Supporting Information

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SI Text

RT-PCR Analysis. Total RNAs were extracted from tissues using TRIzol Reagent (Invitrogen). The cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen) with oligo dTs or random hexamers as a primer. These cDNAs were used as templates for PCRs for 5-HT_{apAC1} (sense, 5′-TGGGATACGC-CAATTCTTTC-3′; antisense, 5′-GCAAGTGAAGT -AAA-GAAGAGATCCA-3′), and S4 (sense, 5′-GACCCTCTGGTG-AAGGTGAA-3′; antisense, 5′-TGGACAGCTTCACACCT-TTG-3′). Amplification was carried out for 28–35 cycles (94°C 15 s; 60°C 15 s; 72°C 30 s). PCR products were visualized on a 2% agarose gel.

Single Cell RT-PCR. Cultured sensory or LSF motor neurons were harvested with RNA*later* (Ambion), and then transferred to 22 μL ice-chilled lysis buffer in PCR tubes, containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, and 20 U RNasin (Promega). The L7 motor neuron was directly pulled from the desheathed abdominal ganglion and transferred to the lysis buffer. After lysis (70°C for 1 min), DNaseI treatment was performed at 37°C for 10 min, by adding DNase treated solution containing 5 μL RNase-free DNaseI (Ambion), 4 μL of 10× DNaseI buffer (Ambion), and 5 μL RNase-free water. DNaseI was inactivated by adding 2 μL EDTA (25 mM/μL) and heat-denaturing (70°C for 5 min). cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) with random hexamers (Invitrogen). A control reaction was performed without adding the reverse transcriptase. The cDNA was used as templates for PCR for 5-HT_{apAC1} (sense1, 5′-ACGGCTCCCTGTATGTCAAC-3′; antisense1, 5′-CCAGATGTCACAGACGATGG-3′), and sensorin (sense, 5′-AACAGAAACAGTCTTTCCCC-3′; antisense, 5′-TCTT-GACTCACCAACTGCC-3′). Templates were amplified using nTaq-Hot (Enzynomics) for 40 cycles (94°C 15 s; 60°C 30 s; 72°C 30 s) except for 5-HT_{apAC1}. In the case of 5-HT_{apAC1}, amplification was carried out for 35 cycles and followed by 35 cycles of nested PCR (sense2, 5′-CAAACGGCACAGACCTGTTC-3′; antisense2, 5′-AACCGCTTCACTATGGCAAC-3′). PCR products were visualized on a 2% agarose gel.

In Situ Hybridization in *Aplysia* Cultured Neurons or Ganglia. In situ hybridization was performed as described previously (1). Briefly, a partial fragment of 5-HT_{apAC1} (# 7 probe, 444 bp from 1,178th nucleotide from the initial ATG for Fig. 1*D*; 3icl probe, 297 bp from 754th nucleotide from the initial ATG for Figs. 1*E*, and 5*A*) was used as a probe. DIG-labeled probes were made by in vitro transcription using a DIG RNA labeling mix (Roche). The DIG-labeled probe was detected using an anti-DIG antibody (Roche), followed by development with NBT/BCIP (Roche).

Transfection, Western Blot, and Immunocytochemistry. Western blot and immunocytochemistry were performed as described previously (1, 2). The entire coding region of 5-HT_{apAC1} was subcloned into pFLAG-CMV2 or p3×FLAG-7.1 (Sigma). To express 5-HT_{apAC1} in *Aplysia* neurons, FLAG-5-HT_{apAC1} was subcloned into pNEXδ (3). These constructs were expressed in HEK293T cells with or without the expression marker pmCherry-N1 (Clontech) using Lipofectamine 2000 (Invitrogen) and were incubated for 1.5 days before experiments. In *Aplysia* sensory neurons, pNEXδ-FLAG-5-HT_{apAC1} and pNEX3-synaptophysin-EGFP were microinjected as described below. To detect the FLAG epitope, an mFLAG M2 antibody (Sigma) was used as a primary antibody and anti-mouse Cy3 (red), or anti-mouse Alexa488 (green) was used as a secondary antibody.

cAMP Assay in HEK293T Cells. To measure the agonist potency, cells were subcultured onto 12-well plates 24 h after transfection and further incubated for 24 h. To avoid the effect of signaling molecules in FBS (FBS) and endogenous PDEs, the media were changed to DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma) 30 min before the drug treatment. Various concentrations of 5-HT were applied in the presence of 0.5 mM IBMX for 30 min. To measure the antagonist potency, the media were replaced with DMEM containing 0.5 mM IBMX and various concentrations of antagonists for 20 min. After antagonist incubation, 10 nM 5-HT was applied for 20 min, and cells were immediately harvested with ice-chilled 100% EtOH and then kept at −20°C. The cAMP assays were performed by Neuronex Inc. using a cAMP RIA kit.

Ca^{2+} Imaging in HEK293T Cells. Either 5-HT_{apAC1} or mouse 5-HT_{2C} was co-transfected with the transfection indicator pmCherry using Lipofectamine 2000. The cells were subcultured onto poly-L-lysine coated 35-mm glass-bottom plates 24 h after transfection and further incubated for 24 h. The media were changed to serum-free DMEM containing 2.5 μg Calcium Green-1 AM. dye (Invitrogen) and incubated for 1 h at room temperature. After washing the cells three times with imaging buffer containing 152 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM Hepes, and 5.6 mM glucose (pH 7.2), 1 μM 5-HT was applied. Data were collected and analyzed with a confocal microscope. The 5-HT-evoked Ca^{2+} response was analyzed by calculating the ratio of mean fluorescence intensity of Calcium Green-1 (F/F0) at each time point (F) and before 5-HT treatment (F0) in the selected region of interest (ROI).

Heterologous Expression and Electrophysiology in *Xenopus* Oocytes. cRNA preparation and microinjection into *Xenopus* oocytes was performed as described previously (4). cRNAs for 5-HT_{apAC1}, Ap oa_1 (5), mouse 5-HT_{2C} (previously termed 5-HT_{1C}) (6), and human CFTR (7) were prepared by in vitro transcription using a MEGAscript kit (Ambion). The cRNAs were qualified by electrophoresis and microinjected into oocytes. Two-electrode voltage clamp was performed with a GeneClamp 500 amplifier (Axon Instruments). The voltage electrode and the current electrode, with the resistances of 1–5 MΩ, were filled with 3M KCl. The oocytes were placed in a chamber perfused with Ca^{2+}-free ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM Hepes, pH 7.5). The holding potential was set at −70 mV. The data were collected on a chart recorder and stored on video cassettes through Digidata (Instrutech) for later analysis.

Double-Stranded RNA Synthesis. cDNA containing a partial ORF (281 bp, 1178th to stop codon) and 3′UTR (163 bp) of 5-HT_{apAC1} was subcloned into pLitmus28i. The construct was linearized and then used as a template for in vitro transcription. Using the MEGAscript RNA*i* kit (Ambion), each strand of RNA was transcribed and then annealed. Double-stranded firefly luciferase (dsLuci) was used as a control.

Intracellular cAMP Level Measurement Using FRET. Briefly, cultured and microinjected *Aplysia* sensory neurons were incubated for 2 days. We performed FRET imaging on a Zeiss (LSM510) confocal laser scanning microscope. For excitation, 458 nm

wavelength was used, and fluorescence emission was collected with BP 480–520 IR (for CFP) and LP 530 (for YFP) emission filters. For the measurement of the fluorescence emission, we selected three regions of interest (ROI) with diameters of 5 μm. The selection was made on the region of the axon hillock because axon hillocks showed the smallest morphological change after 5-HT treatment. Because the binding of cAMP to CFP-Epac(δDEP/CD)-YFP induces a conformational change, reduced FRET indicates the degree of intracellular cAMP elevation. Decreased FRET was converted into an elevated cyan fluorescence protein (CFP)/yellow fluorescent protein (YFP) emission ratio. Measurements were made every 10 s for 600 s. After nine measurements to get the baseline ratio, a 100 μM stock solution of 5-HT in L15/ASW was applied to meet the final concentration of 10 μM. To minimize mechanical artifacts from the application, we applied the stock solution on the edge of the culture plate. In this treatment condition, the drug would have had to diffuse through the bath media to the cell. This technique may account for the time delay between the drug treatment and cAMP increase. Nine measurements, which were made from three ROIs for 30 s, were averaged for statistical analysis.

Microinjection of Plasmids or dsRNA into *Aplysia* Sensory Neurons. Various DNA constructs (500 ng/μL) or dsRNA (500 ng/μL) was mixed with the reporter construct pNEXδ-EGFP (500 ng/μL) and dissolved in the injection solution (Tris-Cl, 10 mM; KCl, 100 mM; fast green, 0.1%; pH 7.3). These solutions were microinjected into *Aplysia* cultured sensory neurons as previously described (8, 9). Three or 4 days after culture, dsRNAs were microinjected into the cells. Cells were incubated 1 or 1.5 days after microinjection.

***Aplysia* Cell Culture and Electrophysiology.** Sensory cell culture and sensory-to-motor coculture were performed as described previously (1, 10). Briefly, sensory neurons were dissociated from sensory clusters of pleural ganglia, and LFS motor neurons were used for sensory-to-motor coculture. Membrane excitability and spike broadening were measured in the sensory neurons of ventrocaudal clusters from pleural ganglia as described elsewhere (5). Briefly, we used the sensory neurons only if the resting potential was between −30 to −45 mV when impaled. To measure the membrane excitability, we injected 0.1–0.5 nA of a current pulse (500 ms) to induce a single action potential. Five minutes after 5-HT treatment, the same amount of current was injected, and the number of action potentials was counted. To measure the spike broadening, a 0.4-nA current pulse was delivered into the cells for 15 ms. The spike duration was measured as the time from the peak of the action potential to 25% of the peak. Spike broadening was measured 2 min after the membrane excitability measurement.

EPSP recordings in sensory-to-motor coculture were performed as previously reported (11, 12). To induce synaptic depression, five (moderately depressed synapse) or 50 (highly depressed synapse) electrical stimuli (ISI = 20 s) were applied to the presynaptic sensory neuron. Ten micromolar of 5-HT (Sigma) was bath-applied for those experiments. For Fig. 6*B*, 10 μM of 5-HT was bath-applied for 1 min, followed by 4 min of wash out using bath solution (1 mL/min). The synapses that initial EPSP was under 2 mV were not used for analysis.

Phylogenetic Analysis. Phylogenetic analysis was performed using ClustalW software.

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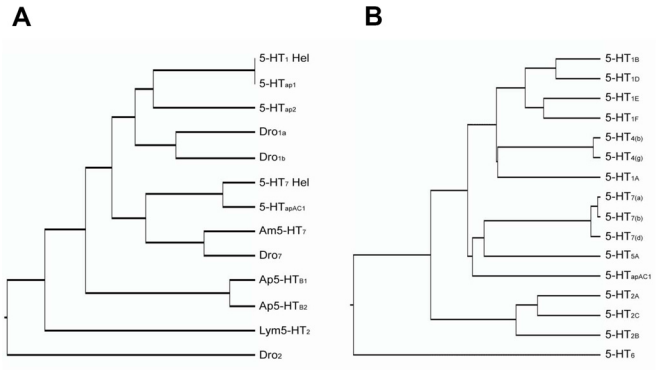

Fig. S1. Phylogenic analysis of 5-HT receptors. Cloned receptor was analyzed with other invertebrate (*A*) and mammalian (*B*) 5-HT receptors using ClustalW software.