further detailed comparison of developmental mechanisms and physiology of these neurons will be needed for better understanding of the evolutionary implication.

A unique feature of Ci-VGLUT-positive network is interconnections between sensory epidermal neurons. The papillar neurons project to RTENs and ATENp project to the DCENs of the tail. The interconnected network of epidermal sensory neurons of ascidian larvae is somewhat reminiscent of the neuroepidermal network found in hemicordates (Lowe et al., 2003). This similarity might be a conserved feature shared by the two organisms, although their developmental modes are greatly diverged.

Prospective glutamatergic neurons in the CNS

In the CNS of the *C. intestinalis* larva, prospective glutamatergic neurons were only detected in the brain vesicle. Interestingly, glutamatergic neurons were not found in the visceral ganglion or caudal nerve cord. In the brain vesicle the sensory cells of the pigmented sensory organs, the otolith and ocellus, appeared to be glutamatergic. The otolith has been proved to be a gravity sense organ (Tsuda et al., 2003a). The afferent synapses of hair cells in the vertebrate inner ear, which are the vertebrate gravity sensor, also use glutamate as a major transmitter (Ottersen et al., 1998). Glutamatergic transmission is likely to be a common feature in chordate gravity sense organs. Similarly, the use of glutamate by the photoreceptor cells is probably a characteristic shared between the ascidian ocellus and the vertebrate retina. These similarities between vertebrates and ascidians suggest that glutamatergic transmission in cranial photo- and mechanosensory cells has a deep evolutionary origin that predates the emergence of the last common ancestor of tunicates and vertebrates. Interestingly, expression of *Ci-VGLUT-GFP* was not found in photoreceptor cells in the posterior region of the ocellus. This raises a possibility that some photoreceptor cells use neurotransmitters other than glutamate. Details of neurotransmitter phenotypes of photoreceptor cells and their physiology are currently under investigation.

Glutamatergic inputs from the ocellus and otolith occur at sites close to each other in the posterior brain vesicle. Axons of the ocellus photoreceptor cells directly innervate this area, while the otolith inputs are mediated by a glutamatergic interneuron(s). Another group of prospective glutamatergic interneurons in the posterior brain vesicle sends axons posteriorly to the visceral ganglion. The glutamatergic interneurons in the *Ciona* brain vesicle may be evolutionary counterparts of glutamatergic interneurons in the vertebrate central nervous system.

Posterior brain vesicle as a processing center that integrates sensory inputs

The present study revealed that axons from most sensory systems in the ascidian larva are glutamatergic and end at the posterior brain vesicle. The posterior brain vesicle contains glutamatergic, cholinergic, and GABAergic neurons, all of which send axons to the visceral ganglion (Horie et al., unpubl. data; Yoshida et al., 2004). In *Ciona* larvae, 10 cholinergic motor neurons (Horie et al., unpubl. data; Takamura et al., 2002; Yoshida et al., 2004) reside in the visceral ganglion and innervate the tail muscle. Thus, the posterior brain vesicle is presumed to serve as a processing center that integrates sensory inputs and modulates motor outputs. With the simple organization of

the sensory and motor systems of the ascidian larva, elucidation of the precise neuronal connections within the posterior brain vesicle should give insight into the mechanisms of the sensory integration and motor control of the simple chordate.

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