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RESEARCH ARTICLE

Development of the GABA-ergic signaling system and its role in larval swimming in sea urchin

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SUMMARY

The present study aimed to elucidate the development and γ-amino butyric acid (GABA)-ergic regulation of larval swimming in the sea urchin *Hemicentrotus pulcherrimus* by cloning glutamate decarboxylase (*Hp-gad*), $GABA_A$ receptor (*Hp-gabrA*) and $GABA_A$ receptor-associated protein (*Hp-gabarap*), and by performing immunohistochemistry. The regulation of larval swimming was increasingly dependent on the GABAergic system, which was active from the 2 days post-fertilization (d.p.f.) pluteus stage onwards. GABA-immunoreactive cells were detected as a subpopulation of secondary mesenchyme cells during gastrulation and eventually constituted the ciliary band and a subpopulation of blastocoelar cells during the pluteus stage. *Hp-gad* transcription was detected by RT-PCR during the period when Hp-Gad-positive cells were seen as a subpopulation of blastocoelar cells and on the apical side of the ciliary band from the 2 d.p.f. pluteus stage. Consistent with these observations, inhibition of GAD with 3-mercaptopropioninc acid inhibited GABA immunoreactivity and larval swimming dose dependently. *Hp-gabrA* amplimers were detected weakly in unfertilized eggs and 4 d.p.f. plutei but strongly from fertilized eggs to 2 d.p.f. plutei, and Hp-GabrA, together with GABA, was localized at the ciliary band in association with dopamine receptor D1 from the two-arm pluteus stage. *Hp-gabarap* transcription and protein expression were detected from the swimming blastula stage. Inhibition of the $GABA_A$ receptor by bicuculline inhibited larval swimming dose dependently. Inhibition of larval swimming by either 3-mercaptopropionic acid or bicuculline was more severe in older larvae (17 and 34 d.p.f. plutei) than in younger ones (1 d.p.f. prism larvae).

Key words: γ-amino butyric acid, $GABA_A$ receptor, GABARAP, GAD, secondary mesenchyme cell, ciliary band.

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INTRODUCTION

The molecule γ-amino butyric acid (GABA) is synthesized by nearly all organisms from bacteria (Ackermann, 1910) to humans (Elliott and Jasper, 1959). The presence of GABA and the similarity of its signaling pathway among a broad range of organisms suggest the importance of its functions (Jelitai and Madarasz, 2005).

GABA is the predominant inhibitory neurotransmitter in the mammalian central nervous system (Tano et al., 2009). After its release from nerve terminals, GABA binds to at least two classes of postsynaptic receptor, $GABA_A$ and $GABA_B$ (Simeone et al., 2003). The $GABA_A$ receptor ($GABA_AR$) and $GABA_AR$-associated protein (GABARAP) were found to be localized close together by immunocytochemical analysis (Wang et al., 1999). Close localization of the GABA biosynthetic enzyme, glutamate decarboxylase (GAD), with GABA and extracellular release of GABA have been reported in sponges (Ramoino et al., 2007).

The swimming activity of sea urchin larvae depends strongly on the larval serotonergic nervous system (Katow et al., 2004; Katow et al., 2007) and dopaminergic regulatory system (Katow et al., 2010). These serotonergic and dopaminergic regulatory systems are regarded as excitatory (Varju et al., 2001; Li et al., 2006). However, it has also been shown that the musculature of isolated tube feet of several species of sea urchin contracts in response to GABA, and the action of GABA is blocked by bicuculline (Florey et al., 1975), which inhibits the function of $GABA_AR$ (Owens and Kriegstein, 2002). Pharmacological and physiological studies revealed the presence of GABA receptors at the echinoderm neuromuscular junction (Devlin, 2001), which indicated the involvement of the GABA signaling system in promoting the mobility of sea urchins. GABA also induces settlement, that is, a cessation of swimming activity, in marine invertebrate larvae such as the planktonic larvae of the red abalone, *Haliotis rufescens* (Morse et al., 1979), and the sea urchin *Strongylocentrotus droebachiensis* (Pearce and Scheibling, 1990), which suggests that this neurotransmitter is also involved in the swimming of marine larvae.

Regarding these previous reports, it is conceivable that the swimming of sea urchin larvae is controlled by the GABA signaling system in combination with the serotonergic nervous system and the dopamine regulation system, which have already been reported to be involved in such control. The sea urchin genome database SpBase (http://www.spbase.org/SpBase/index.php) strongly suggests the presence of the genes and proteins that comprise the GABA signaling system in sea urchin. However, the actual signaling system has not been characterized in sea urchin larvae or adults to date. By referring to SpBase, the aim of the present study was to characterize the localization of GABA, GAD, $GABA_AR$ and GABARAP in embryos and larvae of the sea urchin *Hemicentrotus pulcherrimus*. In addition, we investigated how these components of the GABA signaling system function in larval swimming by analyzing: (1) single- and double-staining immunohistochemistry results obtained by confocal laser scanning microscopy using various combinations of antibodies against components of the GABA signaling system, (2) the gene expression pattern of the relevant proteins, (3) the results of assays of larval swimming activity

conducted in the presence of 3-mercaptopropionic acid (3-MPA, a GAD inhibitor) (van der Heyden and Korf, 1978) and bicuculline (a $GABA_AR$ inhibitor), and (4) previous findings on neuronal systems that regulate larval swimming.

MATERIALS AND METHODS

Incubation of gametes and zygotes

Sea urchins, *H. pulcherrimus* (A. Agassiz), were collected around the Research Center for Marine Biology, Tohoku University, Japan. Gametes were obtained by intracoelomic injection of 0.5 mol l^{-1} KCl. Eggs were inseminated and incubated in filtered seawater (FSW) on a gyratory shaker or stirred gently with a propeller in an incubator at 15 or 18°C until the appropriate developmental stages were reached. Larvae were fed with *Chaetoceros calcitrans* (Nisshin Marine Tech. Ltd, Yokohama, Japan) from 4 days after fertilization until the day described in the text.

RT-PCR

Total RNA was isolated from unfertilized eggs, eggs at 20 min after insemination, 15 h post-fertilization (h.p.f.) swimming blastulae, 18 h.p.f. early gastrulae, 19–20 h.p.f. mid-gastrulae (gastrulation half-completed), 1 day post-fertilization (d.p.f.) prism larvae, and 2, 4 and 34 d.p.f. plutei. The RNA was obtained by dissolving the samples in Isogen (NIPPON GENE, Tokyo, Japan); it was used for PCR with SuperScript II Reverse Transcriptase and Oligo-$d(T)_{12–18}$ Primer (both from Invitrogen, Tokyo, Japan). The following eight primers were used for PCR amplification of GAD (*Hp-gad*), $GABA_AR$ (*Hp-gabrA*), GABARAP (*Hp-gabarap*) and ubiquitin (*Hp-ubi*), respectively (Table 1).

Table 1. Primers

Gene	Direction	Primer sequence (5′–3′)
Hp-gad	Forward	ACTACGCGCCAAAGACCTTC
	Reverse	TCCACGTCTCTTGCATGCTG
Hp-gabrA	Forward	GTCGCCCTGGGCATCACGAC
	Reverse	CGGTTGATGTCCTGCGATGT
Hp-gabarap	Forward	CAAAAGGCAGCCAAAGCAAGA
	Reverse	GCTCCATACACACTTTCATCGG
Hp-ubi	Forward	GAGCTGCGATGTATTTGCCAGATG
	Reverse	TTTGATGGAATAACAAATAACTGATTGCTT

Genes were glutamate decarboxylase (*Hp-gad*), $GABA_A$ receptor (*Hp-gabrA*), $GABA_A$ receptor-associated protein (*Hp-gabarap*) and ubiquitin (*Hp-ubi*) from *Hemicentrotus pulcherrimus*.

DNA sequencing

The *Hp-gabrA* amplimers were purified using a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Ltd, Little Chalfont, Bucks, UK), and these amplimers were sequenced directly. The *Hp-gad* and *Hp-gabarap* amplimers were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and ligated into the vector pGEM-T Easy (Promega). The recombinant plasmids were introduced into *E. coli* JM109 competent cells (Takara Bio. Inc., Tokyo, Japan). The plasmids were recovered using a GenElute Plasmid Mini-Prep Kit (Sigma, St Louis, MO, USA) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and Applied Biosystems 3730xl DNA Analyzer (PE Applied Biosystems, Tokyo, Japan)

Primary antibodies

Primary antibodies against Hp-GabrA and Hp-Gabarap were generated in our laboratory as described below, and those against GAD, GABARAP and GABA were commercially purchased. Epithelial cell-specific Epith-2 monoclonal antibody was produced in our laboratory (Kanoh et al., 2001). Anti-DRD1 antiserum was kindly provided by Dr Suyemitu, Saitama University, Japan. Details about the antisera are described below (see 'Antibody generation' and 'Whole-mount immunohistochemistry' sections).

Antibody generation

Antisera against Hp-GabrA and Hp-Gabarap were raised in accordance with a method described previously (Katow, 2008) using the amino acid sequences Y^{115}AYSEDDVKFKWLENG^{130} from SPU_028560.GR-2 for Hp-GabrA and P^{10}FEKRRQEGEKIR^{22} from SPU_006271.1 for Hp-Gabarap. These predicted partial amino acid sequences of the sea urchin *S. purpuratus* are very similar to those of the present sea urchin *H. pulcherrimus*. Previous analyses of the structure of genes and proteins also indicate a similarity between *S. purpuratus* and *H. pulcherrimus* (Yaguchi and Katow, 2003; Katow et al., 2004; Hara and Katow, 2005; Katow 2008; Katow et al., 2010). The amino acid sequences were chosen on the basis of a Physico-Chemical Profiles analysis within Network Protein Sequence Analysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_pcprof.html), and are specific to $GABA_AR$ protein of invertebrates and GABARAP of both vertebrates and invertebrates according to a BLAST search.

Whole-mount immunohistochemistry

Embryos and larvae were fixed as described before in 4% paraformaldehyde diluted in FSW for 20 min at ambient temperature (Katow et al., 2010) at the 6–7 h.p.f. morula stage, 15 h.p.f. swimming and 17 h.p.f. mesenchyme blastula stage, 18 h.p.f. early gastrula, 20 h.p.f. mid-gastrula, 23 h.p.f. late gastrula or prism larva stage, and 2, 7, 17, 30 and 34 d.p.f. pluteus stage, and were dehydrated in increasing concentrations of ethanol and stored in 70% ethanol at 4°C until use.

For whole-mount immunohistochemistry (IHC), the samples were hydrated in decreasing concentrations of ethanol and 0.1 mol l^{-1} phosphate-buffered saline with 1% Tween-20 (PBST). They were then incubated for 24–48 h at 4°C with anti-GABA monoclonal antibody raised against GABA conjugated to BSA by glutaraldehyde fixation (diluted 1:150 in PBST; GB69 clone, Sigma), an anti-rat GAD ($GAD_{65/67}$) rabbit polyclonal antibody raised against a synthetic peptide comprising the sequence F^{572}LIEEIERLGQDL^{585} (Enzo Life Sciences International, Plymouth Meeting, PA, USA), anti-GABARAP mouse antibodies (diluted 1:500 in PBST) or Epith-2 monoclonal antibody. The primary antibodies were visualized using Alexa Fluor 488-tagged anti-rabbit or Alexa Fluor 488-tagged anti-mouse IgG (diluted 1:500–1000 in PBST; Invitrogen, Paisley, UK) for 2 h at ambient temperature. The samples were counter-stained for nuclear DNA with 1 µmol l^{-1} propidium iodide (PI; SERVA Electrophoresis GmbH, Heidelberg, Germany) for 2–5 min during the final wash in PBST. The antigen peptide of the anti-rat $GAD_{65/67}$ rabbit antibody (F^{572}LIEEIERLGQDL^{585}) has a similar sequence to that of the equivalent peptide in sea urchin GAD, F^{598}MLDEIERLGKPL^{610} (SPU_002857; http://www.spbase.org/SpBase/search/seqPopUp.php?disp=Pep&id=SPU_002857&version=1&sub_version=none). Thus, the immunospecificity of this anti-$GAD_{65/67}$ rabbit antibody was examined by adsorbing the antibody [diluted 1:1000 in Tris-base saline with 1% Tween-20 (TBST)] with 10 mg ml^{-1} antigen peptide for 2 h before use in immunoblotting. The immunospecificity of the other antibodies was examined using antibodies that had been pre-incubated with 3 mg ml^{-1} GABA or glycine (for non-specific binding assay) for the anti-GABA antibody,

the synthetic FEKRRSEGEKI or PFEKRRQEGEKIR peptide for the anti-GABARAP antibody, and the YAYSEDDVKFKWLENG peptide for the anti-$GABA_AR$ antibody. These pre-adsorbed antibodies were substituted for non-adsorbed antibodies and examined as stated above.

Comparative localization of Hp-GabrA and sea urchin dopamine receptor D1 (DRD1) was conducted in 34 d.p.f. plutei using anti-DRD1 rabbit antibody (diluted 1:500 in PBST) (Katow et al., 2010) and the mouse anti-GabrA antiserum as described above.

For double-staining with two different primary antibodies, visualization was performed with Alexa Fluor 488-tagged antibody or Alexa Fluor 594-tagged antibody as described previously (Katow et al., 2010). The samples were examined under a Micro-Radiance Confocal Laser Scanning Microscope (Bio-Rad Microscience, Hemel Hempstead, UK) using 0.1–4 μm thick optical sections. Images were analyzed with the publicly available image analysis software ImageJ (National Institutes of Health, http://rsbweb.nih.gov/ij/), and Adobe Photoshop CS2 software (ver. 9.02, Adobe Systems Inc., San Jose, CA, USA).

Swimming assay of larvae treated with 3-MPA or bicuculline

To examine the involvement of GABA in larval swimming, 3-MPA was applied to 1 d.p.f. prism larvae and 2.5 and 17 d.p.f. plutei. Larvae were treated with 0.25, 0.5 and 1 μmol l^{-1} 3-MPA in FSW for 4 h at 18°C in 5 ml plastic tubes. This incubation time was regarded as sufficient on the basis of a previously reported turnover time (10–14 min) of GABA in mice (Gomes and Trolin, 1982). The inhibition of GABA synthesis by the inhibition of GAD with 3-MPA (Ma et al., 1999) was confirmed by whole-mount IHC. The larvae collected from the bottom 500 μl of each tube using Pasteur pipettes (bottom fraction) represented 'poor swimmers'. The remaining larvae, in the upper 4.5 ml of each tube, were sampled as active swimmers (active fraction). The number of larvae in the two groups was counted under a dissection microscope. Each examination was repeated three times using larvae that had developed from the eggs of three different females. Given that swimming activity varied among the larvae derived from the eggs of three different females, the proportion of active swimmers in the control, which involved incubation in standard FSW, was set as a standard for the calculation of the proportion of active swimmers among 3-MPA-treated larvae (standardization). The total number of larvae used is summarized in Table 2.

To examine the involvement of $GABA_AR$ in the GABA signaling pathway, an inhibitor of $GABA_AR$, (+)-bicuculline (no. 026-1631, Wako Pure Chemical Industries, Osaka, Japan) (Takeuchi and Onodera, 1972) was applied to 1 d.p.f. prism larvae and 2.5, 17 and 34 d.p.f. plutei. Bicuculline was dissolved in DMSO (1 mg ml^{-1} stock solution) and diluted in FSW at 0.3, 3 and 30 μmol l^{-1} immediately before application. Larvae were incubated for 3 h at 18°C. The number of larvae in the bottom fraction and in the active fraction was counted under a dissection microscope as described above, and their proportions were standardized as described above. The total number of larvae in each group is summarized in Table 3. These plutei were derived from three different females and each of the experiments that were conducted using 1 d.p.f. prism larvae and 2.5, 17 and 34 d.p.f. plutei was repeated three times.

Immunoblotting

For immunoblotting, the samples were prepared in accordance with a method described previously (Katow et al., 2004). Unfertilized eggs, eggs at 20 min after insemination, 15 h.p.f. swimming blastulae, 20 h.p.f. early gastrulae, 1 d.p.f. prism larvae, and 2, 3 and 4 d.p.f. plutei were dissolved in lysis buffer (6 mol l^{-1} urea, 1% Nonidet P-40, 10 mmol l^{-1} Tris-HCl, pH 7.6), dehydrated in cold pure ethanol, lyophilized using a VD-800F Vacuum Freeze Dryer (Taitec, Koshigaya, Japan) (lyophilized sample), and stored at −80°C until use.

Table 2. Number of larvae treated with 3-MPA

	Concentration of 3-MPA (μmol l^{-1})			
Developmental stage	0	0.25	0.5	1
1 d.p.f. prism	5051	4402	4889	5207
2.5 d.p.f. two-arm plutei	1337	1374	1189	1017
17 d.p.f. four-arm plutei	545	1027	732	574

3-MPA, 3-mercaptopropionic acid.

The lyophilized samples were dissolved in SDS-PAGE sample buffer at 1 mg ml^{-1}, and separated on 10% SDS-PAGE slab gels under reducing conditions, unless described otherwise in the text. The proteins were transferred electrophoretically to nitrocellulose filters and processed as described before (Katow et al., 2010). The blots were then incubated with the anti-Hp-GabrA or anti-Hp-Gabarap antiserum raised in the present study or rabbit anti-rat GAD antibody (Enzo Life Sciences International Inc.; diluted 1:1000 in TBST) for 2 h. After three washes in TBST (10 min each), the blots were incubated with alkaline phosphatase-tagged goat anti-mouse IgG antibody (Promega; diluted 1:30,000 in TBST) for 1 h. After three washes in TBST (10 min each), the immunoreaction was visualized with the alkaline phosphatase chromogen 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Promega), in accordance with the manufacturer's protocol.

To examine the specificity of the antisera and antibody used, they were diluted 1:1000 in TBST and pre-adsorbed by incubation with 3 and 10 mg ml^{-1} antigen peptide, respectively, before application to the samples for 2 h in small chambers made of Parafilm (American Can Company, Greenwich, CT, USA).

RESULTS

Immunohistochemical localization of GABA

GABA-immunoreactivity (GABA-IR) was not detected in 18 h.p.f. early gastrulae (Fig. 1A), but was found in the cytoplasm of mesenchymal cells on the basal surface of the archenteron in 23 h.p.f. prism larvae (Fig. 1B,C). In 2 d.p.f. plutei, GABA-immunoreactive cells (GABA cells) were spread in the blastocoel (Fig. 1D,E), and the GABA-IR was associated with dots of ~1 μm in diameter at the circumoral ciliary band (Fig. 1F inset). The GABA cells showed a characteristic multipolar shape in the blastocoel (Fig. 1G). A 2 μm thick confocal image of blastocoelar cells that was double-stained with anti-GABA antibody and PI, shows that GABA-IR was detected in the perikaryon (Fig. 1G). Following double-immunostaining with anti-GABA antibody and anti-GAD antibody, the blastocoelar GABA cells were also stained by anti-GAD

Table 3. Number of larvae treated with bicuculline

	Concentration of bicuculline (μmol l^{-1})			
Developmental stage	0	0.3	3	30
1 d.p.f. prism	11,894	10,663	9924	9858
2.5 d.p.f. two-arm plutei	1432	1568	1366	1594
17 d.p.f. four-arm plutei	3742	3575	3233	3522
34 d.p.f. six- to eight-arm plutei	35	38	34	34

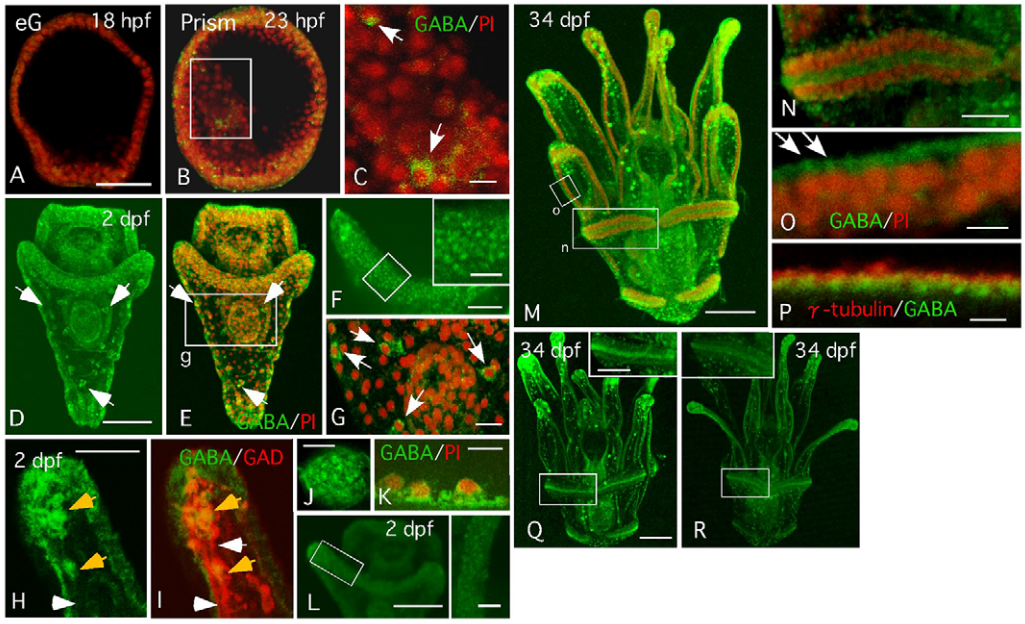

Fig. 1. Whole-mount immunohistochemistry (whole-mount IHC) of γ-aminobutyric acid (GABA) during development. A–C,E,G and K–M were double-stained for GABA-immunoreactivity (GABA-IR; green) and propidium iodide (PI, red). (A) A 2 µm thick optical cross section of an 18 h post-fertilization (h.p.f.) early gastrula (eG). (B) A 2 µm thick optical cross-section of a 23 h.p.f. prism larva. (C) Box from B at higher magnification, showing GABA-IR in mesenchymal cells. Arrows, GABA-immunoreactive mesenchymal cells. (D) A 2 days post-fertilization (d.p.f.) pluteus. Arrows indicate GABA-IR in blastocoelar cells. (E) Double-staining of the same pluteus as in D. Arrows, GABA-immunoreactive blastocoelar cells. (F) Circumoral ciliary band. Box shows association of GABA-IR with 1 µm diameter dots. Inset, boxed area at higher magnification. (G) Boxed area g from E at higher magnification. Arrows indicate GABA-immunoreactive blastocoelar cells. (H) A 2 µm thick stacked image of GABA-immunoreactive blastocoelar cells (yellow arrows). (I) Merged image of H and anti-glutamate decarboxylase (GAD) antibody-stained image (red) and GABA-immunoreactive GAD cells (yellow arrows). White arrowhead in H indicates GABA-negative region that, however, is seen as a GAD-positive cellular process in I. White arrow in I indicates cellular processes between adjacent cells recognized by GAD staining. (J) Circumoral ciliary band and (K) blastocoelar cells of 4 d.p.f. plutei incubated with anti-GABA antibody and glycine. (L) A 2 d.p.f. pluteus stained with pre-adsorbed antiserum; the area outlined by the box is shown at higher magnification to the right. (M) A 34 d.p.f. pluteus. (N) Boxed area n from M at higher magnification. (O) A 0.5 µm thick optical cross-section of boxed area o from M at higher magnification. Arrows indicate association of GABA-IR with ~1 µm diameter dots. (P) A 0.5 µm thick optical cross-section of the double-stained ciliary band. Red, γ-tubulin. Green, GABA-IR. (Q) GABA-IR in a 34 d.p.f. pluteus. Inset, boxed area at higher magnification. (R) A 34 d.p.f. pluteus stained with pre-adsorbed anti-GABA antiserum. Inset, boxed area at higher magnification. Scale bars: A and F, 50 µm; C, 10 µm; D, L, M, Q and R, 100 µm; G, J and K, 20 µm; F inset and H, 5 µm; L inset, N, O inset and P inset, 30 µm; M and N, 2 µm.

antibody and showed a network of GAD-positive cells (GAD cells) with GABA-IR in the perikaryon (Fig. 1H,I). The blastocoelar GABA cells and GABA-immunoreactive dots at the circumoral ciliary band were detected by antibody pre-incubated with glycine (Fig. 1J,K) but not by antibody pre-adsorbed with GABA (Fig. 1L inset), which indicated that the positive signal was specific to the anti-GABA antibody. The immunospecificity of anti-GAD antibody will be described in the following section.

In 34 d.p.f. plutei, major GABA-immunoreactive sites were detected at the ciliary bands of the arms and anterior and posterior epaulets of the trunk (Fig. 1M). The ciliary bands of the arms were each composed of a single row of columnar epithelial cells, and the GABA-immunoreactive site was associated with these cells. In contrast, the epaulets were composed of two rows of columnar epithelial cells, and GABA-IR was detected between these two rows, forming a stripe (Fig. 1M,N). Thus, the ciliary bands of the larval arms and the epaulets were structurally distinct from each other. An optical longitudinal cross-section of the ciliary band of an arm showed GABA-IR on the apical side of the epithelium. The GABA-IR was associated with dots of ~1 µm diameter (Fig. 1O) that resembled those seen at the circumoral ciliary band of 48 h.p.f. plutei (Fig. 1F). On the basis of double-immunostaining with anti-GABA antibody and anti-γ-tubulin antibody, the GABA-IR was localized at the basal side of the tubulin signal, which indicated that GABA-IR was on the basal side of the basal body of the cilia (Fig. 1P). Again, in 34 d.p.f. plutei, the GABA-IR was weakened significantly when pre-adsorbed antibody was used (Fig. 1Q,R).

3-MPA-induced inhibition of GABA-IR and larval swimming

The above findings in relation to the immunohistochemical localization of GABA at the ciliary band suggested the involvement of GABA in ciliary beating, which is responsible for larval swimming activity. To examine this possibility, 17 d.p.f. plutei were treated with 3-MPA and GABA-IR was examined. At this developmental stage, larvae that had not been treated with 3-MPA showed GABA-IR at the ciliary band (Fig. 2A), but those treated with 3-MPA did not (Fig. 2B). To examine whether GABA-IR deficiency resulted in the inhibition of swimming, larvae were treated with 3-MPA from the 1 d.p.f. prism stage, by which time they had formed a preliminary ciliary band, and the 2.5 d.p.f. pluteus stage, by which time they had formed a circumoral ciliary band, through to the 17 d.p.f. pluteus stage, by which time they had developed an extensive ciliary band along the arms. 3-MPA had weak effects on swimming activity in 1 d.p.f. prism larvae, intermediate effects in 2.5 d.p.f. plutei and severe effects in 17 d.p.f. plutei (Fig. 2C). This finding suggests that the GABAergic mechanism has a complex association with larval development or, more specifically, ciliary band organization. It is possible that the increasing dependence of

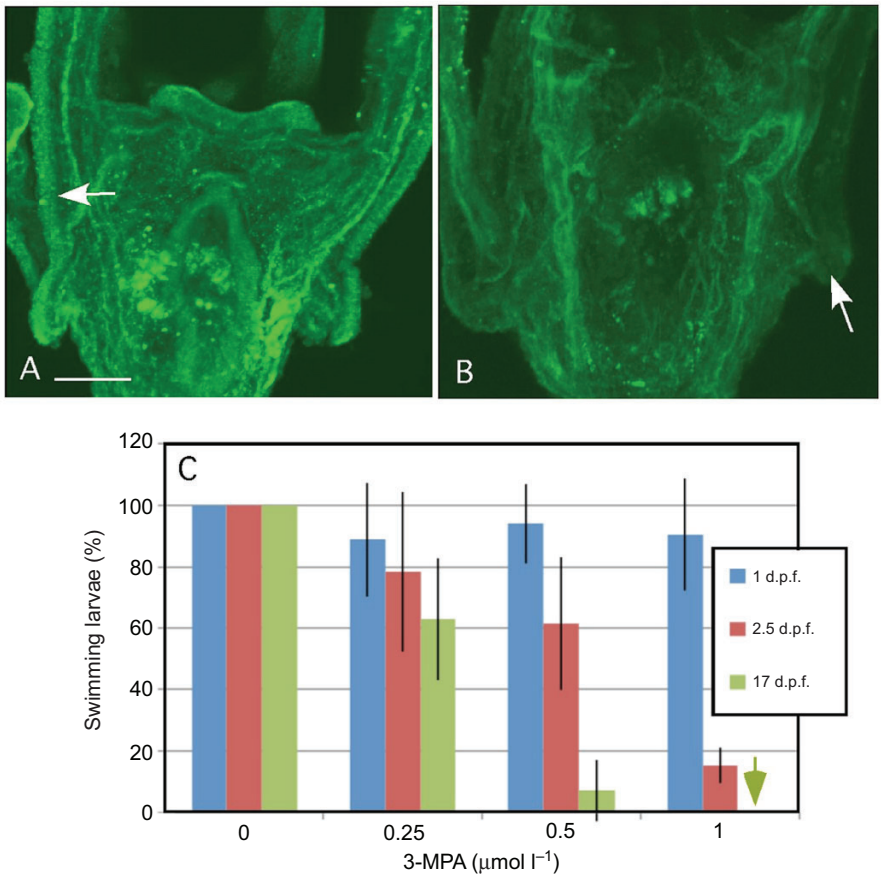

Fig. 2. Inhibition of GABA-IR in 17 d.p.f. plutei and of larval swimming by 3-mercaptopropionic acid (3-MPA). (A) Control pluteus. Arrow, GABA-IR at the ciliary band. Scale bar: 30 μm. (B) 3-MPA-treated pluteus. Arrow, no GABA-IR at the ciliary band. (C) Larval swimming activity of 1 d.p.f. prism larvae, and 2.5 d.p.f. and 17 d.p.f. plutei, shown as the standardized proportion of actively swimming larvae treated with 3-MPA (mean and s.d.). Arrow indicates that 17 d.p.f. plutei did not swim.

ciliary activity on GABA is associated with the increased ability to synthesize GABA. The effect of 3-MPA at a dose sufficient to inhibit larval swimming was not abolished by the exogenous addition of GABA. This might be due to an irreversible effect of 3-MPA above a certain concentration, as has been reported in mammals (Horton and Meldrum, 1973). Alternatively, it could be due to the inability of tonically applied GABA to mimic temporal patterns of GABA release during normal behavior.

GAD expression

To locate the site at which GABA is synthesized, the expression pattern of GAD was analyzed. *Sp-gad*, a GAD homolog gene, has been registered in SpBase (SPU_002857.1). The amino acid sequence contains the peptide F^{598}MLDEIERLGKPL^{610} near the C-terminal end (http://www.spbase.org/SpBase/search/viewAnnoGeneInfo.php?spu_id=SPU_002857); this sequence is quite similar to the antigen of a commercial antibody raised against rat GAD (F^{572}LIEEIERLGQDL^{585}). These two amino acid sequences share 12 identical or similar amino acids out of 13 (positives: 85%, National Center for Biotechnology Information BLAST; http://blast.ncbi.nlm.nih.gov/). In 2.5 d.p.f. plutei, GAD cells were seen in the blastocoel and on the apical surface of the circumoral ciliary band (Fig. 3A). At the 4 d.p.f. pluteus stage, a thin but distinctive linear feature of GAD cells (GAD stripe) was detected on the apical side of the circumoral ciliary band (Fig. 3B). This array was not observed with pre-adsorbed antibody (Fig. 3C), proving support for the proposal that the immunoreactivity represents GAD. The GAD stripe in 4 d.p.f. plutei was composed of four to five bipolar cells (Fig. 3B inset) and each stripe was not connected to the stripes on the other arms (Fig. 3B). The GAD stripe extended further to form a continuous stripe in 17 d.p.f. plutei (Fig. 3D). Thus, organization of the stripe was apparently correlated with increasing sensitivity of the larvae to 3-MPA. On immunoblotting, the anti-GAD antibody bound to a band that corresponded to ~80 kDa (Fig. 3E, lanes 1 and 2), which was close to the predicted molecular mass of Sp-Gad_{67} (70 kDa, SpBase), and the intensity of binding was weakened by pre-adsorption of the antibody (Fig. 3E, lane 3).

According to a BLAST search, the partial DNA sequence of *Hp-gad* was almost identical to that of GAD from the sea urchin *S. purpuratus* (Fig. 3F, *Sp-gad*; E=0, XM_001186613 and XM_779763). Accordingly, the amino acid sequence was similar to that of Sp-Gad_{67} (Fig. 2H; $E=2e^{-119}$, XP_784856.1) and the human GAD1 protein (brain; molecular mass 67 kDa; AAH36552.1, $E=1e^{-73}$). Thus, the GAD homolog of *H. pulcherrimus* was named Hp-Gad (AB713401). Among marine invertebrates, the GAD homologs of the hemichordate *Saccoglossus kowalevskii* (XP_002740674.1, $E=3e^{-71}$) and the lancelet *Branchiostoma floridae* (XP_002592141.1, $E=4e^{-65}$) were highly similar to Hp-Gad. Despite the progressive organization of the GAD stripe, *Hp-gad* amplimers that were detected at ~520 bp from 1 d.p.f. prism larvae remained consistent to 17 d.p.f. plutei (Fig. 3G).

Role of GABA in larval swimming

The above findings strongly suggested the involvement of the GABA system in larval motility. Given that GABAergic function in the brain is mediated mainly by $GABA_A$Rs (Lüscher and Keller, 2004), we used bicuculline, an antagonist of $GABA_A$R (Owens and Kriegstein, 2002; Vanini et al., 2008) to investigate this involvement further. Exposure to bicuculline inhibited the swimming activities of 1 d.p.f. prism larvae, and 2.5, 17 and 34 d.p.f. plutei in a dose-

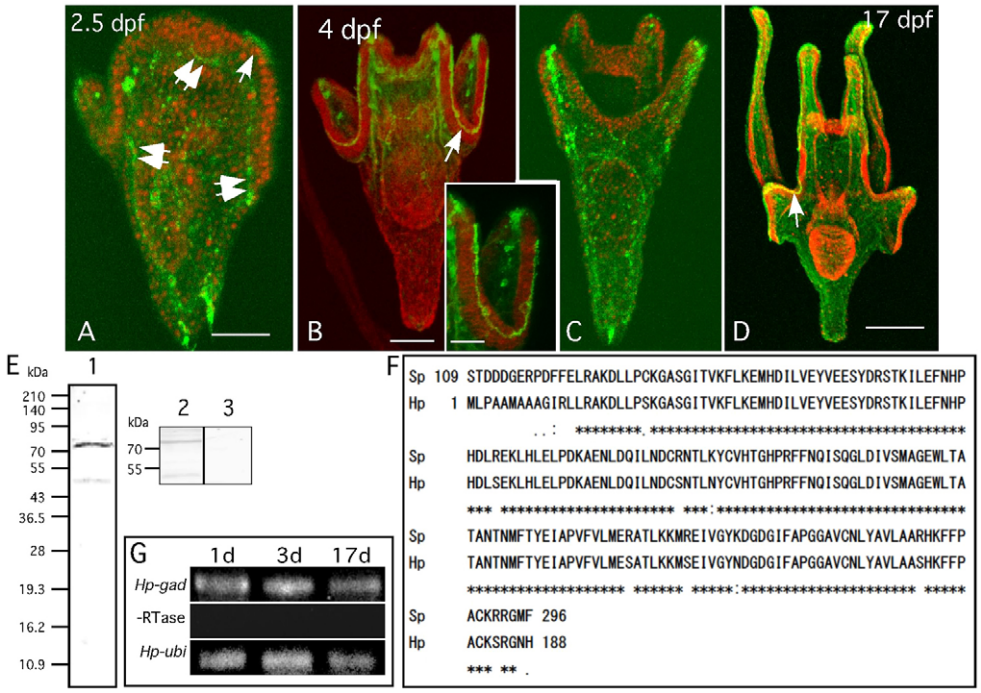

Fig. 3. Whole-mount IHC double-stained with GAD (green) and PI (red; A,C,D) or with Epith-2 (red; B and inset), showing *Hp-gad* transcription pattern and partial amino acid sequence of Hp-GAD and Sp-GAD. (A) Aboral view of 2.5 d.p.f. pluteus. Arrow, apical GAD cells. Double-arrows, blastocoelar GAD cells. (B) A 4 d.p.f. pluteus. Arrow, GAD cells (GAD stripe). Inset, high-magnification image of the area indicated by the arrow. (C) A 4 d.p.f. pluteus. (D) Aboral view of a 17 d.p.f. four-arm pluteus. Arrow, GAD stripe. (E) Immunospecificity test of anti-GAD antibody by immunoblotting. Lanes 1 and 2, unadsorbed antibody; lane 3, pre-adsorbed antibody. (F) Partial amino acid sequences of Hp-Gad (Hp) and Sp-Gad$_{67}$ (Sp). Asterisks indicate identical residues. (G) *Hp-gad* transcription in 1 d.p.f. prism larvae, and 3 and 17 d.p.f. plutei by RT-PCR. –RTase, without reverse transcriptase. *Hp-ubi*, RT-PCR for *Hp-ubiquitin*. Scale bars: A and B, 50 μm; B inset, 100 μm; D, 50 μm.

dependent manner (Fig. 4). The youngest larvae were affected the least, and 2.5 and 17 d.p.f. plutei were inhibited to an intermediate degree. However, although the oldest (34 d.p.f.) plutei were less affected than younger larvae by lower bicuculline concentrations, swimming activity was blocked by 30 μmol l^{-1} bicuculline in all 34 d.p.f. plutei as opposed to in 80% of younger larvae. This suggested that the involvement of the GABA$_A$R-mediated GABA signaling pathway in swimming increased as development progressed.

GABA$_A$R expression

The presence of a GABA$_A$R homolog in sea urchin has been predicted by SpBase. Indeed, in the present study, RT-PCR resulted in an amplimer from *H. pulcherrimus* encoding a partial DNA sequence that was quite similar to GABA$_A$R gene (XM_001186613.1; E=2e^{-112}), as determined by a tblastx BLAST search. The predicted amino acid sequence was also quite similar to Sp-GabrA (Fig. 5A; XP_798340.2, E=2e^{-55}), which in turn is similar to the human GABA$_A$R β-1 subunit precursor (E=2e^{-83}; NP_000803), and has been shown to contain a neurotransmitter-gated ion-channel signal from Cys^{148} to Cys^{162}. Thus, the GABA$_A$R homolog identified in the present study was named Hp-GabrA (AB713402). Among marine invertebrates, Hp-GabrA is similar to GABA$_A$R homologs in the hemichordate *Branchiostoma floridae* (XP_002606735.1, E=5e^{-11}) and abalone, *Haliotis rubra* (ABL10443.1, E=1e^{-07}). *Hp-gabrA* mRNA was not detected in unfertilized eggs, but it was detected after fertilization, which suggested zygotic activity of the gene. The relative intensity of amplified *Hp-gabrA* product then changed little until the 4 d.p.f. pluteus stage, at which point it decreased considerably (Fig. 5B). To examine protein expression of Hp-GabrA, immunoblotting was conducted. The antiserum bound to a band that corresponded to 30 kDa, which was close to the theoretical molecular mass of SPU_028560 (27.7 kDa), under both reducing (Fig. 5C, lane 1) and non-reducing conditions (Fig. 5C, lane 2), which indicated that the protein is not assembled by disulfide bonds. This is consistent with

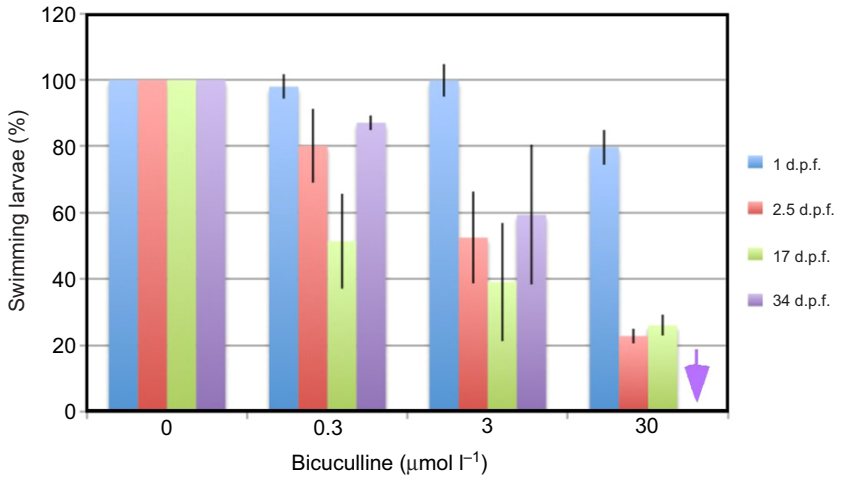

Fig. 4. Inhibition of larval swimming by bicuculline in 1 d.p.f. prism larvae, and plutei of 2.5, 17 and 34 d.p.f., shown as the standardized proportion of actively swimming larvae treated with bicuculline (mean and s.d.). Arrow indicates that 34 d.p.f. plutei did not swim.

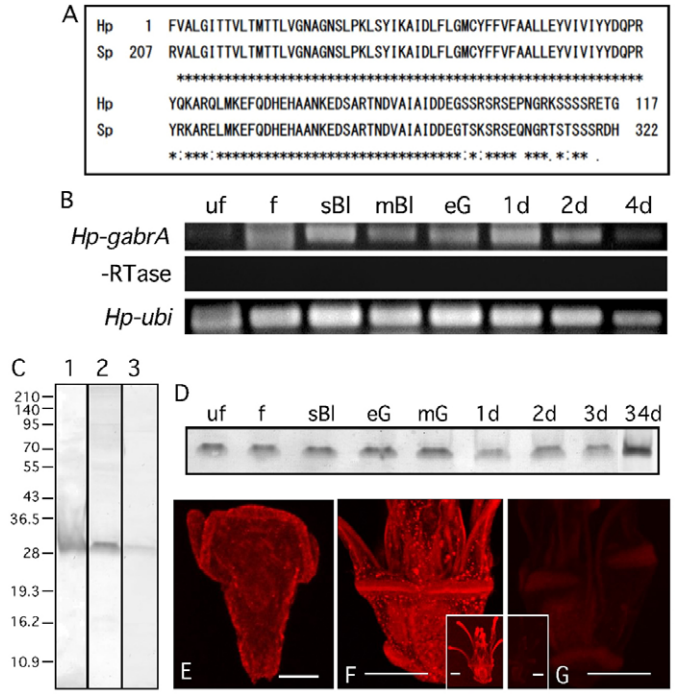

Fig. 5. Alignment of partial amino acid sequences and expression pattern of Hp-GABA$_A$R (Hp-GabrA) by RT-PCR, immunoblotting and whole-mount IHC. (A) Alignment of *H. pulcherrimus* (Hp) and *S. purpuratus* (Sp) indicates high similarity (E=6e^{-50}). (B) RT-PCR of *Hp-gabrA* during development. –RTase, without reverse transcriptase; *Hp-ubi*, RT-PCR for *Hp-ubiquitin*. (C) Immunospecificity tests of anti-Hp-GabrA antiserum by immunoblotting. Lane 1, antiserum bound to the region corresponding to 30 kDa under reducing conditions; lane 2, non-reducing condition; lane 3, pre-adsorbed antibody. (D) Immunoblotting of Hp-GabrA expression during development. uf, unfertilized eggs; f, fertilized eggs; sBl, swimming; mBl, mesenchyme blastulae; eG, early gastrulae; mG, mid-gastrulae; 1d, 1 d.p.f. prism larvae; 2d, 4d and 34d, 2, 4 and 34 d.p.f. plutei. (E–G) Whole-mount IHC of Hp-GabrA. (E) Aboral view of 2 d.p.f. pluteus. (F) Oral view of 34 d.p.f. pluteus. Inset, low-magnification image of the same pluteus. (G) Oral view of 34 d.p.f. pluteus stained by pre-adsorbed antiserum. Inset, low-magnification image of the same pluteus. Scale bars: E, 20 μm; F, F inset, G and G inset, 100 μm.

the known subunit architecture of GABA$_A$R protein (Sigel et al., 2006). The immunoreaction was reduced considerably by pre-adsorption of the antiserum (Fig. 5C, lane 3), supporting the identity of the band as Hp-GabrA. On the basis of immunoblotting of samples from each developmental stage, Hp-GabrA was expressed at a similar intensity from unfertilized eggs to the 34 d.p.f. pluteus stage, which occurs immediately before metamorphosis (Fig. 5D).

Consistently, whole-mount IHC detected Hp-GabrA from the 2 d.p.f. pluteus stage at the circumoral ciliary band (Fig. 5E) to the 34 d.p.f. pluteus stage (Fig. 5F), in which Hp-GabrA was detected strongly at the ciliary bands of the arms, at the epaulets and as dots throughout the body (Fig. 5F). Application of pre-adsorbed antiserum to 34 d.p.f. plutei resulted in no signal (Fig. 5G), which indicated that these structures were seen because of immunospecific binding of the antiserum. However, despite the detection of the protein in unfertilized eggs by immunoblotting, whole-mount IHC did not detect Hp-GabrA expression sites in larvae younger than the 2 d.p.f. pluteus stage.

Expression of GABARAP associated with GABA$_A$R in the pluteus stage

In GABAergic signaling, the GABA signal is transmitted to GABARAP, which binds to the γ2 subunit of GABA$_A$R (Wang et al., 1999; Coyle et al., 2002; Bruneau et al., 2009). To examine whether GABARAP is associated with larval swimming activity, the gene that encodes the GABARAP protein was cloned. The primers that were used for RT-PCR amplified a 220 bp amplimer with a predicted amino acid sequence of 73 amino acids that showed high similarity to the GABARAP protein of *S. purpuratus* (Fig. 6A; Sp-Gabarap, SPU_006271.1). Thus, it was named Hp-Gabarap (AB713403). According to an NCBI/BLAST/xtblastx search, the protein sequence of Hp-Gabarap was also similar to the GABARAP homologs of other marine invertebrates, such as the hemichordate *Saccoglossus kowalevskii* (NM_001171229.1), the starlet sea anemone *Nematostella vectensis* (XM_001634353.1) and the lancelet *Branchiostoma belcheri tsingtauense* (AY616184.1), with E values of 9e^{-40}, 2e^{-38} and 8e^{-38}, respectively. Hp-Gabarap was also similar to the GABARAP homologs of vertebrates, such as frog (AEQ17776, *Hymenochirus curtipes*, E=4e^{-40}) and human (NP_009209, E=5e^{-40}). Transcripts of *Hp-gabarap* were detected from unfertilized eggs to 34 d.p.f. plutei with similar intensity (Fig. 6B).

To examine the pattern of protein expression, an antiserum was generated against Hp-Gabarap. On immunoblotting, the antiserum bound to a band that corresponded to 18 kDa (Fig. 7A, lane 1), which was similar to the size of human GABARAP (17 kDa) (Wang et al., 1999). The band was weakened considerably by pre-adsorption of the antiserum (Fig. 7A, lane 2), supporting the identification of the band as Hp-Gabarap. When samples from each developmental stage were immunoblotted, the protein was detected in all embryos and larvae at and after the swimming

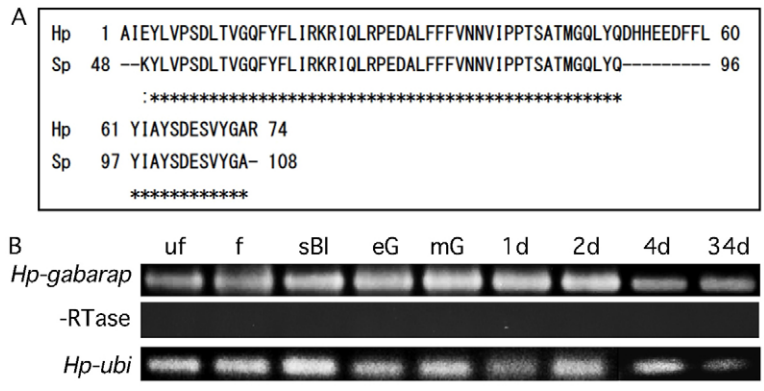

Fig. 6. Alignment of partial amino acid sequences of the GABA$_A$R-associated protein (GABARAP) and the gene transcription pattern of *Hp-Gabarap*. (A) Alignment of *H. pulcherrimus* (Hp) and *S. purpuratus* (Sp) GABARAP. (B) Amplification of *Hp-gabarap* by RT-PCR. uf, unfertilized eggs; f, fertilized eggs; sBl, swimming blastulae; eG, early gastrulae; mG, mid-gastrulae; 1d, 1 d.p.f. prism larvae; 2d, 4d and 34d, 2, 4 and 34 d.p.f. plutei. –RTase, without reverse transcriptase; *Hp-ubi*, RT-PCR for *Hp-ubiquitin*.

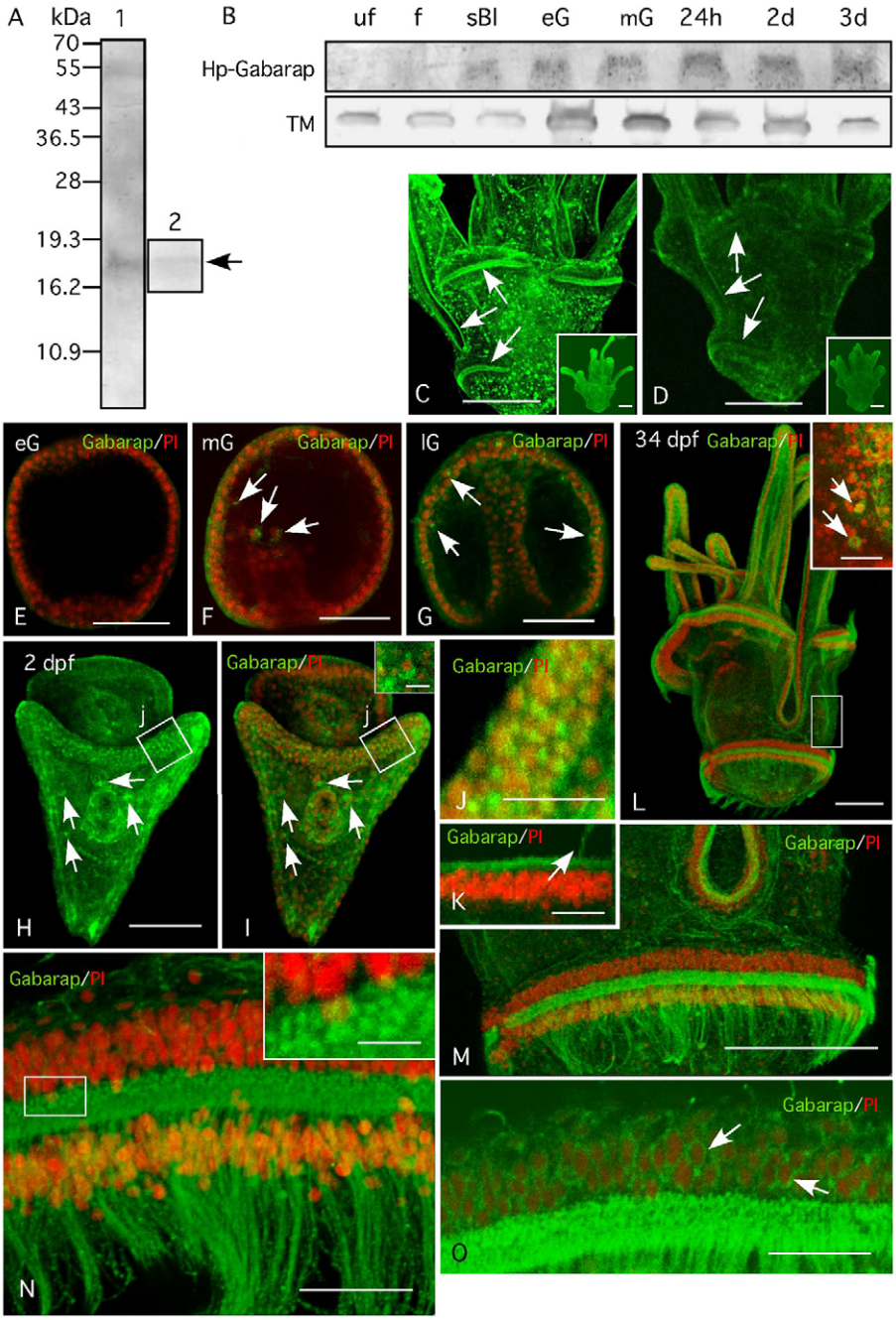

Fig. 7. Expression pattern of Hp-Gabarap during development by immunoblotting and whole-mount IHC. (A) Immunospecificity test of anti-Hp-Gabarap antiserum by immunoblotting. Lane 1, the antiserum bound to a band that corresponded to 18 kDa; lane 2, pre-adsorbed antiserum applied. Arrow, region corresponding to 18 kDa. (B) Immunoblotting of Hp-Gabarap and tropomyosin (TM). uf, unfertilized eggs; f, fertilized eggs; sBl, swimming blastulae; eG, early gastrulae; mG, mid-gastrulae; 24h, 24 h.p.f. prism larvae; 2d and 3d, 2 and 3 d.p.f. plutei. (C) Whole-mount IHC of Hp-Gabarap in a 34 d.p.f. pluteus. Arrows, ciliary band of arms and epaulets. Inset, low-magnification image. (D) Pluteus with pre-adsorbed antiserum. Arrows, ciliary band of arms and epaulets. Inset, low-magnification image. (E–G,I–O) Double-stained whole-mount IHC for Hp-Gabarap (green) and nuclei with PI (red. (E) Early gastrula (eG). (F) Mid-gastrula (mG). Arrows, Hp-Gabarap of blastocoelar cells. (G) Late gastrula (lG). Arrows, Hp-Gabarap-positive cells. (H) Single-stained 2 d.p.f. pluteus with anti-Hp-Gabarap antiserum. Arrows, Hp-Gabarap in blastocoelar cells. Box, Hp-Gabarap at the circumoral ciliary band. (I) Same larvae as in H double-stained with PI. Arrows, Hp-Gabarap of blastocoelar cells. Box, circumoral ciliary band. Inset, high-magnification image of Hp-Gabarap-positive blastocoelar cells. (J) High-magnification image of a part of the circumoral ciliary band indicated by box j in H and I. (K) A 2 µm thick optical cross-section of a ciliary band region. Arrow, cilium. (L) A 34 d.p.f. pluteus. Hp-Gabarap at the ciliary bands of arms and epaulets. Inset, high-magnification image of boxed area. Arrows, Hp-Gabarap of blastocoelar cells. (M) High-magnification image of posterior region of larva shown by L. (N) Higher magnification of posterior epaulet in M. Inset, 1 µm diameter granules at the base of cilia indicated by the box (green). (O) Plasma membrane of the ciliary epithelial cells at the epaulet (arrows). Scale bars: C, D, L and M, 100 µm; E–H, 50 µm; I inset, 10 µm; J and K, 10 µm; L inset and O, 20 µm; N, 30 µm; N inset, 5 µm.

blastula stage (Fig. 7B). Whole-mount IHC localized Hp-Gabarap at the ciliary bands of the arms, at the epaulets and at dots throughout the larval body of 34 d.p.f. plutei, which resembled the localization of Hp-GabrA (Fig. 5F). The intensity of these immunopositive sites was decreased considerably by pre-adsorption of the antiserum (Fig. 7D), which indicated that these features are antiserum specific.

Whole-mount IHC of each developmental stage did not detect Hp-Gabarap in early gastrulae (Fig. 7E), but it was detected in the perikaryon of mesenchymal cells (GABARAP cells) around the tip of the archenteron in mid-gastrulae (Fig. 7F), and then in and around the ectoderm of late gastrulae (Fig. 7G). By the 2 d.p.f. pluteus stage, Hp-Gabarap was detected in the epithelial cells of the circumoral ciliary band (Fig. 7H–J) and the GABARAP cells were dispersed in the blastocoel (Fig. 7H,I and 7I inset). In the cells at the ciliary band, the distribution of Hp-Gabarap was polarized on the apical side of the nucleus (Fig. 7K). In 34 d.p.f. plutei, GABARAP cells were highly organized in the ciliary bands of the arms and epaulets (Fig. 7L), but remained dispersed in the blastocoel (Fig. 7L inset). Unlike in the ciliary bands of the arms, in which GABARAP cells were aligned in a single row (Fig. 7J), the arrangement of GABARAP cells at the epaulets was highly organized to form a two-row epithelium with Hp-Gabarap signal in the middle, from which the cilia extended (Fig. 7M). The cilia themselves were also positive for Hp-Gabarap (Fig. 7L–O). The Hp-Gabarap-positive signal was associated with 1 µm diameter granules in the cytoplasm (Fig. 7N inset) and also with the plasma membrane of the epithelial cells (Fig. 7O). Thus, the distribution of Hp-Gabarap was quite similar to those of GABA-IR (Fig. 1) and Hp-GabrA (Fig. 5E,F).

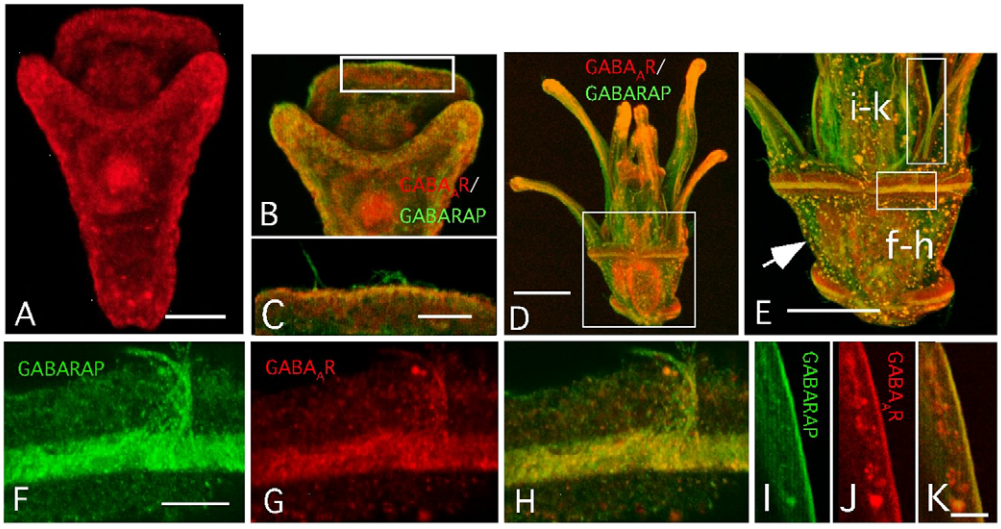

Fig. 8. Double-stained whole-mount IHC of Hp-GabrA ($GABA_AR$, red) and Hp-Gabarap (GABARAP, green) at the ciliary band. (A) Oral view of 2 d.p.f. pluteus. (B) Merged image of the same pluteus as in A. (C) High-magnification image of apical tuft region indicated by the box in B. (D) A 34 d.p.f. pluteus. (E) High-magnification image of trunk region indicated by the box in D. Arrow, dotted Hp-Gabarap and $GABA_AR$. (F–H) High-magnification images of boxes (f–h) in E. (F) Hp-Gabarap. (G) Hp-GabrA. (H) Merged image of F and G. (I–K) High-magnification images (boxes i–k in E) of 2 µm thick optical cross-sections of ciliary band at the arm. (I) Hp-Gabarap. (J) Hp-GabrA. (K) Merged image of I and J. Scale bars: A, 50 µm; C, F and K, 20 µm; D and E, 100 µm.

The close association of sites that expressed Hp-GabrA and Hp-Gabarap was examined by double-immunostaining. In 2 d.p.f. plutei, $GABA_AR$ and GABARAP were localized similarly at the circumoral ciliary band (Fig. 8A,B). The blastocoelar GABARAP cells in 2 d.p.f. plutei were negative for Hp-GabrA (Fig. 5E, Fig. 8A). Close association of these proteins at the ciliary bands remained unaltered until the 34 d.p.f. pluteus stage (Fig. 8D). At this stage, unlike in 2 d.p.f. plutei, in addition to the ciliary band, numerous blastocoelar cells were also stained by both antisera (Fig. 8E). Close association of these two proteins at the ciliary band was detected not only in a pattern parallel to the apical cell surface (Fig. 8F–H) but also in a vertical, apico-basal orientation (Fig. 8I–K), which suggested that these two proteins constitute a closely associated complex.

Co-localization of GABA-IR and Hp-Gabarap at the ciliary band

The double-immunostaining of GABA and Hp-Gabarap in 34 d.p.f. plutei revealed the co-localization of these two molecules at the ciliary bands of the epaulets (Fig. 9A,B) and arms (Fig. 9C). Closer examination located GABA-IR between the two rows of ciliary epithelial cells and an extra posterior fringe of the anterior epaulet (Fig. 9B). An optical longitudinal cross-section at the ciliary band of the arms suggested that these two molecules were co-localized closely in the apical region of the epithelium (Fig. 9C, inset C-1 to C-3), which resembled the subcellular localization of Hp-Gabarap and Hp-GabrA (Fig. 8I–K). Thus, GABA-IR, $GABA_AR$ and GABRAP were located very close together at the ciliary band.

Topological relationship of GABAergic system to regulatory systems involved in larval swimming

In sea urchin larvae, several systems that regulate ciliary movement have been reported; they include the serotonergic nervous system (Yaguchi and Katow, 2003; Katow et al., 2004; Katow et al., 2007) and the dopaminergic system (Katow et al., 2010). In particular, the localization of the dopaminergic system resembles that of the GABAergic system at the ciliary band.

Whole-mount IHC by double-immunostaining of Hp-GabrA and DRD1 in 34 d.p.f. plutei indicated the close co-localization of these proteins at the epaulets (Fig. 10A). Hp-GabrA in the ciliary band was associated with dots of 1–2 µm diameter (Fig. 10B inset), as was DRD1 (Fig. 10C inset) (Katow et al., 2010). The merged images of this site indicated that the two types of dot overlapped perfectly (Fig. 10D inset). Overlapping expression of these two proteins was also seen at the ciliary bands of the arms and dots on the body surface (Fig. 10D). An optical cross-section of the ciliary band also indicated very close localization of Hp-GabrA and DRD1 (Fig. 10E–G). Given that the DRD1 protein is located in close association with the basal body of the cilium (Katow et al., 2010), the present observations suggest that $GABA_AR$ also constitutes a regulatory complex near the basal body of cilia.

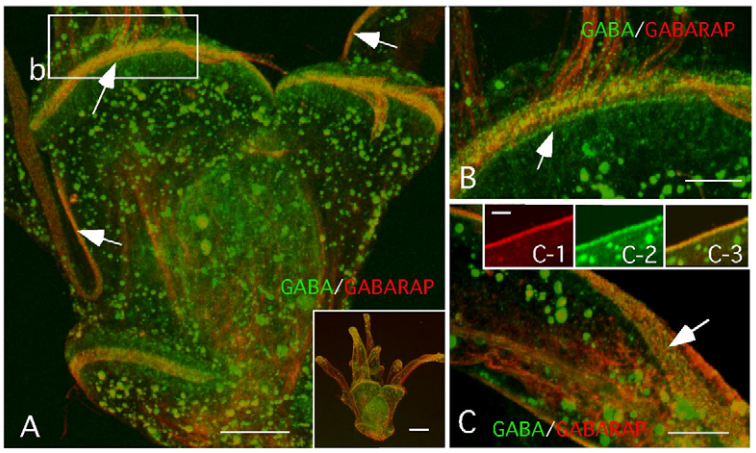

Fig. 9. Double-stained whole-mount IHC of 34 d.p.f. plutei. (A–C) GABA-IR (green) and Hp-Gabarap (GABARAP, red). (A) GABA-IR co-localization with Hp-Gabarap at the ciliary bands (arrows) and dots on the body surface. Inset, low-magnification image of the same pluteus. (B) High-magnification image of the anterior epaulet indicated by box b in A. Arrow, GABA-IR at the posterior fringe of the anterior epaulet without association of Hp-Gabarap. (C) Larval arm. Arrow, co-localization of GABA-IR and Hp-Gabarap. Insets show a 2 µm thick optical cross-section of a ciliary band of the arm: C-1, Hp-Gabarap; C-2, GABA-IR; C-3, merged image. Scale bars: A and A inset, 100 µm; B and C, 50 µm.

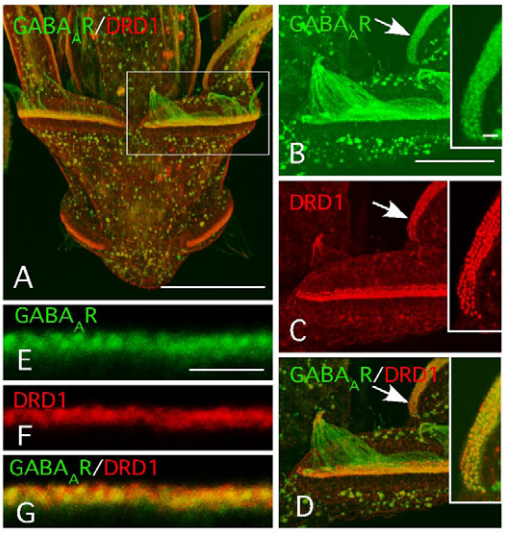

Fig. 10. Double-stained whole-mount IHC of 34 d.p.f. plutei for the Hp-GABA$_A$ receptor (GABA$_A$R, green) and dopamine receptor D1 (DRD1, red). (A) Trunk region. (B–D) High-magnification image of the area indicated by the box in A. Arrow, ciliary band of an arm. (B) Hp-GabrA. (C) DRD1. (D) Merged image of B and C. (E–G) A 0.5 µm thick optical cross-section of an arm. (E) Hp-GabrA. (F) DRD1. (G) Merged image of E and F. Inset in B–D, high-magnification image of the area indicated by the arrow. Scale bars: A, 100 µm; B, 50 µm; B inset and E, 5 µm.

DISCUSSION

GABAergic system for larval swimming

The swimming activity of sea urchin larvae was decreased in a dose-dependent manner by the GABAergic system inhibitors 3-MPA and bicuculline, which suggested that the GABAergic ciliary beating mechanism of marine invertebrates is not distinct from that of vertebrates (Horton and Meldrum, 1973; Xiang et al., 2007). This is consistent with the results obtained for the marine crab *Chasmagnathus granulatus* (Tano et al., 2009), but contrasts with the results of previous studies that were conducted using a GABA binding assay (Mann and Enna, 1980; Lunt, 1991). The partial DNA and protein structures of Hp-Gad, Hp-GabrA and Hp-Gabarap were similar to those of vertebrates, which suggests that their functional mechanisms in vertebrates and invertebrates are highly related, if not identical.

Although the observation of the downregulation of larval swimming by bicuculline suggests the involvement of GABA$_A$Rs, whether these receptors have an excitatory or inhibitory influence in this context has not been elucidated in the present study.

The results of the present study also indicated that sensitivity to 3-MPA and bicuculline increased in a developmental stage-dependent manner. This suggests that the contribution of the GABAergic system to the regulation of larval swimming increases progressively during development. This was supported by the increasingly complex organization of the GABAergic signaling system during development, as seen in the formation of the GAD stripe.

Structure and localization of GAD, GABA$_A$R and GABARAP

In the present pharmaceutical analysis of the involvement of the GABAergic system in larval swimming activity, GABA was localized immunohistochemically at the ciliary band, in particular near the basal body of the cilium. This is the first report of a relationship between GABA-IR and the basal body, apart from a pioneering ultrastructural study of the rat oviduct (Erdö et al., 1986). In the blastocoel, as GABA-IR was detected in perikaryon of GAD-expressing cells that constitute the blastocoelar network, GABA may be synthesized in the same cell. In the ciliary band, however, whereas GAD cells displayed a clear striped pattern, no such feature was associated with GABA-IR. Some degree of non-overlap of GAD and GABA detection has been reported in several species, and there are several possible explanations, of which some are technical and some are biological. Transcellular transport could be a possible biological explanation. This interpretation is somewhat consistent with previous reports, such as one of studies on goldfish (Zucker et al., 1984). Thus, co-localization of GABA and GAD could vary in a manner that depends on the particular organs or tissues in question.

Sp-GabrA contains a neurotransmitter-gated ion-channel signature in the region from Cys^{94} to Cys^{108}, which is similar to those in vertebrates, such as *Xenopus tropicalis* (NP_001096164) and mouse (NP_032095). Thus, the active site of GABA$_A$R is conserved in both vertebrates and invertebrates. Highly specific spatial clustering of GABA$_A$R at the ciliary bands suggests mediation by an anchoring protein (Kneussel and Loebrich, 2007). Although the physical nature of the interaction remains unclear (ISB, 2009), GABA$_A$R clustering requires the intracellular γ2 subunit of the receptor and GABARAP, which mediates GABA$_A$R binding to tubulin and microtubules (Wang et al., 1999; Kittler et al., 2001; Coyle et al., 2002). The γ2 subunit of the human GABA$_A$R is composed of 85 amino acids (AAB25483), which start from His^{1} and end with Ser^{85} (Harkin et al., 2002). However, according to SpBase, no closely related sequence is found in Sp-GabrA (SPU_025266.1). The 80 amino acids at the carboxyl terminal of the receptor starts at Arg^{324} and ends with Ser^{403}, and 42 of these amino acids are either similar to or the same as those of the human γ2 subunit, and it is possible that this segment could fulfill the function of the γ2 subunit.

The protein sequence of sea urchin GABARAP (SPU_006271.1) is quite similar to that of the GABARAP homolog in the hemichordate *Saccoglossus kowalevskii* (NM_001171229.1; E=4e^{-70}), both of which contain a tubulin-binding site at the N-terminus (Knight et al., 2002).

Developmental organization of the GABAergic system

Consistent with the increasing dependence of the swimming activity of larvae on the GABAergic system during development, the cells that showed GABA-IR and expressed Hp-Gad, Hp-GabrA and Hp-Gabarap gradually adopted a highly organized arrangement in the ciliary bands of the arms and epaulets during larval development.

The early site of GABA-IR in the epithelium of the archenteron in 23 h.p.f. prism larvae spread through migration of the cells to the blastocoel and converged with the circumoral ciliary band during the two-arm pluteus stage. This was organized further into a GABA-immunoreactive stripe that formed between the two rows of ciliary epithelial cells of the epaulets in eight-arm plutei. Consistent with this finding, the active migration of GABAergic cells during development has been recognized widely (Jelitai and Madarasz, 2005); for example, telencephalon of the sea lamprey, *Petromyzon marinus* (Pombal et al., 2011), and neocortical projections in mammals (Gilmore and Herrup, 2001). In the sea urchin larvae, the circumoral ciliary band developed extensive accumulation of GABA cells and the larvae acquired sensitivity to GABA-IR deprivation at around the 2 d.p.f. pluteus stage. From this developmental stage, GABA-IR was associated closely with small dots that aligned parallel to the circumoral ciliary band. This pattern

resembled the dotted pattern of dopamine and DRD1 (Katow et al., 2010). Accordingly, in the present study, whole-mount IHC revealed the close association of GABA-IR with the basal body of the cilium. A similar pattern of GABA distribution was reported on the ciliated surface of *Paramecium* (Ramoino et al., 2010). In the rat oviduct in particular, GABA was seen in the cytoplasm of the tubal epithelium along with the ciliated apical surface (Erdö et al., 1986), which is consistent with the present observation of GABA-IR in the cytoplasm of the blastocoelar cells.

Although the mechanism by which GABA in the cytoplasm is transported to the basal bodies of cilia was not addressed in the present study, synthesized GABA may be taken up into vesicles in the cytoplasm and then localized to the basal bodies, as has been reported in *Paramecium* (Ramoino et al., 2010). This is supported by the prediction of the presence of GABA transporter in sea urchin by SpBase (Sp-Gat_2; SPU_000076, etc.). Characteristic localization of GABA-IR in the GABA stripe of the epaulet could occur by the similar GABA transportation process.

The close localization of GABA-IR and Hp-Gad in the blastocoelar cells was consistent with a previous study on the rat cerebral cortex (Rimvall and Martin, 1991) and suggests that GABA is stored, at least temporarily, in the cells that produce it. In the urochordate *Oikopleura*, transcription of the GAD gene occurs 2–3 h earlier than GABA synthesis (Søviknes et al., 2005). It remains to be clarified whether this chronological sequence occurs in sea urchin larvae. The formation of the distinct structure of the GAD stripe on the apical surface of the ciliary bands of the arms occurred in close association with the acquisition of GABA-dependent swimming activity by the larvae, whereas the occurrence of blastocoelar GAD cells seemed to be involved little in GABAergic larval swimming. The striped arrangement of GAD cells near the ciliary band has not been reported in any other animals to date, and thus we consider it to be unique to the plutei of *H. pulcherrimus*. However, it is reminiscent of a similar arrangement of GAD cells that occurs in association with functional change of the GABA system during rat hippocampal formation (Dupuy and Houser, 1996).

The results clearly demonstrated the involvement of the bicuculline-sensitive $GABA_AR$ in larval swimming. There have been contradictory reports that invertebrate $GABA_ARs$ are less sensitive or insensitive to bicuculline (Mann and Enna, 1980; Lunt, 1991). However, $GABA_AR$ of the pond snail (*Lymnea stagnalis*) is bicuculline sensitive (Harvey et al., 1991) and the moth *Manduca sexta* possesses both bicuculline-sensitive and bicuculline-insensitive $GABA_ARs$ in the nervous tissue (Sattelle, 1990). In addition, ciliary reversal induced by muscimol is suppressed effectively by bicuculline in *Paramecium* (Bucci et al., 2005). Thus, sensitivity to bicuculline might not be a crucial feature by which to distinguish vertebrate-type GABA receptors from those of invertebrates, and invertebrate $GABA_ARs$ might not perfectly fit into the vertebrate categories in terms of their mechanisms of action (Wegelius, 2000).

An apparent discrepancy between *Hp-gabrA* transcription and the expression of Hp-GabrA protein was noted in unfertilized eggs and plutei at and after the 4 d.p.f. stage, which might suggest that maternally translated protein remained and suppressed transcription substantially from this stage onwards. There have been few reports on the earliest stage at which $GABA_AR$ gene is transcribed during development, in either vertebrates or invertebrates, except for a report on the expression of a $GABA_AR$-like gene in the freshwater mollusk, *L. stagnalis*, in 10–14 day eggs (Hutton et al., 1993). However, according to our BLAST survey, the *L. stagnalis* gene is rather similar to glycine receptor subunit α-2 of invertebrates, such as that of *Aplasia californica* (NP_001191520, $E=1e^{-98}$), and of vertebrates, such as that of the Nile tilapia *Oreochromis niloticus* (XP_003455513, $E=2e^{-95}$). Glycine receptor subunit α-2 is a member of the same ligand-gated chloride ion channel superfamily as $GABA_AR$ (MacDonald and Olsen, 1994). Thus, the present study could be the first to detect expression of the $GABA_AR$ gene at a very early stage of development.

$GABA_AR$ interacts with a small adaptor protein, GABARAP, which binds to two subunits of the receptor to transduce the GABA signal to the cytoplasm (Coyle et al., 2002). The description herein of the expression pattern of GABARAP during development is to our knowledge the first in invertebrates. Transcription of the *Hp-gabarap* gene was detected from unfertilized eggs through to plutei immediately before metamorphosis, which is similar to the pattern of gene expression reported in amphioxus (*Branchiostoma belcheri tsingtauense*) (Liang et al., 2004). However, unlike in amphioxus embryos, expression of the Hp-Gabarap protein was detected only at or around the swimming blastula stage and continuously thereafter, until immediately before metamorphosis at the eight-arm pluteus stage. Whole-mount IHC revealed that the protein was expressed even later, at the mid-gastrula stage in the perikaryon of secondary mesenchymal cells near the tip of the archenteron and in the blastocoelar space, which strongly suggested the mesenchymal nature and origin of GABARAP-expressing cells. Given that, in murine hippocampal neurons, GABARAP is found predominantly in the perikaryon, which corresponds to the endoplasmic reticulum and Golgi apparatus (Kittler et al., 2001), GABARAP could also be present in these subcellular compartments in the mesenchymal cells. Similar to the GABA system-associated cells described in the present report, the blastocoelar GABARAP cells diverged into two groups: one group remained in the blastocoel and the other merged with the ciliary band, where it shared an identical histological site with the cells that comprise the GABA signaling system. In contrast, the GABARAP cells in the blastocoel might not be involved in neuronal activity, as recognized soon after the discovery of GABA (Akinci and Schofield, 1999).

Interaction between nervous systems involved in larval swimming regulation and the GABAergic system

GABA and dopamine are regarded as being excitatory signals in invertebrates, whereas serotonin is inhibitory (Ukena et al., 1995). Thus, they can act in a complementary manner to regulate ciliary beating, and hence larval swimming. In fact, detailed analysis of the larval swimming behavior of the sea urchin indicates that dopamine and serotonin appear to regulate forward and backward swimming speed in a complementary manner (Mogami et al., 1992), which indicates the occurrence of cross-talk between the receptors of these two neurotransmitters.

The immunohistochemical co-location of $GABA_AR$ and DRD1 suggests the occurrence of cross-talk between these two receptors. $GABA_AR$ and dopamine receptor D5 (DRD5) can be co-immunoprecipitated from mammalian brain samples, which indicates a direct physical association between $GABA_AR$ and DRD5; however, no direct physical association was detected between $GABA_AR$ and DRD1 (Liu et al., 2000). According to a whole-cell patch-clamp recording study using olfactory bulb neurons (Brünig et al., 1999) and striatum (Goffin et al., 2010), suppression of $GABA_AR$ results in activation of DRD1, which indicates functional collaboration between these two receptors.

The close localization of $GABA_A$ receptors and serotonin receptors at the epaulet (Katow et al., 2010) suggests collaboration between these receptors in relation to the regulation of larval swimming. In the cerebellar cortex, serotonin is involved in the

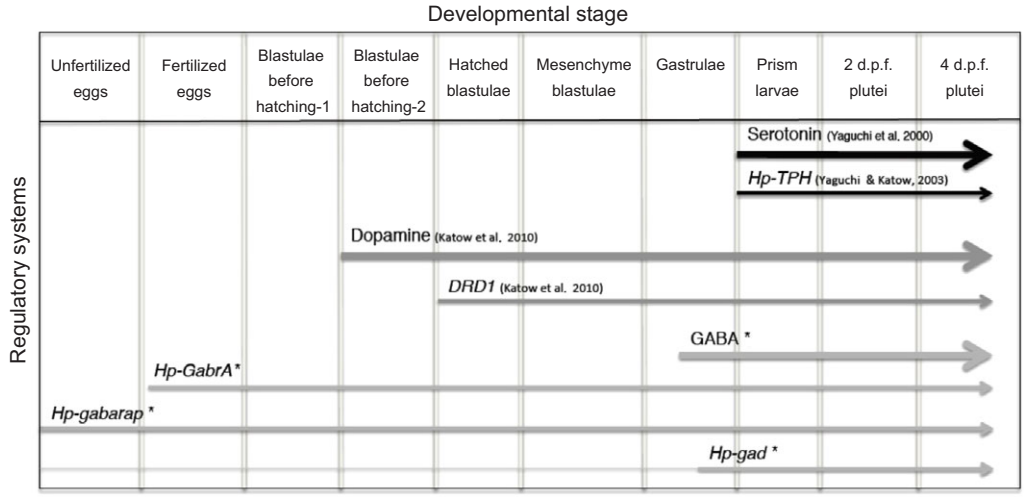

Fig. 11. Summary of the chronological appearance of neuronal regulatory systems in the sea urchin embryos and larvae as currently understood. Thick arrows show periods of expression of serotonin, dopamine and GABA immunoreactivity. Thin arrows show periods of transcription of the genes indicated for each arrow. Representative references are shown above each arrow. The region indicated by a thinner line at the bottom is related to the period of *Hp-gad* transcription, which is putatively assigned on the basis of GABA immunohistochemistry. Blastulae before hatching-1, before acquisition of rotatory movement; blastulae before hatching-2, after acquisition of rotatory movement. *Data from the present study.

modulation of GABAergic transmission, and exogenously applied serotonin induces short-term enhancement of GABAergic transmission between cerebellar interneurons and Purkinje cells (Mitoma and Konishi, 1999).

Previous studies (Mogami et al., 1992; Wada et al., 1997; Yaguchi and Katow, 2003; Katow et al., 2004; Katow et al., 2007; Katow et al., 2010) and the results of the present study show that the increasing complexity of swimming pattern in sea urchin larvae that is associated with progressive development from embryonic stages to larval stages might be associated with an increasing diversity of neural mechanisms involved in the regulation (Fig. 11) and the possible cross-talk among them.

LIST OF SYMBOLS AND ABBREVIATIONS

3-MPA	3-mercaptopropionic acid
d.p.f.	days post-fertilization
DRD1	dopamine receptor D1
FSW	filtered seawater
GABA	γ-aminobutyric acid
GABA-IR	GABA-immunoreactivity
$GABA_AR$	$GABA_A$ receptor (GabrA)
GABARAP	γ-aminobutyric acid A receptor-associated protein (Gabarap)
GAD	glutamate decarboxylase (Gad)
h.p.f.	hours post-fertilization

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AUTHOR CONTRIBUTIONS

H.K. conceived and organized the project and conducted immunochemistry, including raising antibodies, K.A. conducted molecular biology, T.K. conducted the larval swimming assay and immunoblotting, A.Z. conducted larval cultivation and whole-mount IHC, and H.A. supervised and conducted larval cultivation.

COMPETING INTERESTS

No competing interests declared.

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