Identification of a serotonin receptor coupled to adenylyl cyclase involved in learning-related heterosynaptic facilitation in *Aplysia*

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Serotonin (5-HT) plays a critical role in modulating synaptic plasticity in the marine mollusc *Aplysia* and in the mammalian nervous system. In *Aplysia* sensory neurons, 5-HT can activate several signal cascades, including PKA and PKC, presumably via distinct types of G protein-coupled receptors. However, the molecular identities of these receptors have not yet been identified. We here report the cloning and functional characterization of a 5-HT receptor that is positively coupled to adenylyl cyclase in *Aplysia* neurons. The cloned receptor, 5-HT$_{apAC1}$, stimulates the production of cAMP in HEK293T cells and in *Xenopus* oocytes. Moreover, the knockdown of 5-HT$_{apAC1}$ expression by RNA interference blocked 5-HT-induced cAMP production in *Aplysia* sensory neurons and blocked synaptic facilitation in nondepressed or partially depressed sensory-to-motor neuron synapses. These data implicate 5-HT$_{apAC1}$ as a major modulator of learning related synaptic facilitation in the direct sensory to motor neuron pathway of the gill withdrawal reflex.

5-HT receptor | memory | cAMP | protein kinase A

5-Hydroxytryptamine (5-HT), or serotonin, is a key neurotransmitter that modulates a variety of behaviors in both invertebrate and vertebrate animals and is involved in the regulation of mood and mood disorders in humans (1). Serotonin also modulates synaptic plasticity in the marine mollusc *Aplysia* (2, 3). Synaptic facilitation of the connections between sensory and motor neurons of the gill-withdrawal reflex is mediated by 5-HT, and this form of synaptic plasticity has been found to be a critical cellular mechanism of behavioral sensitization (4–6). A number of pharmacological studies have found that, depending on the behavioral history and pattern of sensory stimulation, 5-HT stimulates several downstream signaling pathways, including protein kinase A (PKA), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK), suggesting that serotonin acts on more than one receptor type (2, 3, 7, 8). Of these signaling cascades, the adenylyl cyclase-cAMP-PKA cascade has been most extensively investigated because of its important roles in both behavioral sensitization and synaptic facilitation (3, 4, 9, 10). Historically, this was the initially identified second-messenger system involved in the regulation of synaptic plasticity, behavior, and memory storage (4).

A single pulse of 5-HT activates PKA, which phosphorylates and inactivates potassium channels (11) and subsequently increases synaptic strength at nondepressed synapses. At depressed synapses, however, PKC becomes the major downstream kinase to be activated by a single pulse of 5-HT (8). In addition, repetitive exposures to 5-HT that induce long-term facilitation result in the activation of additional kinases, including MAPK (12), that translocate to the nucleus to induce gene expression. However, the molecular mechanism for this dynamic coupling specificity of downstream signaling pathways is not known.

In vertebrates, seven families of 5-HT receptors have been characterized; six of these include G protein-coupled receptors, and only the 5-HT$_3$ family is composed of ionotropic receptors (13). The G protein-coupled 5-HT receptors are classified on the basis of the second messenger systems to which they are coupled (14). The 5-HT$_1$ and 5-HT$_5$ receptors inhibit adenylyl cyclase, whereas 5-HT$_4$, 5-HT$_6$, and 5-HT$_7$ activate adenylyl cyclase, and the 5-HT$_2$ receptor stimulates phospholipase C (PLC). Molecular evolutionary analyses indicate that primordial 5-HT receptors differentiated into 5-HT$_1$, 5-HT$_2$, and 5-HT$_6$ approximately 800 million years ago (mya) (15). Since vertebrates differentiated from invertebrates 600 mya, one might predict that invertebrates may have 5-HT receptor families that are homologous to at least three subtypes of vertebrate 5-HT receptors: 5-HT$_1$, 5-HT$_2$ and 5-HT$_6$. However, considering that a hundred million years have passed since they have diverged, it is by no means certain that the invertebrate receptors have similar pharmacological characteristics to the vertebrate receptors within each family (16).

Aplysia 5-HT receptors have been characterized mainly by pharmacological means. For example, Abrams et al. characterized the 5-HT receptor(s) that are positively coupled to adenylyl cyclase (5-HT$_{apAC}$) pharmacologically (17, 18). So far, four full-length 5-HT receptors have been cloned in *Aplysia* (19, 20), two of which—5-HT$_{ap1}$ and 5-HT$_{ap2}$—inhibit adenylyl cyclase activity (19, 20). However, no receptor positively coupled to adenylyl cyclase and involved in synaptic facilitation has yet been cloned, leaving questions about the receptors that initiate synaptic facilitation unanswered. Here, we report the molecular cloning and functional characterization of a 5-HT receptor that is positively coupled to adenylyl cyclase in the *Aplysia* nervous system.

Results

The Molecular Cloning of 5-HT$_{apAC1}$ from the *Aplysia* Nervous System. To isolate genes encoding 5-HT receptors that are positively coupled to adenylyl cyclase in *Aplysia*, we designed two degenerative PCR primers based on the peptide sequences of the fifth and seventh transmembrane domains of invertebrate 5-HT$_7$ receptors (Fig. 1*A*). In addition to the highly conserved amino acids sequences, NPXXY, in the seventh transmembrane domains of G protein-coupled receptors, we found that another motif (QIYATL) is strikingly conserved only in invertebrate 5-HT$_7$ receptors. We obtained a 519-bp PCR product showing ≈80% sequence homology with the 5-HT$_7$ receptor of *Helisoma trivolvis*. Using this

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The sequence reported in this paper has been deposited in the GenBank database (accession no. FJ477896).

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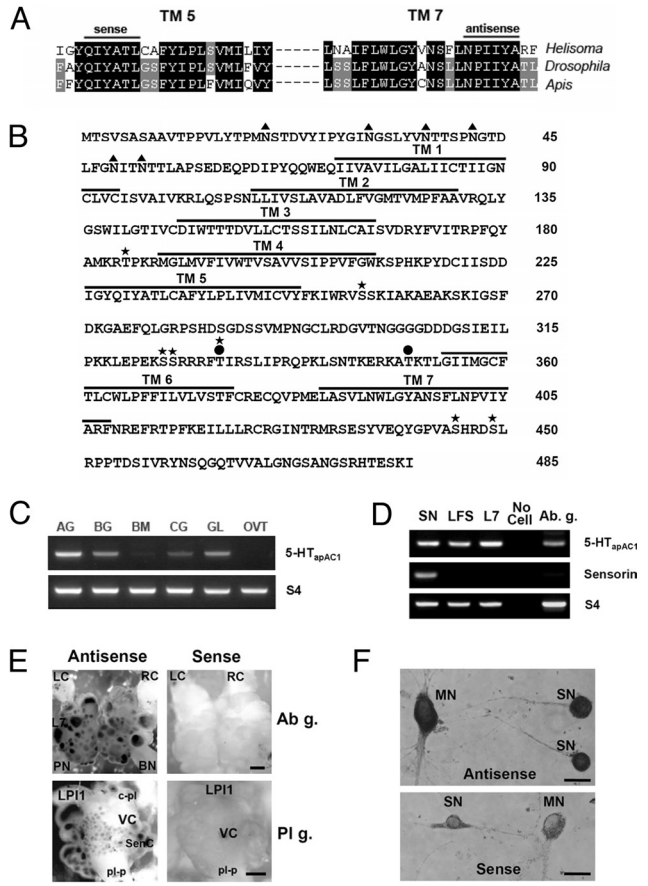

Fig. 1. Molecular cloning and expression pattern of 5-HT_{apAC1}. (*A*) Multiple sequence alignments of TM5 and TM7 of invertebrate 5-HT_7 receptors. (*B*) Deduced amino acid sequence of 5-HT_{apAC1}. Seven transmembrane (TM) domains are indicated and numbered. Predicted phosphorylation sites for PKA (●) and PKC (*) are marked. Triangles (▲) indicate *N*-glycosylation sites. (*C*) Multiple tissue RT-PCR analysis. AG, abdominal ganglia; BG, buccal ganglia; BM, buccal mass; CG, central ganglia (cerebral, pleural and pedal); GL, gill; OVT, ovotestis. (*D*) Single cell RT-PCR analysis of 5-HT_{apAC1}. 5-HT_{apAC1} was expressed in sensory neurons and LFS, L7 motor neurons. The sensory neuron specific gene sensorin was expressed only in sensory cells. The housekeeping gene S4 was used as a control. Total RNA isolated from the whole abdominal ganglion was used as another control. SN, sensory neuron; LSF, LSF motor neuron; L7, L7 motor neuron; Ab. g., total RNA from the abdominal ganglion. (*E*) In situ hybridization of 5-HT_{apAC1} mRNA in sensory clusters. Dorsal abdominal ganglia and left pleural ganglia were shown. 5-HT_{apAC1} was expressed in sensory cells in the sensory cluster in pleural ganglia, as well as in L7 motor neurons. LC, left pleuroabdominal connective; RC, right pleuroabdominal connective; PN, pericardial nerve; BN, branchial nerve; L7, L7 motor neuron; LPI1, left pleural giant neuron; SenC, sensory cluster; c-pl, cerebro-pleural connective; pl-p, pleuropedal connective. (Scale bars, 500 μm for *Upper*; 200 μm *Lower*.) (*F*) In situ hybridization of 5-HT_{apAC1} mRNA in sensory-to-motor coculture. (*Right*) Shown are the hybridization with sense nucleotide probe. (Scale bar, 50 μm.)

fragment as a probe, we screened the *Aplysia kurodai* cDNA library and isolated a full-length cDNA clone, 5-HT_{apAC1}. The putative 1,458 bp ORF encodes a protein of 485 amino acid with a predicted molecular weight of 54 kDa (Fig. 1*B*). Phylogenetic analysis with other invertebrate 5-HT receptors indicates that 5-HT_{apAC1} clusters with 5-HT receptor type 7 from the pond snail, honey bee, and fruit fly (Fig. S1*A*). Our 5-HT_{apAC1} clone also shows a great homology to a partial EST clone, PEG003-C-228120–501 (GenBank accession, EB245546) from *A. californica*, suggesting that this clone is highly conserved between two closely related species of *Aplysia* (21). Comparisons between 5-HT_{apAC1} and mammalian receptors also reveal that the cloned 5-HT_{apAC1} belongs within the 5-HT_7 family of receptors, which is positively coupled to adenylyl cyclase (Fig. S1*B*).

The deduced amino acid sequences of 5-HT_{apAC1} show the hallmarks of known G protein-coupled receptors, including seven transmembrane domains, the tripeptide DRY (Asp-169–Arg-170–Tyr-171) required for G protein coupling (22), and the NPXXY motif (Asn-401–Pro-402–Tyr-405) for receptor desensitization and internalization (23) (Fig. 1*B*). Four potential sites for PKC phosphorylation and two sites for PKA are found within the third intracellular loop. Three additional PKC sites are also found in the second intracellular loop and C-terminal intracellular tail. In addition, six consensus asparagine residues for *N*-glycosylation are found in the extracellular N terminus. 5-HT_{apAC1} also contains, at its C terminus, a putative PDZ-binding motif (Ser-483–Lys-484–Ile-485), suggesting that this receptor may be clustered by scaffolding proteins to form a signaling complex.

We next examined the tissue distribution of the cloned receptor by RT-PCR analysis. For this purpose we used total RNA isolated from the abdominal ganglia, the buccal ganglia, the buccal mass, the central ganglia containing the pleural-pedal ganglia, and the gill and ovotestis of *Aplysia* (Fig. 1*C*). A specific fragment was detected in the CNS tissue, with the strongest signal coming from the abdominal ganglia. The mRNA was also detected in muscle and gill tissue, which is consistent with an earlier study that used an [^{3}H]LSD binding assay (24). To further validate the expression of 5-HT_{apAC1} in specific neurons involved in synaptic facilitation and behavioral sensitization, we performed single cell RT-PCR analysis (Fig. 1*D*). Our results clearly showed that 5-HT_{apAC1} is expressed both in the sensory neurons and in the L7 and LFS motor neurons.

The expression pattern of 5-HT_{apAC1} was further examined by in situ hybridization in the abdominal and pleural ganglia of *A. kurodai*. In situ staining revealed that the cloned receptor is widely expressed in the nervous system, including sensory neurons in the sensory clusters of both abdominal and pleural ganglia, as well as L7 motor neurons in abdominal ganglia (Fig. 1*E*). Expression of 5-HT_{apAC1} in pleural sensory neurons and in LFS motor neurons was also confirmed by in situ hybridization in sensory-to-motor coculture (Fig. 1*F*).

Subcellular Localization of 5-HT_{apAC1}. To investigate the subcellular localization of 5-HT_{apAC1}, we transiently expressed 5-HT_{apAC1} fused with a FLAG tag at its N terminus in HEK293T cells. We confirmed the expression by western blot and immunocytochemistry with an anti-FLAG antibody (Fig. 2 *A* and *B*). Immunocytochemistry analysis revealed the membrane expression of 5-HT_{apAC1} in HEK293T cells (Fig. 2*B*). Overexpressed 5-HT_{apAC1} was detected at the plasma membrane in *Aplysia* sensory neurons (Fig. 2*C*). Importantly, 5-HT_{apAC1} was co-localized with synaptophysin-EGFP at neurites and varicosities in sensory neurons, indicating that 5-HT_{apAC1} is expressed at synapses (Fig. 2*C*).

Stimulation of Adenylyl Cyclase by Heterologously Expressed 5-HT_{apAC1}. To determine whether the heterologously expressed 5-HT_{apAC1} can stimulate cAMP production in response to 5-HT in HEK293T cells, we treated cells with either vehicle or varying concentrations of 5-HT and then measured the amount of cellular cAMP. The addition of 5-HT stimulated the production of cAMP in a dose-dependent manner with an EC_{50} of 6.0 nM ($\log EC_{50} = -8.22 \pm 0.21$), which is comparable to that of other invertebrate 5-HT type 7 receptors, consistent with 5-HT_{apAC1} stimulating cAMP signaling (25, 26) (Fig. 3*A*). This stimulation by 5-HT was specific. Neither dopamine nor octopamine stimulated cAMP accumulation. To further investigate whether 5-HT_{apAC1} can also activate other second messenger cascades such as intracellular Ca^{2+} signaling, we performed Ca^{2+} imaging. As a positive control, we used mouse 5-HT_{2c}, the G_q-coupled 5-HT receptor that stimulates intracellular Ca^{2+} elevation. Exposure to 5-HT (1 μM) triggered a Ca^{2+} signal only in 5-HT_{2c}-expressing cells, not in 5-HT_{apAC1}-expressing cells (F/F_0: 5-HT_{2c}, 1.257 ± 0.026, $n = 6$; 5-HT_{apAC1}, 1.003 ± 0.019, $n = 6$) (Fig. 3*B*).

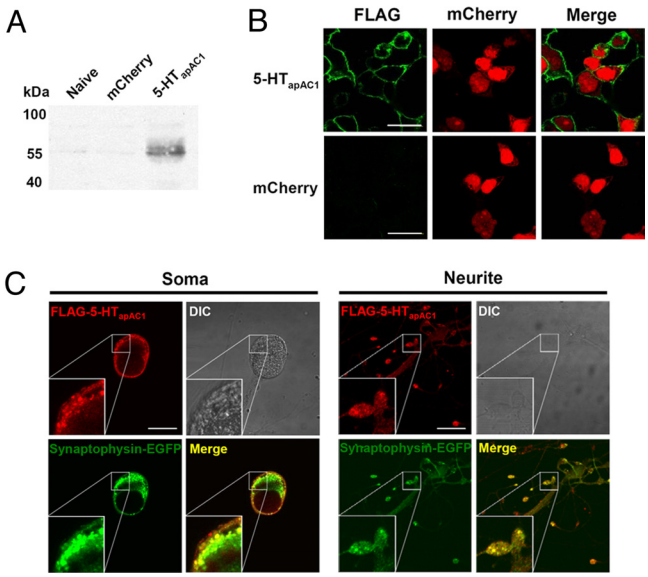

Fig. 2. Subcellular localization of 5-HT_{apAC1}. (*A* and *B*) Expression of FLAG-5-HT_{apAC1} in HEK293T cell. Expression was confirmed by western blot (*A*) and immunocytochemistry (*B*). In immunocytochemistry, FLAG-5-HT_{apAC1} (green) localized in the cytoplasmic membrane. mCherry-N1 (red) was co-transfected as a expression marker and diffusely distributed in the cytosol. (*C*) Co-localization of overexpressed FLAG-5-HT_{apAC1} (red) and synaptophysin-EGFP (green) in *Aplysia* sensory cells co-cultured with LFS motor neurons. Insets show three fold magnification images. FLAG-5-HT_{apAC1} and synaptophysin-EGFP are highly co-localized at synaptic varicosities, and partially co-localized at neurites and the plasma membrane, but not co-localized at the cytosol. (Scale bars, 30 μm.)

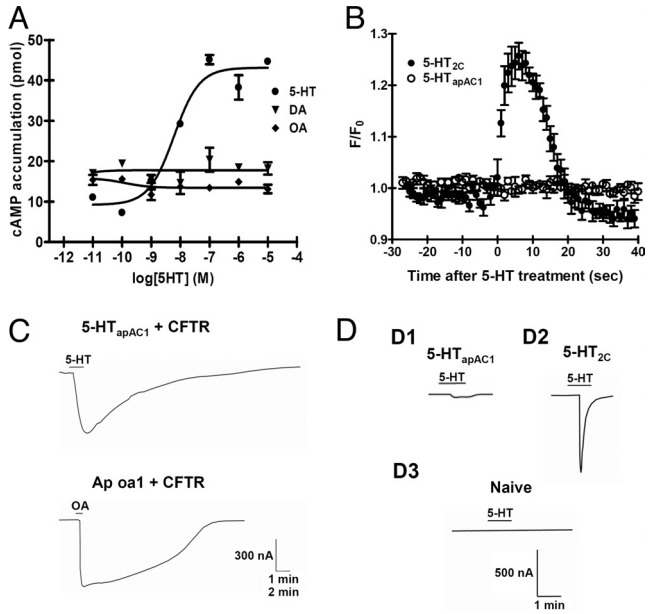

Fig. 3. Coupling specificity of 5-HT_{apAC1}. (*A*) Dose-response curve of 5-HT on cAMP production in HEK293T cells transiently expressing 5-HT_{apAC1}. Various concentrations of 5-HT were applied in the presence of 500 μM 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of the cAMP dependent phosphodiesterase. Neither octopamine (OA) nor dopamine (DA) was able to stimulate cAMP production. (*B*) Ca^{2+} imaging in HEK293T cell overexpressing mouse 5-HT_{2C} or 5-HT_{apAC1}. After loading the calcium dye, Calcium Green-1, the cells were exposed to 1 μM 5-HT and imaged under the confocal microscope. Increase of cellular Ca^{2+} was detected only in cells expressing 5-HT_{2C}, which is known to be coupled to the G_q protein. (*C*) Functional expression and Gs coupling in *Xenopus* oocytes. CFTR cRNA (2.5 ng) was co-injected with either 5-HT_{apAC1} (2.5 ng) or Ap oa_1 cRNA (2.5 ng). CFTR current is known to be activated by cAMP/PKA. Ap oa_1, which is specifically coupled to G_s, was used as a positive control. 5-HT (1 μM) and OA (1 μM) were used to stimulate 5-HT_{apAC1} or Ap oa_1. Scale bars, 300 nA and 1 min for *Upper*, and 300 nA and 2 min for *Lower*. CFTR current was observed in both 5-HT_{apAC1}- and Ap oa_1-injected oocytes. (*D*) 5-HT_{apAC1} does not activate G_q in oocytes. Oocytes were injected with the following cRNAs: 5-HT_{apAC1} (2.5 ng) (*D1*), 5-HT_{2c} cRNA (100 pg) (*D2*), and distilled water (*D3*). (Scale bars, 500 nA and 1 min.) A transient Cl^- current activated by G_q-mediated signaling was observed in 5-HT_{2c}-injected oocytes, not in 5-HT_{apAC1}-injected oocytes.

To examine electrophysiologically the functional expression and G protein coupling specificity of 5-HT_{apAC1}, we expressed 5-HT_{apAC1} in *Xenopus* oocytes with or without the cystic fibrosis transmembrane conductance regulator (CFTR). The CFTR, a Cl^- channel, is activated by PKA and has been used to determine whether a receptor is coupled to the G_s protein (27, 28). Treatment of 1 μM 5-HT induced significant CFTR current (320.6 ± 123.8 nA, $n = 8$) in oocytes injected with 5-HT_{apAC1} and CFTR cRNA (Fig. 3*C*). As a positive control, we coinjected the CFTR with an *Aplysia* octopamine receptor Ap oa_1, which activates adenylyl cyclase (28). The activation of Ap oa_1 with 1 μM octopamine generated a similar CFTR current (565.0 ± 104.1 nA, $n = 8$) (Fig. 3*C*). *Xenopus* oocytes have an endogenous Cl^- channel that is quickly activated by Ca^{2+} after activation of G_q (29). The typical agonist-activated transient inward current was observed in the 5-HT_{2c}-injected oocytes in response to 5-HT treatment (835.0 ± 35.4 nA, $n = 2$). However, 5-HT treatment did not induce such a large and transient Cl^- current in 5-HT_{apAC1} cRNA-injected oocytes (Fig. 3*D*). Taken together, these heterologous expression analyses strongly suggest that the cloned 5-HT_{apAC1} is positively linked specifically to G_s, and not to G_q.

Pharmacological Properties of 5-HT_{apAC1}. To characterize the pharmacological properties of 5-HT_{apAC1}, we examined the effects of known 5-HT receptor antagonists in HEK293T cells in which we had transiently expressed 5-HT_{apAC1} in the presence of 10 nM 5-HT. Of the antagonists tested, the nonselective 5-HT receptor antagonist methiothepin was most effective. Clozapine also blocked 5-HT-induced cAMP production, but with less potency than methiothepin (Fig. 4). Cyproheptadine, spiperone and metergoline, however, were much less effective than methiothepin and clozapine, or not effective at all. These data are consistent with a previous report by Abrams and his colleagues (17) using *Aplysia* CNS membranes, except that in the previous study cyproheptadine and metergoline also blocked 5-HT-induced cAMP production. We also found that SB269970, a specific 5-HT_7 antagonist in mammals (30), was not effective for 5-HT_{apAC1}. SB269970 was also found to be ineffective for Am5-HT_7, a recently cloned invertebrate 5-HTR,

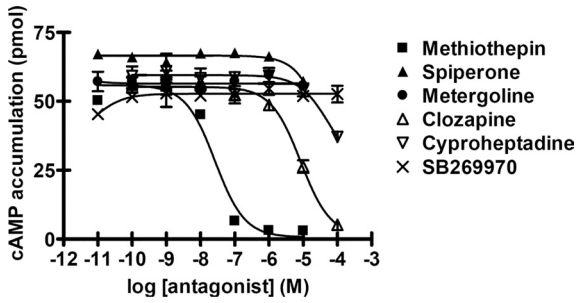

Fig. 4. Pharmacological characterization of 5-HT_{apAC1}. Effects of antagonists on 5-HT stimulated cAMP production were examined in HEK293T cells transiently transfected with 5-HT_{apAC1}. Various concentrations of antagonists were treated in the presence of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) 20 min before 5-HT (10 nM) treatment. Methiothepin and clozapine blocked 5-HT-induced cAMP production with pK_i values of 7.53 and 5.5, respectively. pK_i values were determined by the equation, $K_i = IC_{50}/(1+C/K_d)$, where C is the 5-HT concentration (10 nM) and K_d is the EC_{50} value for 5-HT (6.0 nM). The presented data are representative of at least two independent experiments with each point measured in duplicate (mean ± SEM).

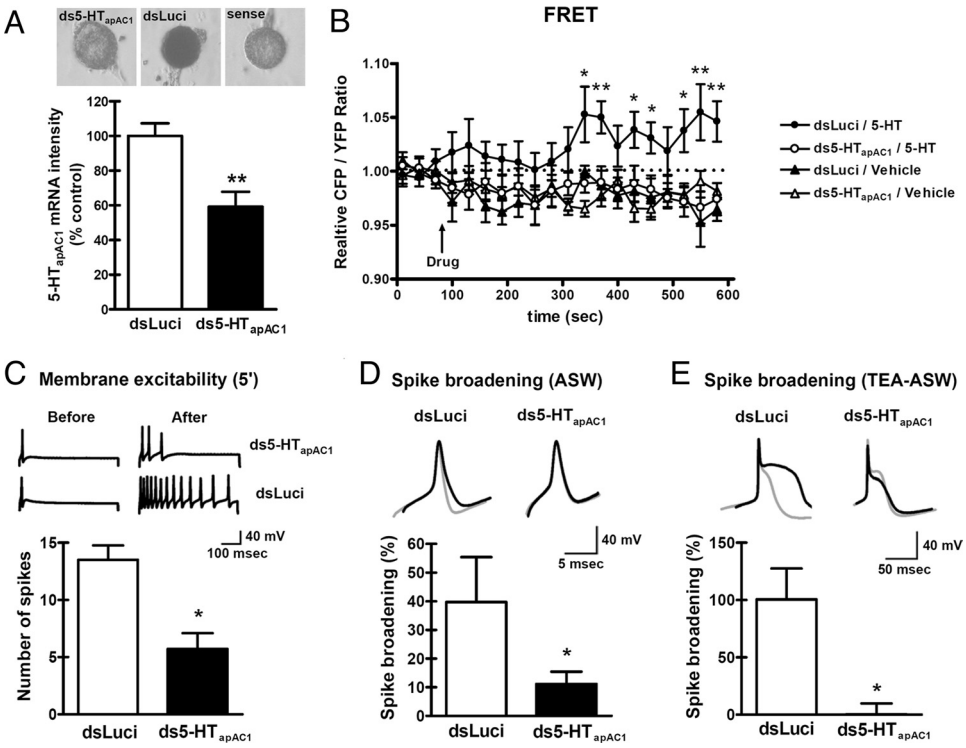

Fig. 5. Blockage of 5-HT$_{apAC1}$ expression impaired the increases in intracellular cAMP, membrane excitability and spike duration by 5-HT. (*A*) Effectiveness of 5-HT$_{apAC1}$ dsRNA was measured by in situ hybridization. Sensory neurons were injected with dsRNA for 5-HT $_{apAC1}$ or with dsLuci as a control. (*B*) ds5-HT$_{apAC1}$ blocked the increase in the intracellular cAMP level stimulated by 5-HT treatment (10 μM). Relative CFP/YFP ratios are measured as an index of the cellular cAMP level. One-way ANOVA followed by a Newman-Keuls post hoc test was performed for each time point. Asterisks indicate significant differences in the post hoc tests between dsLuci/5-HT and ds5-HT$_{apAC1}$/5-HT. *, $P < 0.05$; **, $P < 0.01$. (*C*) ds5-HT$_{apAC1}$ blocked the increase in membrane excitability normally induced by 5-HT treatment (10 μM for 5 min). Representative traces of action potentials during a 500 ms depolarizing pulse are indicated. Unpaired, two-tailed *t* test; *, $P < 0.05$. (*D* and *E*) Spike broadening induced by 5-HT treatment (10 μM for 7 min) was blocked only by ds5-HT$_{apAC1}$ microinjection, not by dsLuci microinjection under normal or TEA/nifedipine-ASW conditions. Representative traces of single action potentials before (gray line), and after 10 μM 5-HT treatment (black line) are indicated. Summary bar graphs are represented as mean ± SEM. Unpaired, two-tailed *t* test; *, $P < 0.05$.

whose sequence is more closely related to 5-HT$_{apAC1}$ than mammalian 5-HT$_7$ (26).

5-HT$_{apAC1}$ Modulates Membrane Excitability and Spike Duration. To investigate the role of endogenous 5-HT$_{apAC1}$ in sensory neurons, we blocked 5-HT$_{apAC1}$ gene expression by injecting double stranded (ds) RNA (ds5-HT$_{apAC1}$) (31). The effectiveness of dsRNA was confirmed by performing in situ hybridization (Fig. 5*A*). As a control, dsLuci was injected into the sensory neuron using EGFP as an expression marker. An antisense RNA against partial 5-HT$_{apAC1}$ was used as a probe (see *Materials and Methods* for the details). In situ hybridization showed that ds5-HT$_{apAC1}$ significantly reduced the expression of 5-HT$_{apAC1}$ in sensory neurons by almost 50% (relative percent of 5-HT$_{apAC1}$ mRNA intensity: dsLuci, 100.0 ± 7.2, $n = 10$; ds5-HT$_{apAC1}$, 59.2 ± 8.7, $n = 18$; $P < 0.01$, Student's *t* test). To determine whether 5-HT$_{apAC1}$ is critically involved in cAMP production in response to 5-HT in *Aplysia* sensory neurons, we monitored changes in the cAMP level using the Epac-based fluorescence resonance energy transfer (FRET) sensor, CFP-Epac(δDEP-CD)-YFP (Fig. 5*B*). Epac is a well-known guanine nucleotide exchange factor (GEF) that is activated by direct binding of cAMP (32). Binding of cAMP to Epac induces conformational changes in Epac, resulting in changes in the CFP/YFP ratio, which serves as a measure of the cAMP level (33). 5-HT (10 μM) increased cAMP level in dsLuci-injected sensory cells. However, there was no significant change in the CFP/YFP ratio in ds5-HT$_{apAC1}$-injected sensory neurons, suggesting that 5-HT$_{apAC1}$ plays a critical role in 5-HT-induced cAMP production in *Aplysia* sensory neurons (Fig. 5*B*).

The activation of adenylyl cyclase by 5-HT is known to modulate K^+ currents to produce an increase in membrane excitability and in spike duration in sensory neurons (8, 11, 28, 34). ds5-HT$_{apAC1}$ significantly suppressed the increase in membrane excitability produced by a 5-min exposure to 10 μM 5-HT (number of spikes: dsLuci, 13.5 ± 1.3, $n = 4$ vs. ds5-HT$_{apAC1}$, 5.7 ± 1.4, $n = 14$; $P < 0.05$, Student's *t* test) (Fig. 5*C*). Similarly, spike broadening was also reduced in ds5-HT$_{apAC1}$-injected sensory neurons as compared with dsLuci-injected control neurons (% increase of spike duration in normal ASW: dsLuci, 39.7 ± 15.7, $n = 5$ vs. ds5-HT$_{apAC1}$, 11.1 ± 4.3, $n = 14$; $P < 0.05$, Student's *t* test) (Fig. 5*D*). To isolate spike broadening, which is specifically mediated by the inhibition of the cAMP dependent-S-type K^+ current, we recorded spike broadening in the presence of 100 mM TEA and 20 μM nifedipine. In the presence of these two drugs, all K^+ currents other than the 5-HT-modulated S-type K^+ currents are blocked (11, 35). Blockage of 5-HT$_{apAC1}$ under these conditions also dramatically impaired the spike broadening (in TEA/nifedipine-ASW: % increase of spike duration: dsLuci, 100.5 ± 27.0, $n = 6$ vs. ds5-HT$_{apAC1}$, 0.2 ± 9.5, $n = 5$; $P < 0.05$, Student's *t* test) (Fig. 5*E*). These results indicate that 5-HT$_{apAC1}$ is required for short-term activation of the cAMP-PKA pathway and for its actions on spike duration and excitability.

Critical Role of 5-HT$_{apAC1}$ in Synaptic Facilitation. Synaptic facilitation produced by 5-HT is state-dependent. PKA plays a major role in synaptic facilitation of a nondepressed synapse that is recruited by behavioral sensitization, whereas PKC activity becomes dominant in facilitation of a depressed synapse involved in dishabituation (8, 28, 36–38). To study the role of 5-HT$_{apAC1}$ in synaptic facilitation in a nondepressed synapse, we first injected ds5-HT$_{apAC1}$ into the presynaptic sensory neuron of a sensory-to-motor neuron synapse. Injection of ds5-HT$_{apAC1}$ did not affect basal synaptic transmission (% change of EPSP: dsLuci, 105.6 ± 53.0, $n = 3$ vs. ds5-HT$_{apAC1}$, 109.8 ± 31.1, $n = 3$; $P = 0.9477$, Student's *t* test). In nondepressed synapses, we examined the effect of knock-down of 5-HT$_{apAC1}$ by dsRNA microinjection in two ways. First, we applied 5-HT (10 μM) for 1 min and then washed out (36, 39). In this condition, knock-down of 5-HT$_{apAC1}$ completely blocked short-term facilitation (% change of EPSP: dsLuci, 85.0 ± 23.9, $n = 7$; vs. ds5-HT$_{apAC1}$, 7.7 ± 16.8, $n = 9$; $P < 0.05$, Student's *t* test) (Fig. 6*A*). Second, we examined a longer application—5 min—of 5-HT exposure. Knock-down of 5-HT$_{apAC1}$ again significantly impaired short-term synaptic facilitation although with their longer exposure it did not fully block synaptic facilitaion (% change of EPSP: dsLuci, 97.1 ± 17.5, $n = 10$ vs. ds5-HT$_{apAC1}$, 31.8 ± 19.7, $n = 12$; $P < 0.05$, Student's *t* test) (Fig. 6*B*).

Next, we stimulated the sensory neuron of the sensory-to-motor

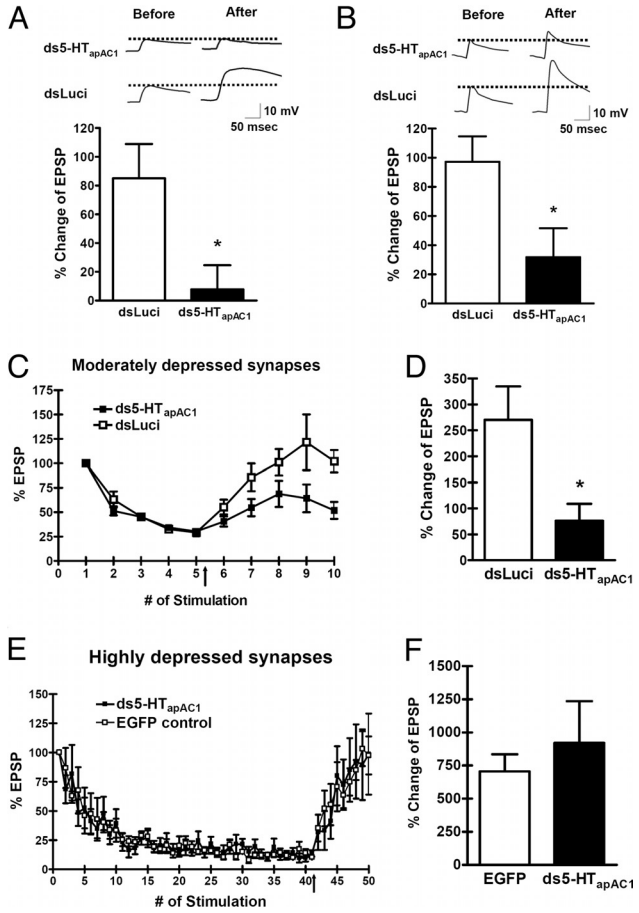

Fig. 6. Blockage of 5-HT$_{apAC1}$ expression impaired short-term facilitation in nondepressed and moderately depressed synapses. (*A* and *B*) Short-term facilitation induced by 5-HT treatment (10 μM, 1 min) followed by 4 min of wash out (*A*) or 5-HT treatment (10 μM, 5 min) (*B*) was significantly reduced both cases in ds5-HT$_{apAC1}$-injected synapses compared to control synapses. EPSP amplitudes were measured both before and 5 min after 5-HT treatment, and the percent of amplitude change was calculated. (*C* and *D*) 5-HT treatment (10 μM, 5 min) reversed a moderate degree of synaptic depression only in control synapses, indicating that cAMP is required for this degree of depression. % Change of EPSP = (mean EPSP at the 9th and 10th stimuli) − (mean EPSP at the 4th and 5th stimuli)/(mean EPSP at the 4th and 5th stimuli) × 100 (%). (*E* and *F*) By contrast, 5-HT treatment reversed a high degree of synaptic depression comparably in both dsRNA-injected and control synapses. Percent change of EPSP = (mean EPSP between the 36th and 40th stimuli) − (mean EPSP between the 46th and 50th stimuli)/(mean EPSP between the 46th and 50th stimuli) × 100 (%). Unpaired, two-tailed *t* test; *, $P < 0.05$.

neuron synapse repeatedly to achieve either a moderate (Fig. 6*C* and *D*) or high degree of synaptic depression (Fig. 6*E* and *F*). In moderately depressed synapses, reversal of synaptic depression by exposure to one pulse of 5-HT was significantly lower in ds5-HT$_{apAC1}$-injected cells than that of dsLuci-injected cells (% change of EPSP: dsLuci, 270.3 ± 64.2, $n = 7$ vs. ds5-HT$_{apAC1}$, 76.2 ± 32.7, $n = 7$; $P < 0.05$, Student's *t* test) (Fig. 6*D*). In highly depressed synapses, one pulse of 5-HT reversed the synaptic depression of ds5-HT$_{apAC1}$-injected synapses as well as EGFP-injected synapses (% change of EPSP: EGFP, 705.4 ± 129.9, $n = 5$ vs. ds5-HT$_{apAC1}$, 920.5 ± 315.8, $n = 4$; $P > 0.05$, Student's *t* test) (Fig. 6*F*). These results demonstrate that 5-HT$_{apAC1}$ is critically involved in short-term facilitation of nondepressed synapses as well as the reversal of moderately depressed synapses, both of which are mediated by the cAMP–PKA pathway. By contrast, 5-HT$_{apAC1}$ may not be involved in the reversal of highly depressed synapses where the reversal depends on PKC.

Discussion

In the present study, we report the cloning and functional characterization of a 5-HT receptor that is positively coupled to adenylyl cyclase in the *Aplysia* nervous system. 5-HT plays a central role in synaptic plasticity in *Aplysia*. Our molecular, pharmacological, and electrophysiological data are consistent with earlier pharmacological studies and reveal that cloned 5-HT$_{apAC1}$ is critically involved in synaptic facilitation via stimulating the production of cAMP (3).

We used degenerative PCR as a cloning strategy. Extensive sequence comparisons with previously cloned invertebrate 5-HT$_7$ receptors enabled us to find a unique motif, QIYATL, in the fifth transmembrane domain (Fig. 1). To our knowledge, this motif has no known function and is not found in other closely related biogenic amine receptors, such as dopamine receptors, or even in other 5-HT receptor subtypes in *Aplysia*. We named the cloned receptor 5-HT$_{apAC1}$, considering that it is a cloned *Aplysia* 5-HT receptor positively coupled to adenylyl cyclase; however, we do not exclude the possibility that other similar receptor subtypes exist in the *Aplysia* nervous system. For example, we found that several 5-HT receptor antagonists that are known to block both cAMP production and synaptic facilitation in other studies are ineffective against 5-HT$_{apAC1}$ expressed in HEK293 cells (7, 17). While this discrepancy may arise from the different experimental conditions, it may imply that more than one subtype of 5-HT receptors can stimulate adenylyl cyclase in the *Aplysia* nervous system. It is also possible, however, that in the previous biochemical and electrophysiological analyses treatments with the antagonists might have influenced crosstalk between different types of 5-HT receptors.

5-HT$_{apAC1}$ also differs pharmacologically from the mammalian 5-HT$_7$ family (Fig. 4). A mammalian 5-HT$_7$ antagonist—SB269970—was ineffective at inhibiting the AC activity of 5-HT$_{apAC1}$. This antagonist is known to also be ineffective in Am5-TH$_7$, which was recently cloned in the invertebrate *Apis mellifera* (26). Furthermore, of several 5-HT receptor antagonists that we tested, only two, methiothepin and clozapine, proved to be effective. The ineffectiveness of mammalian receptor antagonists might be due to the evolutionary divergence of 5-HT receptors between vertebrates and invertebrates over 600 million years. Completion of the ongoing *Aplysia* genome project or extensive analysis of expressed sequence tags will clarify this issue (21).

The endogenous function of 5-HT$_{apAC1}$ was investigated using RNA interference. Our electrophysiological analyses with this technique revealed the critical role of this receptor in learning related heterosynaptic facilitation (Figs. 5 and 6). Due to the lack of an antibody against ds5-HT$_{apAC1}$, we assessed the efficiency of dsRNA by performing in situ hybridization. Interestingly, we found that an ≈40% reduction in mRNA levels was enough to significantly block the cellular and synaptic response to 5-HT treatment. The efficacy of dsRNA on protein expression remains to be examined. If the protein expression is also partially reduced, it may suggest that there is a certain threshold of expression which is necessary for the receptor to be fully functional. We also cannot exclude the possibility of the off-target effects of dsRNA, such as inhibiting other unknown Gs-coupled 5-HT receptors in sensory neurons. So far, however, the only match (PEG003-C-228120–501) for the ds5-HT$_{apAC1}$ sequence in the *Aplysia* EST database (http://www.seahare.org) is thought to be an *A. californica* homolog of 5-HT$_{apAC1}$, suggesting that ds5-HT$_{apAC1}$ specifically knocks down 5-HT$_{apAC1}$.

We also found that 5-HT$_{apAC1}$ dsRNA impaired the 5-HT-induced increases in membrane excitability, spike duration, and synaptic facilitation in nondepressed and moderately depressed synapses. Consistent with earlier pharmacological analysis, we also found that the knock-down of 5-HT$_{apAC1}$ does not reverse the synaptic depression in highly depressed synapses where PKC is critically involved in this reversal (8). Along with calcium imaging data, these results indicate that PKA and PKC are activated by

independent G protein-coupled receptors in *Aplysia* sensory neurons in a state-dependent manner.

Cloning of 5-HT_{apAC1} allows us to revisit several still unsolved questions about the mechanism of synaptic facilitation in *Aplysia*: What happens to the adenylyl cyclase coupled receptor in a highly depressed synapse? Is the receptor desensitized or internalized as the synapse becomes depressed? If so, what are the mechanisms for this functional down-regulation? What are the receptor's binding proteins? Is the same receptor involved in long-term synaptic facilitation? What is the role of the receptor in the postsynaptic neurons? Further molecular, biochemical, and electrophysiological studies on 5-HT_{apAC1} should answer these questions.

Materials and Methods

Molecular Cloning of 5-HT_{apAC1} cDNA. To clone 5-HT_{apAC1} cDNA from *Aplysia kurodai*, we performed degenerative PCR. Two degenerative PCR primers were designed based on the peptide sequences of the highly conserved amino acid sequence, NPXXY in the fifth transmembrane domains, and the QIYATL motif in the seventh transmembrane domains of invertebrate 5-HT_7 receptors (sense, 5′-CARATITAYGCMACICTA-3′; antisense, 5′-TGYRTADATIAYIGGRTT-3′). Amplification was carried out for 3 min at 94°C (one cycle), followed by 35 cycles of 15 s at 94°C, 15 s at 60°C, 1.5 min at 68°C, and a final extension of 5 min at 68°C. The PCR yielded a DNA fragment of 519 bp, showing approximately 80% sequence homology with the *Helisoma trivolvis* 5-HT_7 receptor. Using this fragment as a probe, we screened approximately 2.0×10^5 clones from the *Aplysia kurodai* cDNA library (40) to obtain the entire ORF, and one positive signal was found. This clone was analyzed by DNA sequencing to reveal that it, in fact, contained the entire ORF (5-HT_{apAC1}). The sequence of 5-HT_{apAC1} was subcloned into *Hin*dIII/*Kpn*I-digested pNEXδ (41) to create pNEXδ-5-HT_{apAC1}.

Single Cell RT-PCR. Single cell RT-PCR protocol was modified from the previously described methods (42, 43). Please see *SI Text* for the details.

Intracellular cAMP Level Measurement Using FRET. The construct for monitoring the intracellular cAMP level was a gift from Changjoon Justin Lee (Korea Institute of Science and Technology, Korea), and the analysis was performed as described previously (44). Please see *SI Text* for the details.

***Aplysia* Cell Culture and Electrophysiology.** Sensory cell culture and sensory-to-motor coculture were performed as described previously (45, 46). Please see *SI Text* for the details.

Statistical Analysis. The results are expressed as mean ± SEM. The unpaired Student's *t* test was used for comparison between two groups. One-way ANOVA followed by Newman-Keuls post-hoc test was used for comparison between three- or more groups.

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