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Serotonin stimulates $[Ca^{2+}]_i$ elevation in ciliary ectodermal cells of echinoplutei through a serotonin receptor cell network in the blastocoel

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Summary

A full-length serotonin receptor mRNA from the *5Hthpr* gene was sequenced from larvae of the sea urchin, *Hemicentrotus pulcherrimus*. The DNA sequence was most similar to 5HT-1A of the sea urchin *Strongylocentrotus purpuratus* found by The Sea Urchin Genome Project, and the protein sequence predicted the presence of seven transmembrane domains. Immunohistochemistry with anti-5HThpr antibodies indicated that the protein was expressed on blastocoelar cells that comprised the major blastocoelar network (serotonin receptor cell network). These network cells inserted their processes into the ectoderm in various regions, including the ciliary band region. Serotonin injected into the blastocoel stimulated a transient elevation of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the ectoderm, as detected by Oregon-Green dextran, injected earlier in development. The calcium transient propagated as a wave at about 175 μm s^{-1}, but was not detectable in the serotonin receptor-positive cell network. In larvae treated with *p*-chlorophenylalanine, a potent and irreversible serotonin synthesis inhibitor, serotonin application did not stimulate $[Ca^{2+}]_i$, the serotonin receptor cell network did not develop properly, and the swimming behavior of the larvae was abnormal. However, formation of a different nervous system comprising synaptotagmin-possessed neurites was not affected by *p*-chlorophenylalanine treatment. These results imply that serotonin secreted from the apical ganglion into the blastocoel stimulates the elevation of $[Ca^{2+}]_i$ in the larval ectodermal cells through the serotonin receptor cell network.

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Key words: 5Hthpr, *p*-chlorophenylalanine, sea urchin.

Introduction

The serotonergic nervous system in sea urchins is formed in early larval stages (e.g. Bisgrove and Burke, 1986; Bisgrove and Burke, 1987; Yaguchi and Katow, 2003) and participates, along with dopaminergic neurons, in the regulation of cilia-based larval swimming behavior (Yaguchi and Katow, 2003; Wada et al., 1997). Serotonin application increases larval swimming velocity by 'stabilizing the rhythm of the beating' (Wada et al., 1997), whereas inhibition of serotonin synthesis by pharmacological treatment with *p*-chlorophenylalanine (*p*CPA), a potent and irreversible tryptophan 5-hydroxylase inhibitor (Gal and Whitacre, 1982), severely inhibited spatial larval swimming. In *p*CPA treated larvae, the cilia still beat, and propel the larvae in a 'crawling movement' (Yaguchi and Katow, 2003), but serotonin is implicated in orchestrated ciliary beating. However, it is still unknown how serotonin in the apical ganglion transmits the signal for this orchestration of spatial swimming.

In mammals, serotonergic neurons transmit signals through synapses or by secretion to the target cells that have serotonin receptors (for a review, see Deutch and Roth, 1999). The serotonin receptor is a seven-transmembrane G-protein coupled receptor (Peroutka, 1995), and possesses multiple transmembrane domains as an extracellular signal receptor in both vertebrates (e.g. Barnes and Sharp, 1999) and invertebrates (e.g. Tierney, 2001). In vertebrates, serotonin triggers the elevation of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in target cells (e.g. Jahnel et al., 1993; Saino et al., 2002), probably through the G-protein/adenylate cyclase signal transduction pathway (Brown et al., 2001). The elevation of $[Ca^{2+}]_i$ in ciliary epithelial cells results in increased ciliary beating frequency in invertebrates such as pond snail *Helisoma trivolvis* (Christopher et al., 1999; Doran et al., 2004), mussel *Mytilus edulis* and clam *Spisula solidissima* (Stephens and Prior, 1992), and in vertebrates (Nguyen et al., 2001).

Recently 5HThpr, a serotonin receptor, from plutei of the sea urchin *Hemicentrotus pulcherrimus*, was partially sequenced (Katow et al., 2004). The receptor is localized on the cells that form a network in the blastocoel, but not on the ciliated ectodermal cells that contribute to larval swimming (Katow et

al., 2004). Here, we resolve this conundrum and elucidate the entire coding region of the *5HThpr* gene. To resolve this issue, elevation of $[Ca^{2+}]_i$ was examined in larvae that had been microinjected with Oregon Green dextran 10X, a Ca^{2+} indicator, before fertilization. The larval blastocoel was then treated with serotonin to examine whether such serotonin application transmits any signal that can be detected by $[Ca^{2+}]_i$ elevation. The potential role of the serotonin receptor cell network (SRN) as a mediator of serotonin signaling to the ciliary ectoderm was tested with *p*CPA treatment, $[Ca^{2+}]_i$ examination, and immunohistochemistry using anti-5-HThpr antibodies.

Materials and methods

Gametes of the sea urchin *Hemicentrotus pulcherrimus* A. Agassiz (collected in the vicinity of the Research Center for Marine Biology, Asamushi, Aomori, Japan) were obtained by intra-coelomic injection of 0.5 mol l^{-1} KCl. Fertilized eggs were incubated in filtered seawater (FSW) on a gyratory shaker in an incubator at 18°C, and raised until 60 h post fertilization (60 h.p.f.), the 2-arm pluteus stage.

Sequencing of 5HThpr

Plutei at 60 h.p.f. were collected and dissolved with Isogen (Nippon Gene, Tokyo, Japan) to obtain total RNA. Poly(A)$^+$ RNA was extracted from the total RNA by Oligotex dT-30 Super (Takara, Otsu, Japan). Single strand cDNAs were made from the poly(A)$^+$ RNA by reverse transcriptase, Super Script II (Invitrogen, Tokyo, Japan), and oligo-d(T) primer (Invitrogen). According to the amino acid sequences of serotonin receptors, such as human 1A, *Aplysia*, and Lancelet, the following four degenerate primers were prepared. SRf1; WSNYTNGCNGTNGCNGAYYT, SRf2; YTNATGGT-NGCNGTNYTNGT, SRr1; NSWRTTRAARTANCC-NARCCA, SRr2; DATRAARAANGGNARCCARCA (W; A/T, S; C/G, Y; C/T, N; A/G/C/T, R; A/G). SRf1 and SRr1 were used for first PCR, and SRf2 and SRr2 were used for the nested PCR. After agarose gel electrophoresis, a single band around 800 bp was excised, eluted from the gel, and ligated to pGEM-T Easy Vector (Promega, Madison, WI, USA). Sequencing was conducted using a BigDye terminator cycle sequencing kit with a DNA sequencer model 310 (PE Applied Biosystems, Tokyo, Japan). Based on this sequence, the following four primers were designed: SR 5′-1; GAGATCCA-CACATCACAGAGT, SR 5′-2; ACTACCACAATCTCTT-TAGTC, SR 3′-2; TACCTGGTCAGATTCAGGAGA, SR 3′-3; AAGACTCTTGGGATTGTCACT.

To obtain the 5′ and 3′ termini of *5HThpr*, the reverse transcription was carried out with SR 5′-1 and oligo-d(T) primer combined with Adaptor sequence (Invitrogen), respectively. SR 5′-2 for 5′ termini, and SR 3′-2 and SR 3′-3 for 3′ termini, were used for first and second PCR with Adaptor primer (Invitrogen).

The 5HThpr protein sequences of the seven transmembrane domains from Scallop (*Mizuhopecten yessoensis*, accession number, AB209935), Aplysia (*Aplysia californica*, accession number, AF372526), Fugu (*Takifugu rubripes*, accession number, CAA65175), and sea urchin (*Strongylocentrotus purpuratus*, protein id='XP_780260.1) were aligned with the *H. pulcherrimus* 5HThpr using CLASTAL W. Domain structure, protein sorting signals and transmembrane structure of 5HThpr were analyzed by open database programs PROSITE (http://www.expasy.ch/prosite/), PSORT (http://psort.ims.u-tokyo.ac.jp/), and SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuimenu0.html) or TMpred (http://www.ch.embnet.org/software/TMPRED_form.html, respectively.

Whole-mount immunohistochemistry

To examine the structural relationship of the serotonin receptor cell network (SRN) with the ectoderm in 48 h.p.f. plutei, *p*CPA (Sigma, St Louis, MO, USA) was applied at 2 μg ml^{-1} to 17 h.p.f. mesenchyme blastulae until the 48 h.p.f. pluteus stage. The larvae were then fixed with 4% paraformaldehyde in FSW for 3 h for anti-5Hthpr antibodies or 15 min for 1E11, an anti-synaptotagmin monoclonal antibody (Burke et al., 2006). Embryos were then dehydrated through a series of increasing concentrations of ethanol from 30% to 70% and stored in 70% ethanol at 4°C. The samples were then hydrated in a series of decreasing concentrations of ethanol and finally in phosphate-buffered saline with 0.1% (v/v) Tween-20 (PBST), incubated with mouse anti-5HThpr antibodies (Katow et al., 2004) diluted at 1:200 in PBST or with 1E11 monoclonal antibody (hybridoma culture medium without dilution). After washing the samples with PBST 3 times (10 min each), the primary antibodies were detected with Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) or Alexa Fluor 594-conjugated goat anti-mouse IgG (H+L) antibodies (both from Molecular Probes Inc., Eugene, OR, USA) diluted in PBST 1:500 for 2 h. The anti-5HThpr antibodies were raised in mice against the synthetic peptide whose amino acid sequence was deduced from *5HThpr* DNA sequenced in our laboratory (Katow et al., 2004). After washing the samples in PBST 3 times (10 min each), they were examined under a Nikon epi-fluorescence microscope (Nikon, Tokyo, Japan). Aliquots of pluteus samples were double stained with rabbit anti-serotonin antibodies (Sigma), as stated above, after staining with anti-5HThpr antibodies. The anti-serotonin antibody-binding sites were visualized with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) or Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L) antibodies (both from Molecular Probes Inc.).

Detection of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) in plutei

To detect $[Ca^{2+}]_i$ in larvae, unfertilized eggs were microinjected with 5 mg ml^{-1} Oregon Green dextran 10X (Molecular Probes Inc.), a fluorescent Ca^{2+} indicator dye, at 2% of the total volume of an egg, and raised at 18°C in a dark incubator until the 48 h.p.f. pluteus stage. The plutei were then hooked to the tip of glass needles to prevent movement during serotonin microinjection and detection of $[Ca^{2+}]_i$-stimulated Oregon Green fluorescence excitation under a fluorescent microscope. Serotonin was diluted in FSW at 10 mmol l^{-1} and positioned in the micropipette between two oil droplets. Then,

64 pl of serotonin was microinjected into the blastocoel of the plutei as described previously (Kyozuka et al., 1998). The final concentration of serotonin microinjected into the blastocoel was about 250 μmol l^{-1}, based on a calculation that the average volume of the blastocoel is about 2.5 nl. Fluorescence intensity was recorded with a computer-controlled photomultiplier system (OSP-3, Olympus, Tokyo, Japan). The microinjection of FSW without serotonin did not trigger excitation of Oregon Green dextran 10X. Aliquots of embryos microinjected with Oregon Green dextran 10X were treated with 2 μmol l^{-1} of *p*CPA from the 17 h.p.f. mesenchyme blastula stage until the 48 h.p.f. pluteus stage, microinjected with serotonin, and examined for the occurrence of Oregon Green dextran 10X excitation as stated above.

Fluorescence images of videotape were converted into digital images and processed using NIH Image (a public domain image processing software for the Macintosh computer). The sequential digitized images, each of which was an average of four successive images, were captured at intervals of 0.5 s. To examine transient elevation of $[Ca^{2+}]_i$ near the surface of plutei, the values of average fluorescence intensities calculated in the region were normalized by dividing them by the resting value. To analyze the detailed spatio-temporal propagation of $[Ca^{2+}]_i$ elevation in the larvae, sequential fluorescence images were normalized by dividing them by the resting image immediately before the injection in a pixel-to-pixel manner and expressing them with pseudo color images, with red for the highest $[Ca^{2+}]_i$ followed by yellow, green, light blue and to the lowest $[Ca^{2+}]_i$ with deep blue. Since the fluorescent Ca^{2+} indicator was introduced into unfertilized eggs and excited after 2 days in culture by microinjecting serotonin, the intensity was weaker in some larvae than in ordinary usage when Ca^{2+} excitatory stimulation is applied immediately after microinjection of the Ca^{2+} indicator. However, we confirmed that the dye was able to respond to Ca^{2+} stimulation with intensified fluorescence by application of 20 μmol l^{-1} A23187 instead of serotonin (data not shown). The experiment was repeated with 11 larvae for control and 6 larvae for *p*CPA-treated. The number of ectodermal cells along the major $[Ca^{2+}]_i$ wave propagation route was counted in 48 h.p.f. larvae that were stained with 4′,6-diamidino-2-phenylindole.

Results

Gene structure of 5HThpr

The *5HThpr* cDNA comprised 1395 bp of the open reading frame (ORF) between 543 bp of 5′ untranslated region (UTR) and 254 bp of 3′ UTR regions (Fig. 1, DNA Data Bank of Japan, accession number AB248086). The ORF encoded 465 amino acids, and showed high similarity to the serotonin receptors of *S. purpuratus* 5HT-1A (e value=0), Scallop ($9e^{-67}$),

A

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1    ATTACCTTGTTATGTTAGTGGTGCATGCATACTGTCAGTGGAACAATATCACAAGGTAGC   60
61   TGAAGTACACCAAGCAACAAAGCCAATTAGTAAATCAAGGAGAAGTACACACACATGACT  120
121  GACCCTTCAATCATACGTATCGTCAAGGGGTATTCACTCTTACAAGTAATTCAACCACTT  180
181  AGGAGACTCTGTGCATGATACCCATTGATTATGCAAAAGACCACATCTGGCATCATCCCA  240
241  AGACCTTCTGTTTGATTGCAGAAAATGAACTAGCACTCTGAGAAGCTAGCGAAGATCAAA  300
301  AGTACCATTTCCCTTGAAGGACCTCTCATATACAGATGCGTAGTAGTTGAGATTTCTGTT  360
361  AGACCTGCATTTTTTTTAAAACTGAGAGTGTTTAAAAACTATTGCAAAACATTGTTATAT  420
421  TCATTTTGAAGGAGGGAACTTCAACACTGACTTAGCAAACGGATCCAGACAGTCATACTT  480
481  AGTCATGGCTGATTTTGCTTCTGAGGCAACTGTGACAAATACTGAGACTGTGGACTATGT  540
1        M  A  D  F  A  S  E  A  T  V  T  N  T  E  T  V  D  Y  V    19
541  GGAAGCATTAGACAGTAGCTATGATTGGGTGATCACCAATAATGTGACGGAGAATGAGAC  600
20   E  A  L  D  S  S  Y  D  W  V  I  T  N  N  V  T  E  N  E  T    39
601  CATAGAAATCAAGGTAGCCACAGTTCTGCTGAGCATCGGACTGGGCTTCATCATCATTGG  660
40   I  E  I  K  V  A  T  V  L  L  S  I  G  L  G  F  I  I  I  G    59
661  GACAATCTTCCGGAAATGCATTAACGCTCACTGCGATCTCCAAACATCGCCCCCTCCAAAC  720
60   T  I  F  G  N  A  L  T  L  T  A  I  S  K  H  R  P  L  Q  T    79
721  ACCCCAGAACCACTTGATAGCATCCTTGGCTGTTGCTGATCTGATGGTGGCAATTCTGGT  780
80   P  Q  N  H  L  I  A  S  L  A  V  A  D  L  M  V  A  I  L  V    99
781  GATGCCTTTGAGCTTGACTAAAGAGATTGTGGTAGTCTGGATATTTGGCCCCATACTCTG  840
100  M  P  L  S  L  T  K  E  I  V  V  V  W  I  F  G  P  I  L  C   119
841  TGATGTGTGGATCTCACTGGATGTTCTCCTGTGCACCGCCAGTATCCTCAGCCTCTGTAT  900
120  D  V  W  I  S  L  D  V  L  L  C  T  A  S  I  L  S  L  C  M   139
901  GATATCCCTTGATCGATTCTGGGCCATCACAAAACCCATCAAGTATCCAAAGTACCGGAC  960
140  I  S  L  D  R  F  W  A  I  T  K  P  I  K  Y  P  K  Y  R  T   159
961  AGCGCGTACCATGGTAATCCTTATCGCCTTGGCCTGGTTCCTCTCTAGTATCATTTCTAC 1020
160  A  R  T  M  V  I  L  I  A  L  A  W  F  L  S  S  I  I  S  T   179
1021 CTCACCATTCTTCGGCTGGCGTGGCAACCAGAGCATCGACGCTTACACCTGTCAGATTAG 1080
180  S  P  F  F  G  W  R  G  N  Q  S  I  D  A  Y  T  C  Q  I  S   199
1081 CCAGAATATTGCCTACACCATCTTCTCCACCTTTGGTGCCTATTTCATCCCTATGACAAT 1140
200  Q  N  I  A  Y  T  I  F  S  T  F  G  A  Y  F  I  P  M  T  I   219
1141 CATGATGATCGTCTATGCGAGAATATATTGTGAGGCACGTAAGAGAATCCGCGGTAAAAC 1200
220  M  M  I  V  Y  A  R  I  Y  C  E  A  R  K  R  I  R  G  K  T   239
1201 ATTCAACAGGGGGTGTCAGCCCTTAAACAGCGCTGTGAACTATCGAGAAACAGCAAATTA 1260
240  F  N  R  G  C  Q  P  L  N  S  A  V  N  Y  R  E  T  A  N  Y   259
1261 TTGCGATAGAAAAGATTCTGAGGAGTCGAATAAATCTCCTATTGTCAAATGTGATCCAGC 1320
260  C  D  R  K  D  S  E  E  S  N  K  S  P  I  V  K  C  D  P  A   279
1321 ATCTGACAATCACAAAGGAGGCGTCTTTGATGGATCAACGTTGTCTCACAGCATGCTCGC 1380
280  S  D  N  H  K  G  G  V  F  D  G  S  T  L  S  H  S  M  L  A   299
1381 TGCATCAGCTCCAAACCTTCAGAACCAACACAAAGTGGGAGAGGACGTCAAGAAGGATAC 1440
300  A  S  A  P  N  L  Q  N  Q  H  K  V  G  E  D  V  K  K  D  T   319
1441 CTTTGTTACGTCACTGACTGTACCAGGGCAGCACCAAGCAACGTTTACCTCAACATCTCT 1500
320  F  V  T  S  L  T  V  P  G  Q  H  Q  A  T  F  T  S  T  S  L   339
1501 CCGCGAGATCAACTCCAACGAGAGTCCGTCTTCTCAACGAAGTCTCCTTTCATCCCCAAG 1560
340  R  E  I  N  S  N  E  S  P  S  S  Q  R  S  L  L  S  S  P  S   359
1561 CCCCCTACCTGGTCAGATTCAGGAGAGGGAGCGCAGGAGACGCGCAATGGCTACTGCCAG 1620
360  P  L  P  G  Q  I  Q  E  R  E  R  R  R  R  A  M  A  T  A  R   379
1621 AGAGCATCGCGCCACAAAGACTCTTGGGATTGTCACTGGAGCCTTTCTCGTGTGCTGGTT 1680
380  E  H  R  A  T  K  T  L  G  I  V  T  G  A  F  L  V  C  W  L   399
1681 ACCATTTTTTCTCCATGCTCTTATCGTGCCCCTATGTGGTGAAGCGTGCAACGTCCCTCG 1740
400  P  F  F  L  H  A  L  I  V  P  L  C  G  E  A  C  N  V  P  R   419
1741 GTCGTTGGAAAGCATCTTCCTTTGGCTTGGCTACTTCAATTCCATGCTTAATCCCATCAT 1800
420  S  L  E  S  I  F  L  W  L  G  Y  F  N  S  M  L  N  P  I  I   439
1801 ATACACCAAATTTAACCAAGACTTTCAGAAGGCCTTCAAGAAACTTCTGCATTGTAAAGA 1860
440  Y  T  K  F  N  Q  D  F  Q  K  A  F  K  K  L  L  H  C  K  E   459
1861 GAGCAAGTCTCAATCCTTGTGAGGACAGCTAATCACAAATCTCCAGCGTATTTGTGTTAA 1920
460  S  K  S  Q  S  L  *                                          465
1921 GTCTTGCTCACCTAAAGGAAACACCTCACCTACCATTGACGCGACTATGTGCATTAATTT 1980
1981 TGCCATGGACTAACAGTGCTCATTATCAAAAGACAATATAAAGAACCTTTAAAAAAAAAA 2040
2041 AAAAAAAGTACTAGTCGACGCGTGGCCACATACCTGGTTACCTGGTCAGATTCAGGAGAC 2100
2101 ACATATAACATCTTATTGGAGTGTCTCATACATTGTACATGTAAATTAAATAATCGGTCA 2160
2161 TAAAAAAAGGGAACCAAAAAAAAAAAAAAAAAA  2194
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B

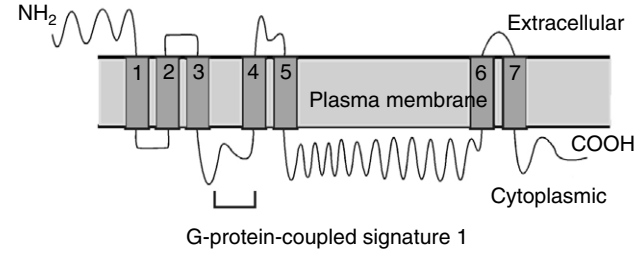

Fig. 1. Sequence of 5HThpr gene and protein and expected secondary structure. (A) *5HThpr* gene consists of 2194 nucleotides comprising 543 bp of 5′ untranslated region (UTR), 1395 bp of coding region and 254 bp of 3′ UTR. The open reading frame encoded 465 amino acids that contained seven predicted transmembrane domains (shaded boxes) and an expected G-protein-coupled signature domain (underlined). Three potential *N*-glycosylation sites were shown (open boxes). (B) Schematic presentation of predicted secondary structure of 5HThpr based on analysis by SOSUI software program. First six transmembrane domains (1–6) from the N terminus (NH_2) are primary helix, and seventh domain (7) nearest to the C terminus (COOH) is a secondary helix. A G-protein-coupled signature 1 locates between the third and fourth transmembrane domains. Cytoplasmic, inside the cell; extracellular, outside the cell.

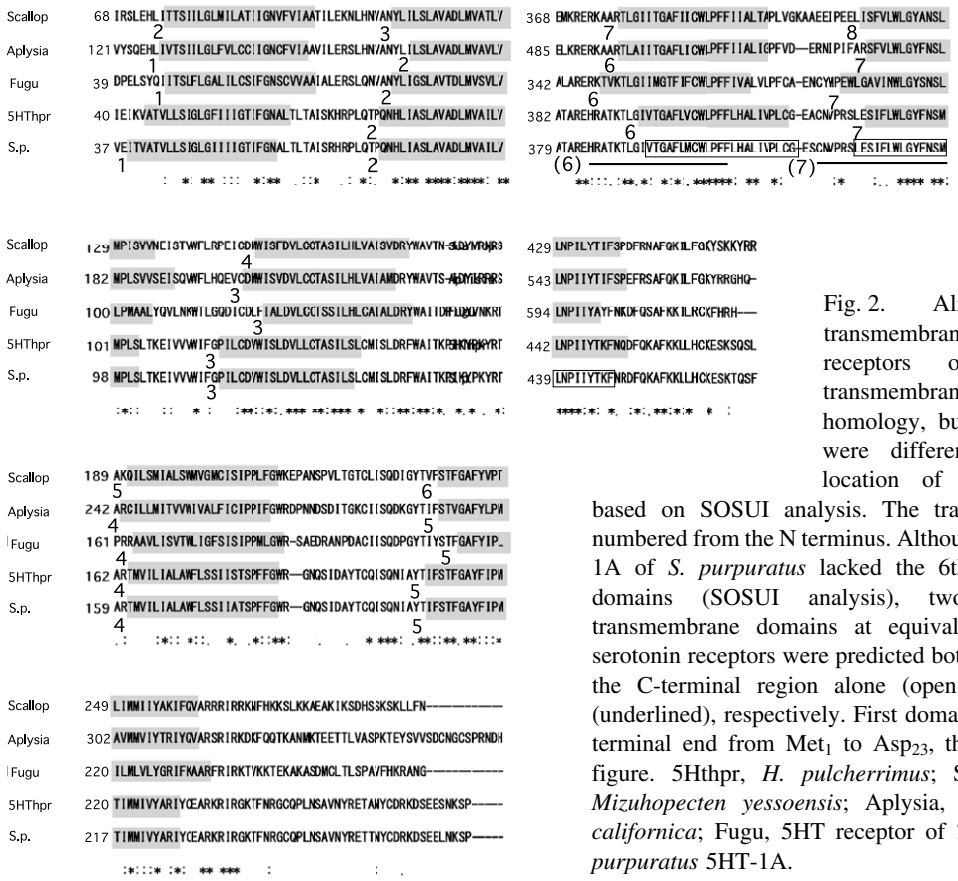

Fig. 2. Aligned sequences of transmembrane domains of serotonin receptors of five species. All transmembrane domains showed high homology, but the number of domains were different. Shaded boxes show location of transmembrane domains, based on SOSUI analysis. The transmembrane domains are numbered from the N terminus. Although serotonin receptor 5HT-1A of *S. purpuratus* lacked the 6th and 7th transmembrane domains (SOSUI analysis), two additional C-terminal transmembrane domains at equivalent regions to the other serotonin receptors were predicted both by SOSUI analysis using the C-terminal region alone (open rectangles) and TMpred (underlined), respectively. First domain of Scallop was at the N-terminal end from Met_1 to Asp_{23}, thus was not shown in this figure. 5Hthpr, *H. pulcherrimus*; Scallop, 5HT receptor of *Mizuhopecten yessoensis*; Aplysia, 5HT receptor of *Aplysia californica*; Fugu, 5HT receptor of *Takifugu rubripes*; S.p., *S. purpuratus* 5HT-1A.

Aplysia ($9e^{-61}$), and Fugu ($2e^{-63}$), particularly at the transmembrane domains (Fig. 2). Protein analysis by PROSITE and SOSUI showed that 5HThpr is composed of three extracellular *N*-glycosylation sites at the N-terminal region (Asn_{33} to Glu_{36}, Asn_{37} to Ile_{40}, and Asn_{188} to Ile_{191}), a cytoplasmic G-protein-coupled domain (Ala_{132} to Ile_{148}), and seven-transmembrane domains (1: Ala_{45} to Thr_{67}, 2: Gln_{81} to Ser_{103}, 3: Pro_{116} to Thr_{131}, 4: Thr_{162} to Gly_{184}, 5: Ile_{206} to Ile_{227}, 6: Ile_{389} to Cys_{411}, and 7: Ser_{423} to Gln_{445}) (Fig. 1). SOSUI analysis also showed that the first six transmembrane domains were primary helices and the last domain was a secondary helix. PSORT-expected 5HThpr had no signal peptide, suggesting it locates at the plasma membrane independent of the signal peptide system, like the serotonin receptors of the other animals.

The number of predicted transmembrane domains by SOSUI analysis, however, was different among them. The serotonin receptors of *Aplysia*, *Fugu* and the 5HThpr each have seven transmembrane domains, while that of Scallop has eight. Although 5HT-1A of *S. purpuratus* showed the highest similarity to 5HThpr, unlike 5HThpr, it contains only five predicted transmembrane domains. SOSUI analysis did not predict the two C-terminal transmembrane domains in the *S. purpuratus* 5HT-1A, even though the amino acid sequence is very similar to the 6th and 7th transmembrane domains of 5HThpr (Fig. 2). However, SOSUI analysis of the *S. purpuratus* 5HT-1A, using only the C-terminal sequence (from Val_{361} to Phe_{463}) predicted the region as two transmembrane domains (Fig. 2, open rectangles).

About 30 potential serotonin receptors were predicted in *S. purpuratus* based on The Sea Urchin Genome Project using GLEAN3 (GOMS Language Evaluation and Analysis, http://www.ulb.ac.be/di/gom/mavvyve/goms.pdf#search='GLEAN3'). However, they turned out to be shared by many non-serotonin receptor transmembrane proteins, and BLAST search analysis using non-transmembrane domains reduced this number to four serotonin receptor subtypes, including those homologues to 5HT-1A (GLEAN3_18826), 5HT-1F (GLEAN3_25436), 5HT-2C (GLEAN3_25436) and 5HT-7 (GLEAN3_05097). However, except for the *S. purpuratus* 5HT-1A, that is the 5HThpr homologue, no RNA of any other serotonin receptor homologue has been cloned to date, suggesting that three serotonin receptors may be not transcribed during the larval period of the sea urchin. According to computation of relative molecular mass based on the present deduced amino acid sequence by ExPasy analysis (http://us.expasy.org/tools/pi_tool.html), the predicted relative molecular mass of 5HThpr was 51 889.08 Da, and was similar to those of other animals (cf. Katow et al., 2004).

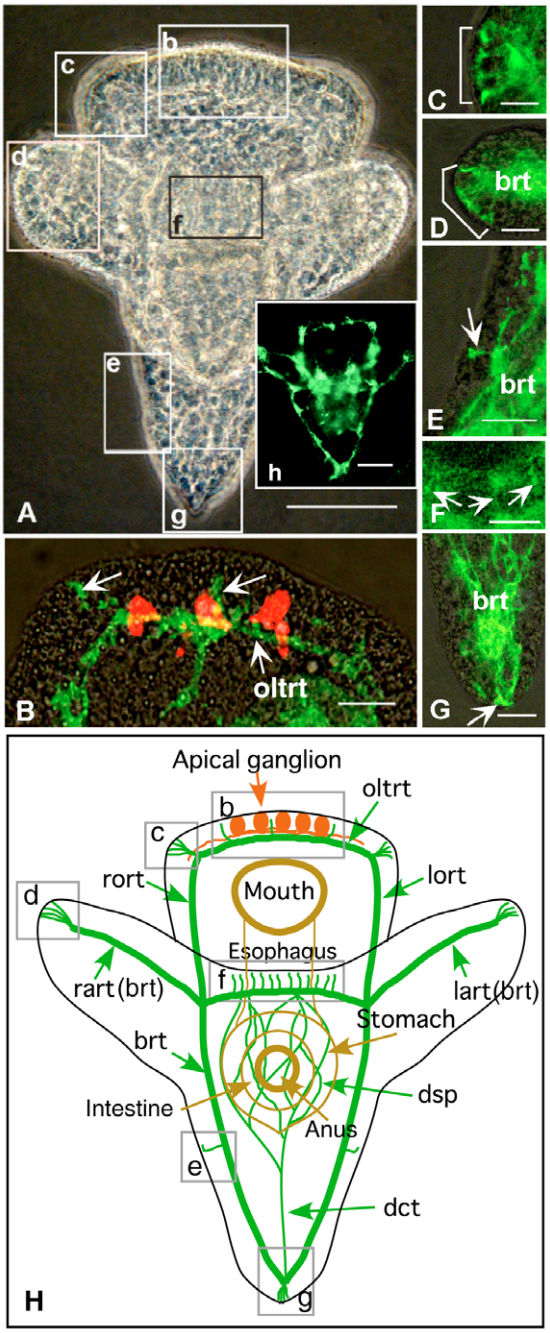

Fig. 3. (A–G) Immunohistochemical localization of serotonin receptor cell processes (fibers) inserted into larval ectoderm. In plutei at 48 h post-fertilization (h.p.f.), fibers from serotonin receptor cell network are inserted into the ectoderm around the apical ganglion (Ab,B, arrows, H), extending from the oral lobe transverse tract ('oltrt' in B,H), at the left and right corners of oral lobe (Ac,C,H), at the tip of left and right arms (Ad,D,H), at the middle region of posterior body on both left and right sides (Ae,E,H), at ventral ciliary band (Af,F, arrows, H), and at the tip of posterior end of the body (Ag,G, arrow, H). Bars, 100 μm (A,h), 30 μm (B,E,F), 40 μm (C,D) and 20 μm (G). (H) Schematic ventral view of 48 h.p.f. pluteus larva summarizing major tracts of the serotonin receptor cell network and the sites where fibers are inserted into the ectoderm (green lines), based on present and previous observations (Katow et al., 2004). Letters b–g in gray rectangles correspond to the white rectangles in A and insets in C–G. This scheme shows tracts of serotonin receptor network shown by whole-mount immunohistochemistry (Ah), including left and right oral rod tracts (lort and rort), left and right anal rod tracts (lart and rart), dorsal stomach plexus (dsp) and dorsal central tract (dct). Digestive tracts are shown with light brown line.

Connection between ectoderm and serotonin receptor cell network (SRN)

The SRN comprises five major tracts that include left and right oral rod tracts, left and right body rod tracts, an oral lobe transverse tract and two SRN plexuses that include dorsal esophagus serotonin receptor cell plexus and dorsal stomach plexus (Katow et al., 2004), and inserted cell processes (fibers) into the larval ectoderm at nine places (SRN-ectoderm connection sites) as will be described as follows (Fig. 3). The major SRN-ectoderm connection sites were (1) around the apical ganglion with thin fibers from the oral lobe transverse tract (oltrt) (Fig. 3Ab,B,arrows, H), (2) at the left and right corners of the oral lobe ectoderm with multiple fibers from 'oltrt' (Fig. 3Ac,C,H), (3) at the tips of the left and right larval arms with multiple fibers extended from anal rod tracts [Fig. 3Ad,D,H,(art)], (4) at the middle of left and right sides of posterior trunk with single fiber with bulged head from body rod tracts (brt) (Fig. 3Ae,E,H), (5) inbetween two larval arms (Fig. 3Af,F,arrows, H) and (6) at the posterior end of larva with fibers from 'brt' (Fig. 3Ag,G,arrow, H). Most of these regions of ectoderm contain the ciliary band, except at the middle of the posterior trunk region and posterior end of the larva. The middle of the posterior trunk region has scattered body surface cilia (Hara et al., 2003). SRN fibers inserted at the both corners of oral lobe and at the tip of larval arms were characteristically branched to show a 'forkhead' feature (Fig. 3C,D,H), whereas at the middle of the posterior trunk region was only a single-headed fiber in the ectoderm (Fig. 3E,arrow, H). Many SRN fibers inserted into the ventral ectoderm in between two larval arms resembled a comb-like feature (Fig. 3F,arrows, H). These fibers suggest that SRN extends neurites to signal the ectoderm, particularly to the ciliary band region.

Elevation of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the ectoderm by serotonin

In sea urchin larvae, serotonin cells at the apical ganglion inserted neurites into the ciliary band ectoderm and blastocoelar space (e.g. Bisgrove and Burke, 1987). However, neither at the ciliary band ectoderm nor in the blastocoelar space, did these nervous terminals constitute synapses (Nakajima et al., 1993), suggesting that serotonin is secreted into the blastocoelar space from the nerve ends. Thus, to mimic this manner of *in vivo* serotonin delivery, the neurotransmitter was microinjected to the blastocoelar space.

Eggs injected with Oregon Green dextran 10X, fertilized and incubated in dark room, developed, at least, to 48 h.p.f. 2-arm pluteus larva stage. Microinjection of serotonin into the blastocoel stimulated $[Ca^{2+}]_i$ elevation in the ectoderm

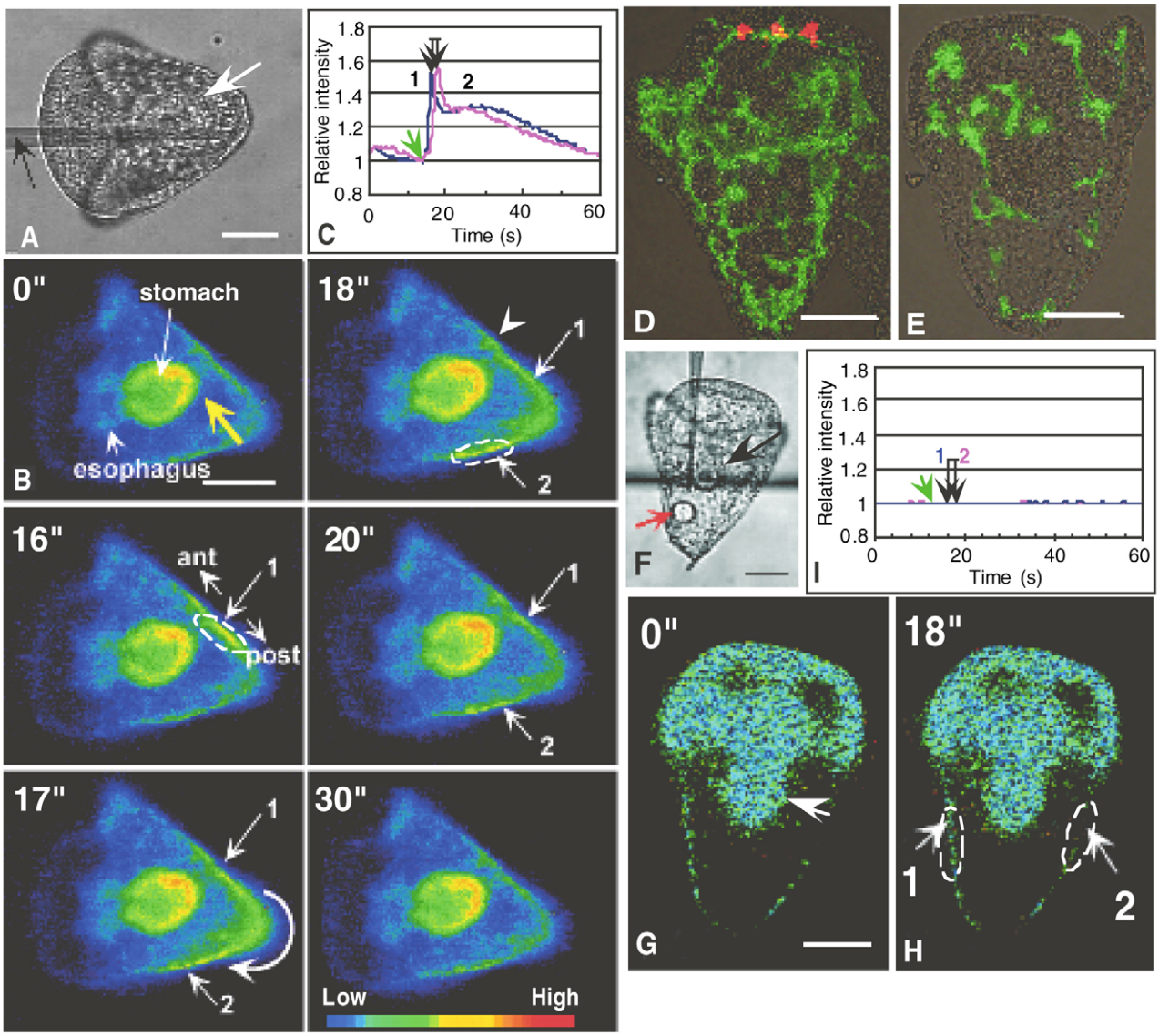

Fig. 4. Elevation of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) by exogenous serotonin microinjected to the blastocoel. (A) Ventral view of a larva hooked at the tip of a glass needle (black arrow) near stomach (white arrow). (B) Propagation of $[Ca^{2+}]_i$ wave in a same pluteus larva as in A. Microinjection of serotonin into the blastocoel near stomach (B, 0″, yellow arrow) triggered elevation of $[Ca^{2+}]_i$ on the larval surface. The micropipette was kept inserted to the blastocoel without releasing serotonin for 15 s to monitor $[Ca^{2+}]_i$ level before serotonin application. Initial elevation of $[Ca^{2+}]_i$ occurred near serotonin injection site immediately after onset of serotonin release (B,16″, arrow 1), that transiently propagated posteriorly for about 350 μm in 2 s (C, double-headed arrow) on the left side (B, 16″, post), then traveled to the right side of larva by 17 s (B, 17″, arrow 2 and curved arrow), and the other one anteriorly (B, 16″, ant) for 50 μm before diminishing earlier than the posterior propagation at 20 s (B, 20″). The numbers shown on upper left corner in (B) show the time in second from when micropipette was inserted (time zero, 0′) and thereafter. Transient $[Ca^{2+}]_i$ elevation returned close to visual time zero level in 30 s (B, 30″), and to background level in 60 s as shown by a time-course of the wave intensity (C). $[Ca^{2+}]_i$ elevation occurred soon after the completion of serotonin releasing (C, green arrow). A second fluorescence wave toward the anterior direction diminished in 2 s after the initial $[Ca^{2+}]_i$ elevation (B, from 16″ to 18″, 18″ arrowhead). Rainbow-colored bar shows relative intensity of fluorescence, as described in Materials and methods. (C) Entire time-course of $[Ca^{2+}]_i$ elevation from 15 s before onset of serotonin release (time zero, green arrow) to 60 s. (D) Double stained immunohistochemistry of serotonin (red) and serotonin receptor cell network (green). The serotonin receptor cell network is a major structure in the blastocoelar space. (E) Double stained immunohistochemistry of serotonin (negative immunoreaction) and serotonin receptor cell network (green) of *p*-chlorophenylalanine (*p*CPA)-treated larva. *p*CPA inhibited serotonin synthesis at the apical ganglion and perturbed the formation of serotonin receptor cell network. (F) Same *p*CPA-treated larvae as that used to examine $[Ca^{2+}]_i$ elevation. Red arrow shows an oil droplet from the glass micropipette, and is used as a marker of injection. The stomach is smaller than that of normal larvae (black arrow). (G) *p*CPA-treated larva lacked intrinsic high level of $[Ca^{2+}]_i$ at stomach (0′, arrow). (H) 18 s after serotonin injection, no elevation of $[Ca^{2+}]_i$ occurred. (I) Time course of $[Ca^{2+}]_i$ elevation in pCPA-treated larva. No $[Ca^{2+}]_i$ elevation occurred. Bars, 100 μm.

immediately after the injection (16 s) in the region of the injection site (Fig. 4B, 16″, arrow 1), but not in the SRN. The major fluorescence triggered by the elevation of $[Ca^{2+}]_i$ apparently propagated about 350 μm in 2 s that encompassed 27±5 ectodermal cells (N=5), initially toward the posterior end of the larval body and then anteriorly on the opposite side of the larval body within 20 s (Fig. 4B, 16″ to 20″, arrow 2; C, pink line). Although the intensity of the fluorescence at the injection site decreased rapidly in 1 s after the first elevation of $[Ca^{2+}]_i$ (Fig. 4C, blue line), the leading edge of intensive fluorescence continued to propagate as a wave. Relatively high $[Ca^{2+}]_i$ levels remained on the larval surface until it returned to the initial background level by 60 s (Fig. 4C). Propagation of a minor fluorescence wave also occurred from the injection site toward the anterior region of the larval body (Fig. 4B, 16″, ant). This anterior wave propagated about 50 μm, and diminished 1 s later than was seen earlier than the major posterior wave (Fig. 4B, 17″). The present study also detected an intrinsically high $[Ca^{2+}]_i$ level around the stomach which, however, did not apparently respond to serotonin (Fig. 4B).

Calcium ions are known to activate ciliary beating in larval swimming. To examine whether serotonin signals the elevation of $[Ca^{2+}]_i$ in the ciliary ectoderm, we used *p*CPA, an inhibitor of serotonin synthesis. Levels of *p*CPA that severely inhibit larval swimming activity (2 mmol l^{-1}) (Yaguchi and Katow, 2003) were added to the culture medium of 17 h.p.f. mesenchyme blastulae, in which differentiation of serotonin ganglion was not yet observed. In all *p*CPA-treated larvae examined in this study, serotonin synthesis was severely inhibited (Fig. 4E), and the intrinsic high level of $[Ca^{2+}]_i$ was not seen in these larval stomachs (Fig. 4H) whose size was characteristically smaller than that of control larvae (Fig. 4A, black arrow, 4F, white arrow). However, they crawled on the bottom of culture dishes as was previously reported (Yaguchi and Katow, 2003). Thus, the lack of intrinsically high $[Ca^{2+}]_i$ in the stomach does not affect viability. In these embryos, the elevation of $[Ca^{2+}]_i$ in the ectoderm never occurred by microinjection of serotonin (Fig. 4I,G,H). Immunohistochemistry revealed astonishingly severely disrupted SRN conformation in *p*CPA-treated larvae. In these larvae, considerably fewer serotonin receptor cells were seen, and were scattered in the blastocoel with few intercellular connections among them. Thus, most of the major SRN tracts were not formed, and they comprised few detectible SRN-ectoderm connection sites (Fig. 4E).

The echinolarvae also have another nervous system immediately beneath the ciliary band ectoderm. This system possesses synaptotagmin (Fig. 5A), and is implicated in participation of the nervous system for larval swimming. However, *p*CPA treatment did not affect the formation of this alternative nervous system (Fig. 5B), showing that this nervous system is not sensitive to serotonin deprivation and does not participate in larval swimming. Thus, the present observation strongly suggested that the transient elevation of $[Ca^{2+}]_i$ in the ectoderm occurs in the presence of an intact SRN structure with sufficient SRN-ectoderm connection sites, and that the larval swimming activity needs intact SRN that transmits serotonin signaling to the ectoderm to stimulate $[Ca^{2+}]_i$ elevation. Although we cannot exclude potential participation of another nervous system that deploys unspecified neurotransmitters other than serotonin and regulates the larval spatial swimming behavior, we do not have any observations to suggest such a possibility to date.

Discussion

Serotonin microinjected into the blastocoel of sea urchin larvae triggered a transient elevation of $[Ca^{2+}]_i$ in the ectodermal cells that bidirectionally propagated beyond intercellular boundaries. Perturbation of SRN structure by *p*CPA inhibited the transient elevation of $[Ca^{2+}]_i$ in the ectoderm, suggesting that SRN transmitted serotonin signal to the ectoderm, probably through its fibers inserted into nine regions near the ciliary band of the ectoderm. From the previous observation, *p*CPA does not inhibit ciliary beating itself, and yet severely inhibits larval spatial swimming behavior that is, however, prevented by application of exogenous serotonin (Yaguchi and Katow, 2003). SRN formation failed in *p*CPA treatment, but that is also rescued by the presence of exogenous serotonin (H.K., unpublished observation). These observations suggest that serotonin-triggered $[Ca^{2+}]_i$ elevation in the ectoderm may be mediated by SRN, and its propagation is likely involved in 'stabilizing the rhythm of ciliary beating' for larval spatial swimming (Wada et al., 1997).

Based upon the previous pharmacological studies, 'pre-nervous' serotonin is involved in early cleavage of sea urchin development (Renaud et al., 1983; Shmukler, 1993; Shmukler

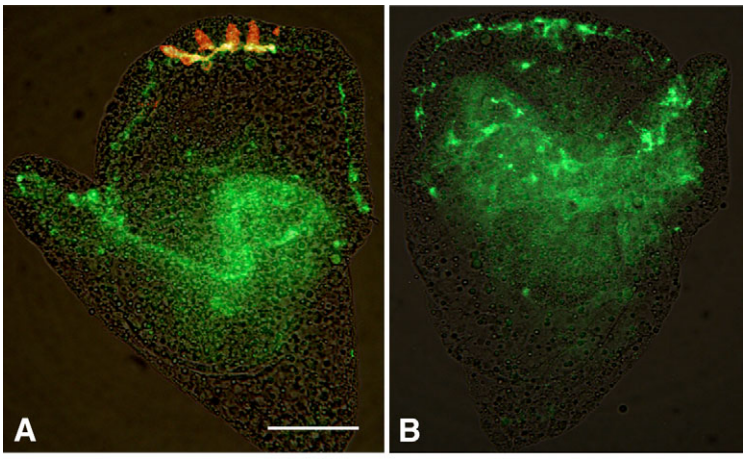

Fig. 5. Double-stained immunohistochemistry of serotonin (red) and synaptotagmin (green). (A) In intact larva, serotonin was synthesized at the apical ganglion (red) and synaptotagmin-possessed nerve cell network lined along the ciliary band (green). (B) In *p*CPA-treated larva, the apical ganglion did not produce serotonin, but formation of the synaptotagmin-possessing nerve cell network (green) was not affected (B). Bar, 100 μm.

and Tosti, 2001), and serotonin receptors have been suggested to be in the plasma membrane of blastomeres during cleavage period (Shmukler, 1993; Shmukler and Tosti, 2001). Serotonin is involved in ciliary beating regulation in embryos such as gastrulae (Soliman, 1983). Our previous immunoblotting conducted using anti-5HThpr antibodies detects very weak serotonin receptor expression soon after fertilization that further weakens until the late gastrula stage before the serotonergic nervous system morphologically emerges. At and after prism stage, however, distinctively intensive immunoreaction of 5HThpr reappears, at least through the pluteus stage (Katow et al., 2004). On the other hand, immunohistochemistry does not locate serotonin receptors at any particular region of the egg or the embryos before the prism stage, but at and after prism stage a part of secondary mesenchyme cells expresses 5HThpr until, at least, the pluteus stage (Katow et al., 2004). These observations suggest that the subtype of serotonin receptor participating in very early embryogenetic periods, such as during cleavage, may be different from the 5-HThpr that we have studied in larvae.

Nevertheless, when serotonin is released during the cleavage stages, the sea urchin blastomeres respond to the neurotransmitter with elevation of $[Ca^{2+}]_i$ (Shmukler et al., 1999), as was reported in mammalian cells (e.g. Jahnel et al., 1993; Saino et al., 2002; Ulrich et al., 2003). Serotonin is also suggested to activate contraction of the muscle that is surrounding the esophagus of sea urchin larvae by stimulating a strong influx of Ca^{2+} to the muscle cells (Gustafson, 1991). The present observation of a serotonin-triggered transient elevation of $[Ca^{2+}]_i$ in the ectoderm is the first report in sea urchin larvae and this signaling requires the presence of an intact SRN in the blastocoel. Although SRN extends fibers around the muscle cells at the esophagus (Katow et al., 2004), the present observation barely detected elevation of $[Ca^{2+}]_i$ in the muscle cells by microinjected serotonin (Fig. 4B), suggesting that the intensity of $[Ca^{2+}]_i$ elevation in muscle cells, even if it occurred, was below the level detectable by the present technique.

The characteristic property found in the present $[Ca^{2+}]_i$ elevation in the ectoderm was the propagation of a $[Ca^{2+}]_i$ wave beyond intercellular borders with a velocity of 175 $\mu m\ s^{-1}$ in the posterior regions of the larval body. The regions where initial elevation of $[Ca^{2+}]_i$ occurred were closely associated with the presence of SRN-ectoderm connection sites, such as at the middle of posterior ectoderm (Fig. 3E,H, Fig. 4B). The present observation showed that the propagation of $[Ca^{2+}]_i$ elevation was led by a high $[Ca^{2+}]_i$ edge. The leading edge of the present $[Ca^{2+}]_i$ wave in the ectoderm was not as sharp as those seen in eggs at fertilization. However, since ectodermal cells are less than 1/10 of the diameter of an oocyte [about 110 μm and often the subject of $[Ca^{2+}]_i$ wave propagation studies (e.g. Kyozuka et al., 1998)], the leading edge of the $[Ca^{2+}]_i$ wave in the ectodermal cells was considered to be distinctively sharp. This particular manner of $[Ca^{2+}]_i$ wave propagation in the ectoderm may implicate the occurrence of 'regeneration' of $[Ca^{2+}]_i$ elevation at each ectodermal cell. This could be triggered by an intensity of $[Ca^{2+}]_i$-derived signal that surpasses a certain threshold rather than simple diffusion of serotonin in the blastocoel which, unlike the present observation, creates a decreasing gradient of $[Ca^{2+}]_i$ intensity from the initial elevation site to the leading edge of $[Ca^{2+}]_i$ wave, probably with weak fluorescence. Diffusion of Ca^{2+} from the previous cell to the next through gap junctions (Braet et al., 2003) also seems to be an unlikely mechanism in sea urchin larvae, because gap junctions were not found in the ectoderm (Katow and Solursh, 1980), and connexin-like proteins have not been found in the Sea Urchin Genome Resources to date (Sea Urchin Genome Sequencing Consortium, 2006).

In *Xenopus* egg activation, the signal transmitter from serotonin-activated SRN cells needs to activate the cytoplasmic signal transduction pathways to elevate $[Ca^{2+}]_i$ that are augmented *via* Ca^{2+}-stimulated formation of inositol-1,4,5-trisphosphate, as was seen in the protein kinase C (PKC) wave that follows the $[Ca^{2+}]_i$ wave (Larabell et al., 2004). PKC-related break down of inositol phospholipids occurs in association with desmosomes (Kitajima et al., 1992), the intercellular junction also found in sea urchin ectoderm (Katow and Solursh, 1980). Thus, desmosomes could be involved in the present intercellular propagation of $[Ca^{2+}]_i$ elevation in the ciliary epithelium.

Internal application of serotonin to *p*CPA-treated, and thus SRN-perturbed, larvae did not stimulate $[Ca^{2+}]_i$ elevation in ectodermal cells, suggesting a role of SRN as a mediator of serotonin signal from the apical ganglion to the ectodermal cells. This pathway resembles serotonergic interneurons of mollusk *Tritonia diomedea* (Sakurai and Katz, 2003) or serotonergic sensory-motor neurons of the pond snail *Helisoma trivolvsi* (Kuang et al., 2002). A study of the gastropod mollusks, *Aplysia*, has also shown that the giant serotonergic cells can act as peripheral modulator neurons, as well as interneurons, and in this way they can affect their target organs at more than one level (Rozsa, 1984). The abnormal morphology of the *p*CPA-treated larvae included a smaller digestive organ with severely decreased intrinsic levels of $[Ca^{2+}]_i$ (Fig. 4G). This observation implicated the involvement of serotonin in the morphogenetic process of digestive organs, as was suggested by the previous observation that in the larvae under the presence of excess concentration of serotonin the neurotransmitter not only prevents *p*CPA-induced perturbation of SRN formation but also develops hyper-branching of SRN (H.K., unpublished observation).

Although possible participation of other subtypes of serotonin receptors in the ectodermal $[Ca^{2+}]_i$ regulation in sea urchin larvae is not excluded, and GLEAN3 predicated the presence of at least three other types of 5HT receptors, there has been no report of their mRNA other than the 5HThpr homolog to date. It also possible to predict the involvement of other non-serotonergic nervous systems in the regulation of larval spatial swimming behavior and $[Ca^{2+}]_i$ elevation in the ectoderm, such as the nervous system that has synaptotagmin and appears about the same developmental period in the larvae

as the serotonergic system (Burke et al., 2006). The present observation, however, indicated it was unlikely, because conformation of the synaptotagmin-possessed nervous system was not affected by *p*CPA (Fig. 5). Sea urchin larvae also develop dopaminergic, GABAergic (Bisgrove and Burke, 1986; Bisgrove and Burke, 1987) and peptidergic nervous systems (Beer et al., 2001). These nervous systems, however, appear in later larval stages (after 4-arm larva stage), and thus are not present in the early 2-arm larva stage, excluding possible participation of these nervous systems in the present larval swimming behavior and $[Ca^{2+}]_i$ elevation in the ectoderm observed here.

The absence of 5HThpr on the ectodermal cells themselves may explain why 5HThpr-possessed SRN is required and the SRN-deprived larvae did not respond to internally applied serotonin. The perturbation of SRN by *p*CPA was prevented to some extent by simultaneous external application of serotonin, implicating externally applied serotonin also stimulates larvae as has been previously reported (Wada et al., 1997; Yaguchi and Katow, 2003). This stimulation pathway may be carried out through SRN fibers pierced through the ectoderm in places (Fig. 3C–H).

Although 5HThpr had been predicted to have a strong similarity in its partial amino acid sequence to *Aplysia* 5-HT2 (Katow et al., 2004), the present homology search based on the entire ORF sequence showed that 5HThpr is much more similar to *S. purpuratus* 5HT-1A than to *Aplysia* 5-HT2.

The present study thus strongly suggests that serotonin secreted from the apical ganglion is received by 5HThpr on SRN cells, and then transmitted to the ectoderm through SRN fibers inserted into the ectoderm. Unlike intra-ectodermal signal transmission mechanism, intra-SRN signaling may not be mediated by $[Ca^{2+}]_i$, and thus may involve signal transduction pathways such as G-protein/CREB/CRE pathways (Brown et al., 2001). Such conversion of signaling media may occur at the SRN–ectoderm connection sites and have yet to be examined in detail. Furthermore, the mechanism of intercellular propagation of high $[Ca^{2+}]_i$, area in ectoderm ought to be addressed in near future.

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References

Barnes, N. M. and Sharp, T. (1999). A review of central 5-HT receptors and their function. *Neuropharmacology* **38**, 1083-1152.

Beer, A. J., Moss, C. and Thorndyke, M. C. (2001). Development of serotonin-like and SALMFamide-like immunoreactivity in the nervous system of the sea urchin *Psammechinus miliaris*. *Biol. Bull*. **200**, 268-280.

Bisgrove, B. W. and Burke, R. D. (1986). Development of serotonergic neurons in embryos of the sea urchin, *S. purpuratus*. *Dev. Growth Differ*. **28**, 557-569.

Bisgrove, B. W. and Burke, R. D. (1987). Development of the nervous system of the pluteus larva of *Strongylocentrotus droebachiensis*. *Cell Tissue Res*. **248**, 335-343.

Braet, K., Vandamme, W., Martin, P. E., Evans, W. H. and Leybaert, L. (2003). Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26. *Cell Calcium* **33**, 37-48.

Brown, M., Keynes, R. and Lumsden, A. (2001). *The Developing Brain*. New York: Oxford University Press.

Burke, R., Osborne, L., Wang, D., Murabe, N., Yaguchi, S. and Nakajima, Y. (2006). Neuron-specific expression of a synaptotagmin gene in the sea urchin, *Strongylocentrotus purpuratus*. *J. Comp. Neurol.* **496**, 244-251.

Christopher, K. J., Young, K. G., Chang, J. P. and Goldberg, J. I. (1999). Involvement of protein kinase C in 5-HT-stimulated ciliary activity in *Helisoma trivolvis* embryos. *J. Physiol.* **515**, 511-522.

Deutch, A. Y. and Roth, R. H. (1999). Neurotransmitters. In *Fundamental Neuroscience* (ed. M. J. Zigmond, F. E. Bloom, S. C. Landis, J. L. Roberts and L. R. Squire), pp. 193-234. San Diego: Academic Press.

Doran, S. A., Koss, R., Tran, C. H., Christopher, K. J., Gallin, W. J. and Goldberg, J. I. (2004). Effect of serotonin on ciliary beating and intracellular calcium concentration in identified populations of embryonic ciliary cells. *J. Exp. Biol.* **207**, 1415-1429.

Gal, E. M. and Whitacre, D. H. (1982). Mechanism of irreversible inactivation of phenylalanine-4- and tryptophan-5-hydroxylases by [4-36Cl, 2-14C]*p*-chlorophenylalanine: a revision. *Neurochem. Res*. **7**, 13-26.

Gustafson, T. (1991). Pharmacological control of muscular activity in the sea urchin larva-IV. Effects of monoamines and adenosine. *Comp. Biochem. Physiol*. **98C**, 307-315.

Hara, Y., Kuraishi, R., Uemura, I. and Katow, H. (2003). Asymmetric formation and possible function of the primary pore canal in plutei of *Temnopleurus hardwicki*. *Dev. Growth Differ*. **45**, 295-308.

Jahnel, U., Nawrath, H., Rupp, J. and Ochi, R. (1993). L-type calcium channel activity in human atrial myocytes as influenced by 5-HT. *Naunyn Schmiedebergs Arch. Pharmacol*. **348**, 396-402.

Katow, H. and Solursh, M. (1980). Ultrastructure of primary mesenchyme cell ingression in the sea urchin *Lytechinus pictus*. *J. Exp. Zool.* **213**, 231-246.

Katow, H., Yaguchi, S., Kiyomoto, M. and Washio, M. (2004). The 5-HT receptor cell is a new member of secondary mesenchyme cell descendants and forms a major blastocoelar network in sea urchin larvae. *Mech. Dev*. **121**, 325-337.

Kitajima, Y., Owaribe, K., Nishizawa, Y. and Yaoita, H. (1992). Control of the distribution of hemidesmosome components in cultured keratinocytes: Ca^{2+} and phorbol esters. *J. Dermatol*. **19**, 770-773.

Kuang, S., Doran, S. A., Wilson, R. J., Goss, G. G. and Goldberg, J. I. (2002). Serotonergic sensory-motor neurons mediate a behavioral response to hypoxia in pond snail embryos. *J. Neurobiol*. **52**, 73-83.

Kyozuka, K., Deguchi, R., Mohri, T. and Miyazaki, S. (1998). Injection of sperm extract mimics spatiotemporal dynamics of Ca^{2+} responses and progression of meiosis at fertilization of ascidian oocytes. *Development* **125**, 4099-4105.

Larabell, C. A., Rowning, B. A. and Moon, R. T. (2004). A PKC wave follows the calcium wave after activation of Xenopus eggs. *Differentiation* **72**, 41-47.

Nakajima, Y., Noda, Y. and Burke, R. D. (1993). The structure and development of the apical ganglion in the sea urchin pluteus larvae of *Strongylocentrotus droebachiensis* and *Mespilia globules*. *Dev. Growth Differ*. **35**, 531-538.

Nguyen, T., Chin, W. C., O'Brien, J. A., Verdugo, P. and Berger, A. J. (2001). Intracellular pathways regulating ciliary beating of rat brain ependymal cells. *J. Physiol*. **531**, 131-140.

Peroutka, S. J. (1995). 5HT receptors: past, present and future. *Trends Neurosci*. **18**, 68-69.

Renaud, F., Parisi, E., Capasso, A. and De Prisco, P. (1983). On the role of serotonin and 5-methoxy-tryptamine in the regulation of cell division in sea urchin eggs. *Dev. Biol*. **98**, 37-46.

Rozsa, K. S. (1984). The pharmacology of molluscan neurons. *Prog. Neurobiol*. **23**, 79-150.

Saino, T., Matsuura, M. and Satoh, Y. (2002). Application of real-time confocal microscopy to intracellular calcium ion dynamics in rat arterioles. *Histochem. Cell Biol*. **117**, 295-305.

Sakurai, A. and Katz, P. S. (2003). Spike timing-dependent serotonergic neuromodulation of synaptic strength intrinsic to a central pattern generator circuit. *J. Neurosci*. **23**, 10745-10755.

Sea Urchin Genome Sequencing Consortium (2006). The genome of the sea urchin *Strongylocentrotus purpuratus*. *Science* **314**, 941-952.

Shmukler, Y. B. (1993). Possibility of membrane reception of neurotransmitter in sea urchin early embryos. *Comp. Biochem. Physiol.* **106C**, 269-273.

Shmukler, Y. B. and Tosti, E. (2001). Serotoninergic processes in cells of early embryos of the sea urchin *Paracentrotus lividus*. [Russian] *Ross. Fiziol. Zh. Im. I. M. Sechenova* **87**, 1557-1564.

Shmukler, Y. B., Buznikov, G. A. and Whitaker, M. J. (1999). Action of serotonin antagonists on cytoplasmic calcium levels in early embryos of sea urchin *Lytechinus pictus*. *Int. J. Dev. Biol.* **43**, 179-182.

Soliman, S. (1983). Pharmacological control of ciliary activity in the young sea urchin larva. Effects of monoaminergic agents. *Comp. Biochem. Physiol.* **76C**, 181-191.

Stephens, R. E. and Prior, G. (1992). Dynein from serotonin-activated cilia and flagella: extraction characteristics and distinct sites for cAMP-dependent protein phosphorylation. *J. Cell Sci.* **103**, 999-1012.

Tierney, A. J. (2001). Structure and function of invertebrate 5-HT receptors: a review. *Comp. Biochem. Physiol.* **128A**, 791-804.

Ulrich, M. L., Rotzinger, S., Asghar, S. J., Jurasz, P., Tanay, V. A., Dunn, S. M., Radomski, M., Greenshaw, A. and Silverstone, P. H. (2003). Effects of dextroamphetamine, lithium chloride, sodium valproate and carbamazepine on intraplatelet Ca^{2+} levels. *J. Psychiatry Neurosci.* **28**, 115-125.

Wada, Y., Mogami, Y. and Naba, S. (1997). Modification of ciliary beating in sea urchin larvae induced by neurotransmitters: beat-plane rotation and control of frequency fluctuation. *J. Exp. Biol.* **200**, 9-18.

Yaguchi, S. and Katow, H. (2003). Expression of tryptophan 5-hydroxylase gene during sea urchin neurogenesis and role of serotonergic nervous system in larval behavior. *J. Comp. Neurol.* **466**, 219-229.