Serotonin Inhibits Neuronal Excitability by Activating Two-Pore Domain K$^+$ Channels in the Entorhinal Cortex

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ABSTRACT

The entorhinal cortex (EC) is regarded as the gateway to the hippocampus; the superficial layers (layers I–III) of the EC convey the cortical input projections to the hippocampus, whereas deep layers of the EC relay hippocampal output projections back to the superficial layers of the EC or to other cortical regions. The superficial layers of the EC receive strong serotonergic projections from the raphe nuclei. However, the function of serotonin in the EC is still elusive. In the present study, we examined the molecular and cellular mechanisms underlying serotonin-mediated inhibition of the neuronal excitability in the superficial layers (layers II and III) of the EC. Application of serotonin inhibited the excitability of stellate and pyramidal neurons in the superficial layers of the EC by activating the TWIK-1 type of the two-pore domain K$^+$ channels. The effects of 5-HT were mediated via 5-HT$_{1A}$ receptors and required the function of Gα_{i3} subunit and protein kinase A. Serotonin-mediated inhibition of EC activity resulted in an inhibition of hippocampal function. Our study provides a cellular mechanism that might at least partially explain the roles of serotonin in many physiological functions and neurological diseases.

The entorhinal cortex (EC) is regarded as the gateway to the hippocampus because it mediates the majority of connections between the hippocampus and other cortical areas. Sensory inputs converge onto the superficial layers (layers I–III) of the EC, which give rise to dense projections to the hippocampus; the axons of the stellate neurons in layer II of the EC form the perforant path that innervates the dentate gyrus and commissura anterior 3, whereas the pyramidal neurons in layer II/III provide the primary input to commissura anterior 1 regions (Steward and Scoville, 1976; Witter et al., 1989). On the other hand, neurons in the deep layers of the EC (layers IV–VI) relay a large portion of hippocampal output projections back to the superficial layers of the EC (van Haeften et al., 2003) and to other cortical areas (Witter et al., 1989). The EC is part of a network that aids in the consolidation and recall of memories (for review, see Steffenach et al., 2005). Neuronal pathology and atrophy of the EC are potential contributors to Alzheimer's disease (Kotzbauer et al., 2001) and schizophrenia (Prasad et al., 2004). Furthermore, the EC plays an important role in the induction and maintenance of temporal lobe epilepsy (Spencer and Spencer, 1994).

The EC receives information from both the cortical mantle and the brain stem. The raphe nuclei send strong serotonergic projections to the superficial layers of the EC (Bobillier et al., 1975), where high densities of serotonin [5-hydroxytryptamine (5-HT)] receptors are expressed (Pazos and Palacios, 1985). Although the exact actions of 5-HT in the EC remain to be explored, application of 5-HT to this region generally

ABBREVIATIONS: EC, entorhinal cortex; 5-HT, 5-hydroxytryptamine; TTX, tetrodotoxin; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EPSC, excitatory postsynaptic current; QX-314, *N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide; sEPSC, spontaneous EPSC; mEPSC, miniature EPSC; HEK, human embryonic kidney; Rp-cAMPs, *R*p-diastereomer of cyclic adenosine 3′,5′-phosphorothioate; 8-OH-DPAT, 8-hydroxy-2-dipropylaminotetralin·HBr; GDP-β-S, guanosine-5′-*O*-(2-thiodiphosphate); SKF 96365, 1-(β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenethyl)-1*H*-imidazole; UH-301, (7*S*)-7-(dipropylamino)-4-fluoro-5,6,7,8-tetrahydronaphthalen-1-ol; ICS-205930, [(1*S*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl]-1*H*-indole-3-carboxylate; ZD 7288, 4-(*N*-ethyl-*N*-phenylamino)-1,2-dimethyl-6-(methylamino)pyrimidinium; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; TWIK, tandem pore domain weak inwardly rectifying K$^+$ channel; THIK, TWIK-related halothane-inhibited K$^+$ channel; TREK, TWIK-related K$^+$ channel; TASK, TWIK-related acid-sensing K$^+$ channel; TALK, TWIK-related alkaline-activated K$^+$ channel; TRESK, TWIK-related spinal cord K$^+$ channel; PKA, protein kinase A.

inhibits synaptic transmission (Schmitz et al., 1998) and neuronal excitability, possibly via activating K$^+$ channels (Grünschlag et al., 1997; Ma et al., 2006). However, the ionic and signaling mechanisms underlying 5-HT-mediated inhibition in the EC have not been elucidated. In the present study, we aimed to reveal the molecular mechanisms underlying 5-HT-mediated inhibition of the neuronal excitability in the superficial layers (layer II–III) of the EC because neurons in this area provide the major innervations to the hippocampus. Changes in neuronal activity in this region are likely to have profound influence on the functions of the hippocampus. Our results indicate that 5-HT inhibits the excitability of stellate and pyramidal neurons in the superficial layers of the EC by activating the TWIK-1 type of the two-pore domain K$^+$ channels. The effects of 5-HT are mediated via 5-HT$_{1A}$ receptors and require the function of Gα_{i3} subunit and protein kinase A.

Materials and Methods

Slice Preparation. Horizontal brain slices (400 μm) including the EC, subiculum, and hippocampus were cut using a vibrating blade microtome (VT1000S; Leica, Wetzlar, Germany) usually from 15- to 22-day-old Sprague-Dawley rats as described previously (Deng et al., 2006; Deng and Lei, 2007). After being deeply anesthetized with isoflurane, rats were decapitated and their brains were dissected out in ice-cold saline solution that contained 130 mM NaCl, 24 mM NaHCO$_3$, 3.5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 0.5 mM CaCl$_2$, 5.0 mM MgCl$_2$, and 10 mM glucose, saturated with 95% O$_2$ and 5% CO$_2$, pH 7.4. Slices were initially incubated in the above solution at 35°C for 40 min for recovery and then kept at room temperature ($\sim$24°C) until use. All animal procedures conformed to the guidelines approved by the University of North Dakota Animal Care and Use Committee.

Whole-Cell Recordings from Stellate and Pyramidal Neurons in Layers II–III of the EC. Whole-cell patch-clamp recordings using an Axopatch 200B or a Multiclamp 700B (Molecular Devices, Sunnyvale, CA) in current- or voltage-clamp mode were made from stellate neurons in layer II or pyramidal neurons in layer III of the EC visually identified with infrared video microscopy (BX51WI; Olympus, Tokyo, Japan) and differential interference contrast optics (Deng and Lei, 2007). Unless stated otherwise, recording electrodes were filled with 130 mM K$^+$-gluconate, 0.5 mM EGTA, 2 mM MgCl$_2$, 5 mM NaCl, 2 mM ATP$_2$Na, 0.4 mM GTPNa, and 10 mM HEPES, pH 7.4. The extracellular solution comprised 130 mM NaCl, 24 mM NaHCO$_3$, 3.5 mM KCl, 1.25 mM NaHPO$_4$, 2.5 mM CaCl$_2$, 1.5 mM MgCl$_2$, and 10 mM glucose, saturated with 95% O$_2$ and 5% CO$_2$, pH 7.4. Stellate and pyramidal neurons were identified by their location, shape, and electrophysiological properties. Stellate neurons are usually located in layer II or the border of layer II and III, and they have larger and polygonal soma with a variable number of main dendrites radiating out from the cell body but are devoid of a clearly dominant dendrite (Deng and Lei, 2007; Fig. 1, A and B). These neurons have unique electrophysiological properties; i.e., hyperpolarizing current pulse injection always caused the membrane potential to attain an early peak and then "sag" to a steady-state level (Alonso and Klink, 1993; Deng and Lei, 2007; Fig. 1C). Pyramidal neurons have a pyramidal or elongated soma with dendrites oriented in a bidirectional way: one (sometimes two) thick apical dendrite that runs to the surface of the cortex and several (three to five) basal dendrites extending toward the deeper layers. Pyramidal neurons in layer III usually lack the depolarizing voltage sag in response to hyperpolarizing current pulses (Fig. 1E). After formation of whole-cell recording, we initially recorded the voltage responses by injecting currents from +0.4 to −1 nA at an interval of −0.1 nA (Fig. 1, C and E) in current-clamp. We calculated the percentage of the sag by the equa-

tion [(peak − steady-state)/peak × 100%]. Together with the morphology and location of the neurons, we defined cells exhibiting >20% depolarizing voltage sag in response to −1 nA hyperpolarizing current injection as stellate neurons (Deng and Lei, 2007). Data were filtered at 2 kHz, digitized at 10 kHz, acquired on-line and analyzed after-line using pCLAMP 9 software (Molecular Devices).

Recordings of Action Potentials. Action potential firing was recorded from stellate neurons in layer II or pyramidal neurons in layer III of the EC with the above intracellular solution containing K$^+$-gluconate. Because dialysis of K$^+$-containing internal solution into cells can change the resting membrane potential and influence action potential firing, we waited for $\sim$15 min after the formation of whole-cell recordings to allow the resting membrane potential to stabilize. By and large, for most of the cells a positive current injec-
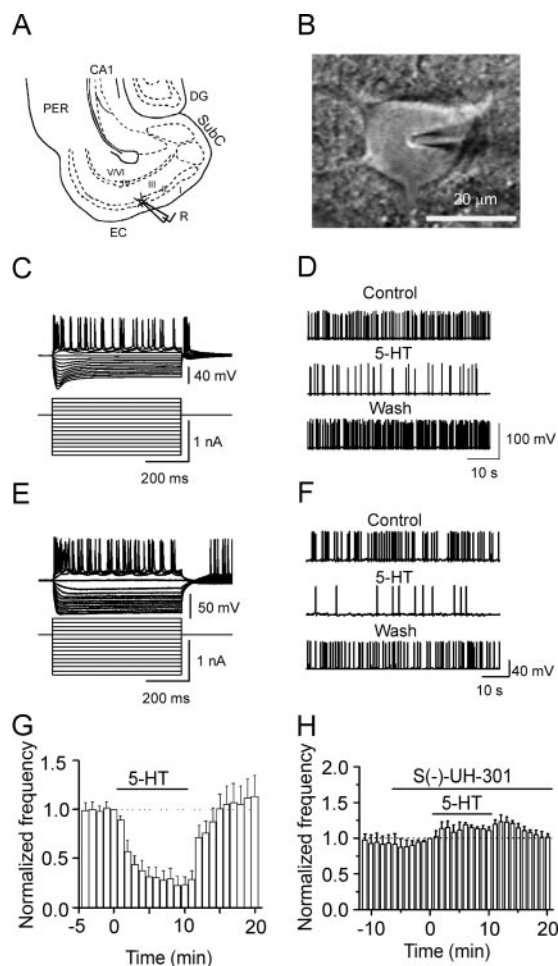

Fig. 1. 5-HT reduces neuronal excitability in the superficial layers of the EC via activation of 5-HT$_{1A}$ receptors. A, schematic illustration of the recording location in the EC. DG, dentate gyrus; Sub, subiculum; PER, perirhinal; R, recording electrode. B, a stellate neuron identified under an infrared video microscopy. C, voltage responses (top) generated by current injection from +0.4 to −1 nA at an interval of −0.1 nA (bottom) recorded from a stellate neuron in layer II. Note the depolarizing voltage sag in response to hyperpolarizing current pulses. D, action potential firing recorded before, during, and after the application 5-HT (1 μM) from a stellate neuron. E, voltage responses (top) generated by current injection from +0.4 to −1 nA at an interval of −0.1 nA (bottom) recorded from a pyramidal neuron in layer III. Note that there was no depolarizing voltage sag in response to hyperpolarizing current pulses. F, action potential firing recorded before, during, and after the application 5-HT (1 μM) from the pyramidal neuron. G, pooled time course of the action potential firing frequency recorded from eight stellate and six pyramidal neurons before, during, and after the application of 5-HT (1 μM). H, 5-HT-mediated inhibition of action potential firing was blocked in the presence of 5-HT$_{1A}$ inhibitor, $S(-)$-UH-301 (10 μM, n = 7).

tion was needed to bring the membrane potential to ∼−50 mV to induce action potential firing. 5-HT was applied after the action potential firing had been stable for 5∼10 min. To avoid potential desensitization induced by repeated applications of 5-HT, one slice was limited to only one application of 5-HT. The frequency of the action potentials was calculated by Mini Analysis 6.0.1.

Recordings of Holding Current. Holding current at −55 mV was recorded in the extracellular solution containing TTX (0.5 μM) to block action potential firing. Because gradual dialysis of K^+ into cells changed the holding current, we began our recordings after waiting for ∼15 min from the formation of whole-cell configuration. Holding currents at −55 mV were recorded every 3 s and then averaged per minute. We subtracted the average of the holding current recorded for the last minute before the application of 5-HT from those recorded at different time points to zero the basal level of the holding current for better comparison.

Recordings of AMPA Receptor-Mediated Spontaneous and Miniature EPSCs from Dentate Gyrus Granule Cells. Potassium gluconate in the above intracellular solution was replaced with the same concentration of cesium gluconate and QX-314 (1 mM) was included in the pipette solution to block action potential firing. To record AMPA receptor-mediated spontaneous EPSCs (sEPSCs), the above external solution was supplemented with bicuculline methobromide (10 μM) to block GABA$_A$ receptor responses. Miniature EPSCs (mEPSCs) were recorded by including TTX (0.5 μM) in the above external solution to block action potential-dependent responses. The recorded sEPSCs and mEPSCs were subsequently analyzed by Mini Analysis 6.0.1 (Synaptosoft Inc., Decatur, GA). Each detected event was inspected visually to exclude obvious artifacts before analysis. Mean amplitude, frequency, cumulative amplitude, and frequency histograms were calculated by this program.

Construction of Voltage-Current Curves. Voltage-current curves were constructed from identified stellate neurons in layer II of the EC. K^+-gluconate internal solution was used and the external solution contained 0.5 μM TTX, 100 μM CdCl$_2$, 10 μM 2,3-dihydroxy-6,7-dinitroquinoxaline, 50 μM dl-2-amino-5-phosphonovalerate, and 10 μM bicuculline. Voltage-current relationship was obtained by using a ramp protocol from −140 to −40 mV at a rate of 0.1 mV/ms. We compared the voltage-current curves recorded before and during the application of 5-HT for 5 to 10 min.

Cotransfection of HEK293 Cells with 5-HT$_{1A}$ Receptors and TASK or TWIK-1 Channels. cDNA constructs containing the N-terminal green fluorescent protein-tagged rat TASK-1 (GenBank accession number AF031384) or TASK-3 (GenBank accession number AF391084) channels subcloned into pEGFP (Clontech, Mountain View, CA) were kindly provided by Dr. Douglas A. Bayliss (University of Virginia, Charlottesville, VA; Berg et al., 2004). A cDNA construct containing the human 5-hydroxytryptamine 1A (5-HT$_{1A}$) receptor subtype (GenBank accession number AB041403) subcloned into pcDNA3.1 (Clontech) was purchased from UMR cDNA Resource Center (http://www.cdna.org). The full-length human TWIK-1 potassium channel construct (GenBank accession number NM_002245.2) subcloned into pCMV6-XL4 was purchased from OriGene (Rockville, MD). The green fluorescent protein (pGreenLantern; Invitrogen) was cotransfected with TWIK-1 channels or TWIK-1 channels plus 5-HT$_{1A}$ receptors for the identification of transfected cells. Dulbecco's minimum essential medium and fetal bovine serum was purchased from Atlanta Biologicals (Atlanta, GA). Cell culture grade penicillin and streptomycin was purchased from Mediatech, Inc (Herndon, VA). Human embryonic kidney 293 cells obtained from American Type Culture Collection (Manassas, VA) were maintained in Dulbecco's minimum essential medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml). Confluent HEK293 cells were washed in Hanks' balanced salt solution, trypsinized, and seeded at the appropriate density in 35-mm dishes to ensure 50 to 80% cell confluence within 24 h. Transient transfection of the cDNA constructs was performed after 24 h with *Trans*IT-293 transfection reagent according to the manufacturer's protocol

(Mirus Bio Corporation, Madison, WI) using a 3-μl reagent per 2 μg of cDNA ratio for the transfection cocktail. Transfected HEK293 cells were subsequently used for electrophysiological recordings 24 to 48 h after transfection.

Recordings from Transfected HEK293 Cells. Holding current at −55 mV was recorded from the HEK293 cells that showed fluorescence under a fluorescence microscope (Olympus 1 × 70) by whole-cell recordings. The extracellular solution contained 130 mM NaCl, 3 mM KCl, 2 mM MgCl$_2$, 2 mM CaCl$_2$, 1.25 mM NaH$_2$PO$_4$, 10 mM HEPES, and 10 mM glucose. pH was adjusted to 7.4 using NaOH and HCl. The above K-gluconate internal solution was used for this experiment. A continuous gravity perfusion system (flow, 5–7 ml/min) was used to change solutions.

Data Analysis. Data are presented as the means ± S.E.M. Concentration-response curve of 5-HT was fit by Hill equation: $I = I_{max} \times \{1/[1 + (EC_{50}/[ligand])^{n_H}]\}$, where I_{max} is the maximum response, EC_{50} is the concentration of ligand producing a half-maximal response, and n_H is the Hill coefficient. Student's paired or unpaired t test or analysis of variance was used for statistical analysis as appropriate; P values are reported throughout the text and significance was set as $P < 0.05$. For sEPSC or mEPSC cumulative probability plots, events recorded 5 min before and 5 min during the application of 5-HT were selected. Same bin size (100 ms for frequency and 1 pA for amplitude) was used to analyze data from control and 5-HT treatment. Kolmogorov-Smirnoff test was used to assess the significance of the cumulative probability plots. N number in the text represents the cells examined.

Chemicals. Rp-cAMPs, 8-hydroxy-2-dipropylaminotetralin·HBr (8-OH-DPAT), tertiapin, guanosine-5′-O-(2-thiodiphosphate) (GDP-β-S), anti-Gα_{i1}, anti-Gα_{i2}, anti-Gα_{i3}, anti-Gα_o, anti-Gα_z and anti-Gβ antibodies were from BIOMOL Research Laboratories (Plymouth Meeting, PA). SKF 96365, $S(-)$-UH-301, cyproheptadine, ICS-205930, and ZD 7288 were purchased from Tocris Cookson Inc. (Ellisville, MO). 5-HT and other chemicals were products of Sigma-Aldrich (St. Louis, MO).

Results

5-HT Reduced Action Potential Firing in Stellate and Pyramidal Neurons of the EC. We initially examined the effects of 5-HT on the excitability of stellate neurons in layer II and pyramidal neurons in layer III of the EC by recording action potential firing from these neurons. A schematic illustration of the EC appears in Fig. 1A. Stellate neurons were identified by their location (Fig. 1A), morphology (Fig. 1B), and electrophysiological property (depolarizing voltage sag in response to hyperpolarizing current pulses, Fig. 1C). Application of 5-HT (1 μM) reversibly reduced the frequency of action potentials to 14 ± 13% of control ($n = 8$, $p = 0.003$, Fig. 1D), suggesting that 5-HT inhibits the excitability of stellate neurons. Figure 1E showed the current-voltage responses recorded from a pyramidal neuron in layer III. This cell exhibited no noticeable depolarizing voltage sag in response to hyperpolarizing current injection. Likewise, application of 5-HT (1 μM) to pyramidal neurons reversibly inhibited the frequency of action potential firing to 26 ± 12% of control ($n = 6$, $p = 0.004$, Fig. 1F). Because 5-HT had the same effects on both stellate and pyramidal neurons, the data from stellate and pyramidal neurons were pooled (22 ± 10% of control, $n = 14$, $p < 0.001$, Fig. 1G). These data indicate that 5-HT inhibits neuronal excitability in both stellate and pyramidal neurons in the EC. For the rest of the experiments, we focused on stellate neurons to determine the underlying cellular and molecular mechanisms. The effect of 5-HT was mediated by 5-HT$_{1A}$ receptors, because application of the 5-HT$_{1A}$ receptor inhibitor $S(-)$-UH-301 (10 μM)

blocked 5-HT-induced depression of action potential firing ($108 \pm 10\%$ of control, $n = 7$, $p = 0.39$, Fig. 1H).

5-HT Generated Hyperpolarization. Reduction in action potential firing could be attributed to 5-HT-induced membrane hyperpolarization. We therefore used current clamp and recorded the resting membrane potential in the presence of TTX (0.5 μM). Application of 5-HT (1 μM) reversibly generated a membrane hyperpolarization (control, -55.42 ± 0.03 mV; 5-HT, -57.44 ± 0.05 mV, $n = 8$, $p < 0.001$; Fig. 2, A and B). We then used voltage-clamp and recorded the holding current at -55 mV, a potential close to the resting membrane potential. Application of 5-HT (1 μM) generated an outward holding current (70.0 ± 3.7 pA, $n = 8$,

$p = 0.003$; Fig. 2C). The EC$_{50}$ value was measured to be 0.48 μM (Fig. 2D). We also examined the role of 5-HT$_{1A}$ receptors in 5-HT-induced increases in outward holding current. Application of the 5-HT$_{1A}$ receptor antagonist $S(-)$-UH-301 (10 μM) generated an inward holding current (-43.7 ± 14.7 pA, $n = 6$, $p = 0.04$; Fig. 2E) suggesting that endogenously released 5-HT has a tonic hyperpolarizing effect in stellate neurons. In the presence of $S(-)$-UH-301 (10 μM), application of 5-HT (1 μM) for 10 min failed to significant change the holding current (6.8 ± 4.4 pA, $n = 6$, $p = 0.19$; Fig. 2E). Consistent with this result, application of 8-OH-DPAT (5 μM), a 5-HT$_{1A}$ receptor agonist, generated an increase in outward holding current (66.5 ± 4.7 pA, $n = 5$, $p < 0.001$; Fig. 2F). Together, these results indicate that 5-HT reduced neuronal excitability in the superficial layers of the EC via activation of 5-HT$_{1A}$ receptors, consistent with the observations that 5-HT$_{1A}$ receptors are expressed in this region (Wright et al., 1995).

In addition to expressing 5-HT$_{1A}$ receptors, the EC also expresses 5-HT$_{1C}$ (Wright et al., 1995), 5-HT$_2$ (Wright et al., 1995), and 5-HT$_3$ (Kilpatrick et al., 1987) receptors, although the types of cells that express these receptors remains unclear. We next tested whether 5-HT-induced hyperpolarization was related to the function of these receptors. The function of 5-HT$_{1C}$, 5-HT$_2$, or 5-HT$_3$ was unnecessary because 5-HT-induced increase in outward holding current was not significantly changed by coapplication of cyproheptadine (10 μM, an antagonist to both 5-HT$_{1C}$ and 5-HT$_2$ receptor, 76.5 ± 11.8 pA, $n = 5$, $p = 0.65$, Fig. 2F) or ICS-205930 (2 nM, an antagonist to 5-HT$_3$ receptors, 72.0 ± 7.4 pA, $n = 6$, $p = 0.54$, Fig. 2F).

5-HT-Induced Hyperpolarization Was Not Mediated by Inhibition of H- or Other Cation Channels. We next examined whether 5-HT generated membrane hyperpolarization via inhibition of H-channels by using two H-channel inhibitors, CsCl or ZD 7288. H-channel currents (Ih) were recorded by hyperpolarization for 1 s from -50 to -140 mV at an interval of -10 mV every 10 s (Fig. 3, A1 and B1). The external solution contained TTX (0.5 μM). Ih was measured by subtracting the instantaneous current evoked at the beginning of the voltage protocol from the steady-state current at the end of the protocol. Under these conditions, inclusion of 3 mM CsCl in the extracellular solution inhibited Ih to $4.7 \pm 2.3\%$ of control ($n = 5$, $p < 0.001$, Fig. 3A1). In the presence of CsCl (3 mM), application of 5-HT (1 μM) still increased the outward holding current to 63.1 ± 5.4 pA ($n = 5$, $p < 0.001$; Fig. 3A2). Likewise, inclusion of ZD 7288 (50 μM) in the extracellular solution inhibited Ih to $6.3 \pm 2.4\%$ of control ($n = 5$, $p < 0.001$; Fig. 3B1) but failed to block 5-HT-induced increase in outward holding current (52.5 ± 7.6 pA, $n = 8$, $p < 0.001$; Fig. 3B2). Together, these results suggest that a 5-HT-induced increase in outward holding current is not mediated by inhibition of H-channels. The function of other cation channels was unlikely to be involved because 5-HT-induced increase in outward holding current was not changed by the following treatments; replacing extracellular Na$^+$ with the same concentration of NMDG (61.4 ± 6.1 pA, $n = 7$, $p < 0.001$; Fig. 3C), application of cation channel blockers Gd^{3+} (10 μM, 60.3 ± 6.5 pA, $n = 6$, $p < 0.001$; Fig. 3D) or La^{3+} (10 μM, 68.1 ± 7.4 pA, $n = 6$, $p < 0.001$; Fig. 3D) and SKF96365 (50 μM, 61.3 ± 10.2 pA, $n = 6$,

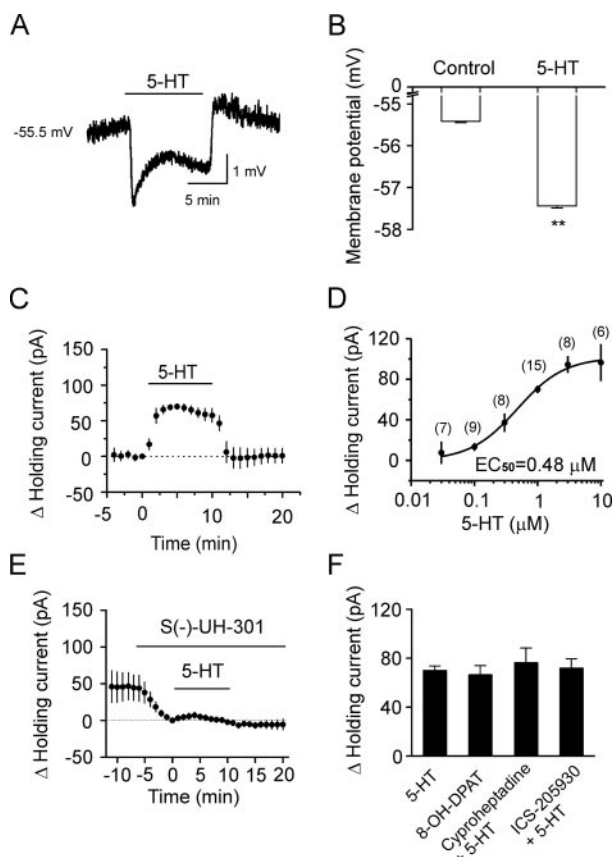**Fig. 2.** Activation of 5-HT$_{1A}$ receptors generates membrane hyperpolarization in the EC. A, application of 5-HT (1 μM) generated membrane hyperpolarization in a stellate neuron. Resting membrane potential was recorded in the presence of TTX (0.5 μM) in current-clamp. B, summarized data for 5-HT-induced changes in resting membrane potentials ($n = 8$). C, application of 5-HT induced an outward holding current in stellate neurons ($n = 8$). Stellate neurons were held at -55 mV, and the holding currents were recorded in the presence of TTX (0.5 μM) every 3 s. The holding currents were averaged every minute and 5-HT-induced changes in holding currents were calculated by subtracting the averaged holding currents at each min from the holding current just before the application of 5-HT. D, concentration-response curve of 5-HT-induced changes in holding current. Numbers in the parenthesis are number of cells recorded. E, 5-HT-induced changes in holding current were blocked in the presence of $S(-)$-UH-301 (10 μM) suggesting the involvement of 5-HT$_{1A}$ receptors ($n = 6$). Note that application of the 5-HT$_{1A}$ receptor antagonist, $S(-)$UH-301 alone induced an inward holding current suggesting that there was tonic hyperpolarization mediated by endogenously released 5-HT. F, application of the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT (5 μM) generated an outward holding current in stellate neurons ($n = 5$), whereas application of antagonists for 5-HT$_{1C}$ and 5-HT$_2$ receptors, cyproheptadine (10 μM, $n = 5$), or 5-HT$_3$ receptors, ICS-205930 (2 nM, $n = 6$) had no effects on 5-HT-induced increase in outward holding current.

$p = 0.002$; Fig. 3D), or omitting extracellular Ca^{2+} (63.7 ± 13.4 pA, $n = 7$, $p = 0.003$; Fig. 3D).

5-HT Activated Two-Pore Domain K^+ Channels. We next examined the roles of K^+ channels because 5-HT could activate the background K^+ channels to generate membrane hyperpolarization. We measured the reversal potential of the 5-HT-generated current using a ramp protocol (from −140 to −40 mV) before and during the application of 5-HT. Application of 5-HT (1 μM) in the presence of 3.5 mM K^+ induced a net current that had a reversal potential of −91.4 ± 4.2 mV ($n = 8$, Fig. 4A), identical to the theoretical K^+ reversal potential calculated by the Nernst equation (−92.2 mV) suggesting that 5-HT produces membrane hyperpolarization by activating a background K^+ conductance. The net current generated by 5-HT was inwardly rectifying when the membrane potentials were positive to −60 mV (Fig. 4A, inset). Further evidence to support the involvement of K^+ channels was that elevating the extracellular K^+ concentration to 10 mM significantly reduced 5-HT-induced outward holding current (11.4 ± 4.3 pA, $n = 5$, $p < 0.001$, Student's unpaired t test) compared with that recorded in the presence of normal (3.5 mM) K^+ (70.01 ± 3.72, $n = 8$, Fig. 4B).

We then identified the properties of the K^+ channels involved in 5-HT-induced hyperpolarization. 5-HT-induced increase in outward holding current was not blocked by application of tetraethylammonium (TEA; 10 mM, 69.2 ± 9.9 pA, $n = 7$, $p = 0.43$; Fig. 4B), 4-aminopyridine (4-AP; 2 mM, 66.1 ± 8.5 pA, $n = 6$, $p = 0.39$; Fig. 4B), or Cs^+ (3 mM, 63.7 ± 6.8 pA, $n = 6$, $p = 0.93$; Fig. 4B), suggesting that 5-HT-activated K^+ channels are insensitive to the classic K^+ channel blockers. Although the inward rectifier K^+ channels are involved in controlling the resting membrane potential, they are unlikely to be the channels activated by 5-HT because these channels are also sensitive to TEA and Cs^+. Moreover, application of the specific inward rectifier K^+ channel blocker tertiapin (50 nM) failed to block the effect of 5-HT (72.2 ± 10.4 pA, $n = 8$, $p = 0.11$; Fig. 4B).

The two pore-domain K^+ channels (K_{2P}) are involved in controlling resting membrane potential and they are insensitive to the classic K^+ channel blockers (TEA, 4-AP, Cs^+). We next examined the roles of K_{2P} in 5-HT-induced membrane hyperpolarization. K_{2P} channels can be grouped according to sequence and functional similarities into six subfamilies: TWIK, THIK, TREK, TASK, TALK, and TRESK (Bayliss et al., 2003; Lesage, 2003), some of which are sensitive to Ba^{2+} or the local anesthetic bupivacaine. We therefore tested the roles of Ba^{2+} and bupivacaine in 5-HT-induced membrane hyperpolarization. Inclusion of Ba^{2+} (2 mM) in
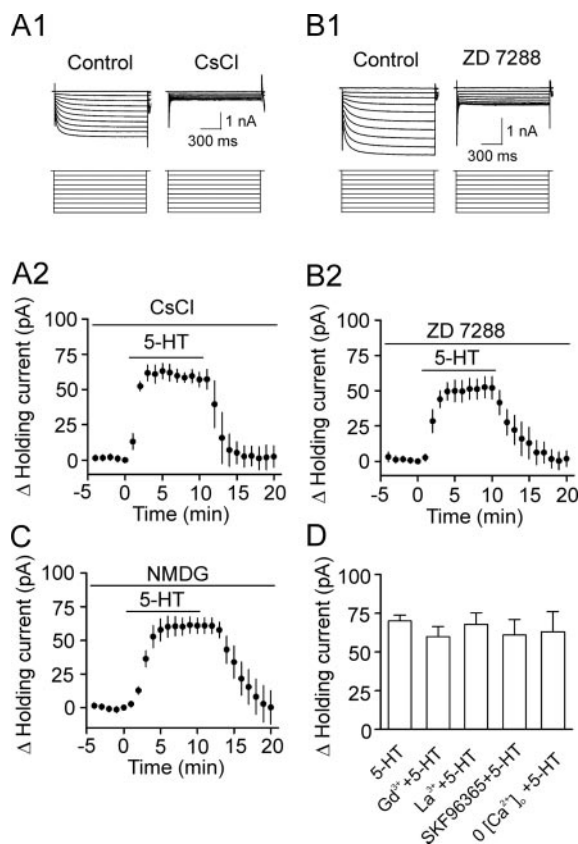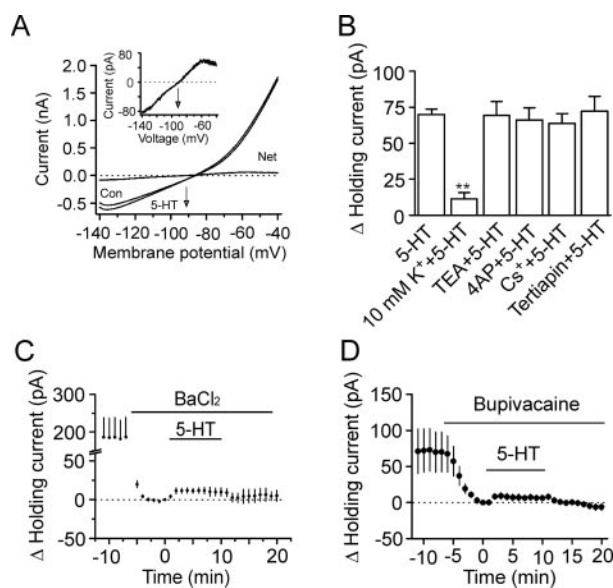

Fig. 3. 5-HT-mediated increase in outward holding current is not related to H- or other cation channels. A1, extracellular application of CsCl (3 mM) blocked H-channel currents. Top, H-currents; bottom, voltage paradigm. A2, In the presence of CsCl (3 mM), application of 5-HT (1 μM) still induced an increase in outward holding current ($n = 5$). B1, application of ZD 7288 (50 μM) blocked H-channels in stellate neurons. B2, application 5-HT (1 μM) in the presence of ZD 7288 (50 μM) still induced an outward holding current ($n = 8$). C, replacing extracellular Na^+ with the same concentration of NMDG failed to block 5-HT (1 μM)-induced increases in outward holding current ($n = 7$). D, 5-HT-induced increase in outward holding current was not sensitive to extracellular application of cation channel blockers (Gd^{3+}, 10 μM, $n = 6$; Lan^{3+}, 10 μM, $n = 6$; SKF96365, 50 μM, $n = 6$) or omission of extracellular Ca^{2+} ($n = 7$).

Fig. 4. 5-HT-mediated hyperpolarization is mediated by activating K_{2P} channels. A, voltage-current relationship induced by a ramp protocol from −140 to −40 mV at a rate of 0.1 mV/ms before and during the application of 5-HT (1 μM). Subtraction of the current before from the current during the application of 5-HT generated a net current induced by 5-HT. The traces were averages from 8 cells. Note that the reversal potential was ~−91 mV, close to the calculated K^+ reversal potential (−92.2 mV). The inset was 5-HT-induced net current in an enlarged scale. Note that 5-HT-induced net current was inwardly rectifying when the potentials were positive to −60 mV. B, 5-HT-induced increase in outward holding current was insensitive to the classic K^+ channel blockers (TEA, 10 mM, $n = 7$; 4-AP, 2 mM, $n = 6$; Cs^+, 3 mM, $n = 6$; tertiapin, 50 nM, $n = 8$) but reduced when the extracelluar K^+ concentration was increased to 10 mM ($n = 5$, $p < 0.001$). C, inclusion of Ba^{2+} (2 mM) in the extracellular solution induced an inward holding current per se, but blocked 5-HT-induced increase in outward holding current ($n = 6$, $p = 0.08$). D, inclusion of bupivacaine (200 μM) in the extracellular solution induced an inward holding current and blocked 5-HT-induced increase in outward holding current ($n = 6$, $p = 0.06$).

the extracellular solution by itself generated an inward holding current (-186.5 ± 52.2 pA, $n = 6$, $p = 0.04$; Fig. 4C) suggesting that the Ba^{2+}-sensitive K$_{2P}$ channels are involved in controlling resting membrane potential in stellate neurons. However, in the presence of Ba^{2+}, application of 5-HT (1 μM) failed to significantly induce an outward holding current (9.9 ± 5.3 pA, $n = 6$, $p = 0.08$; Fig. 4C), suggesting that 5-HT-induced membrane hyperpolarization is Ba^{2+}-sensitive. Likewise, application of bupivacaine (200 μM) alone induced a large inward holding current (-67.5 ± 20.6 pA, $n = 6$, $p = 0.02$; Fig. 4D) and blocked 5-HT-induced increase in outward holding current (9.1 ± 3.9 pA, $n = 6$, $p = 0.06$; Fig. 4D), suggesting that 5-HT-induced membrane hyperpolarization is bupivacaine-sensitive as well. Together, these results suggest that 5-HT generates membrane hyperpolarization by activating K$_{2P}$ channels.

TASK and TREK Channels Were Not Involved in 5-HT-Induced Hyperpolarization. Among the K$_{2P}$ channels, TASK-1 (Han et al., 2002), TASK-3 (Kim et al., 2000; Han et al., 2002), TREK-1 (Fink et al., 1996), TREK-2 (Han et al., 2002), TWIK-1 (Lesage et al., 1996), and TRESK (Sano et al., 2003; Kang et al., 2004) are sensitive to Ba^{2+}. Bupivacaine also inhibits TASK, TREK-1, TWIK-1, and the K$_{2P}$ channels isolated from yeast (*TOK1*) or *Drosophila melanogaster* (*ORK1*) (Kindler et al., 1999). We then performed more experiments to identify the type of K$_{2P}$ channels involved in 5-HT-induced hyperpolarization. TRESK is unlikely to be

involved because it is expressed in the spinal cord and other organs but not in the brain, and the voltage-current relationship of TRESK channels is outwardly rectifying (Sano et al., 2003; Kang et al., 2004), which is different from the voltage-current relationship of 5-HT-induced net current (inwardly rectifying, Fig. 4A). We therefore focused on TASK-1, TASK-3, TREK-1, TREK-2, and TWIK-1. One important property of the TASK channels is their high sensitivity to acid (Bayliss et al., 2003; Berg et al., 2004). Lowering the pH of the extracellular solution from 7.4 to 6.4 generated an inward holding current (-41.1 ± 9.8 pA, $n = 7$, $p = 0.006$; Fig. 5A) suggesting that TASK channels or acid-sensitive ion channels participate in controlling the resting membrane potential. However, application of 5-HT (1 μM) still produced a comparable outward holding current (68.3 ± 13.9 pA, $n = 7$, $p = 0.003$; Fig. 5A) suggesting that the 5-HT-induced current is not acid-sensitive. We further tested the roles of TASK channels by using their selective inhibitors. We examined the effects of one TASK-1 channel inhibitor, anandamide (10 μM; Bayliss et al., 2003), and two TASK-3 blockers, Zn^{2+} (100 μM; Bayliss et al., 2003) and ruthenium red (10 μM; Bayliss et al., 2003). However, none of these inhibitors blocked 5-HT-induced increase in outward holding current (Fig. 5B). In native hypoglossal motoneurons (Berg et al., 2004), TASK-1 and TASK-3 form heterodimers (TASK-1/TASK-3) that are insensitive to the inhibitors of TASK-1 or TASK-3 homodimers when they are applied separately. We
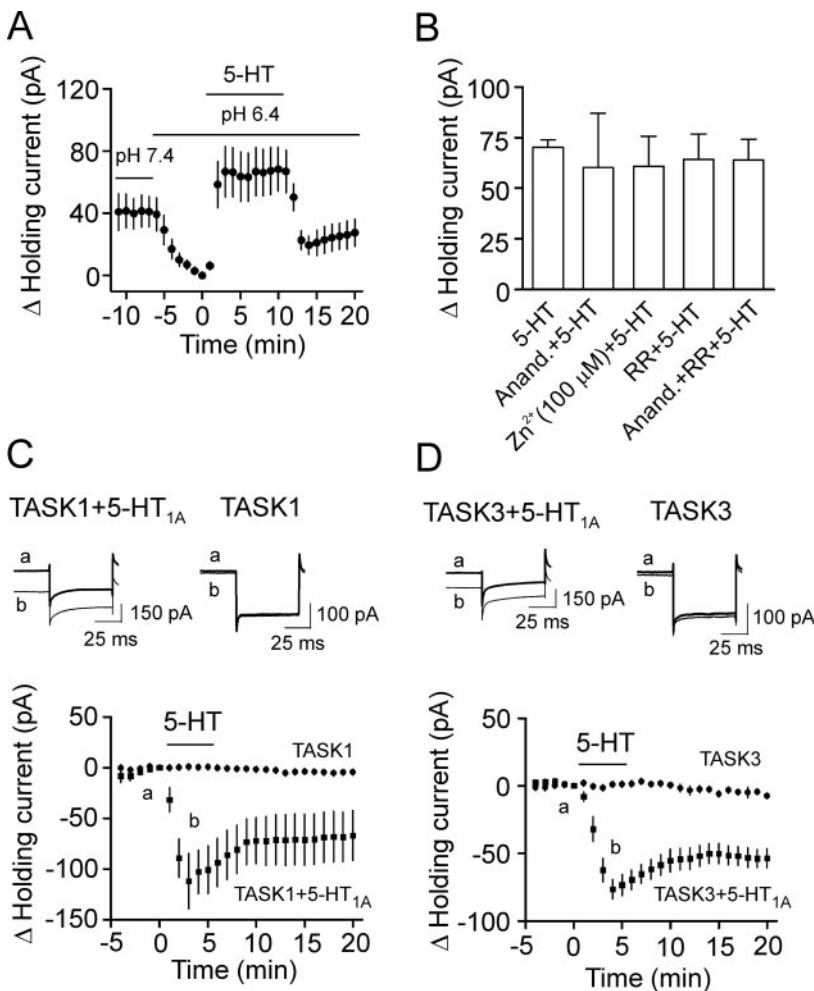
Fig. 5. TASK channels are not involved in 5-HT-induced hyperpolarization in stellate neurons. A, lowering the extracellular pH from 7.4 to 6.4 induced an inward holding current but failed to block 5-HT-induced increase in outward holding current ($n = 7$). B, application of antagonists to TASK-1, anandamide (10 μM, $n = 6$), or TASK-3, Zn^{2+} (100 μM, $n = 7$), ruthenium red (RR, 10 μM, $n = 9$) or coapplication of anandamide and ruthenium red ($n = 6$) failed to change 5-HT-mediated increase in outward holding current. C, application of 5-HT (1 μM) induced an inward holding current in HEK293 cells cotransfected with 5-HT$_{1A}$ receptors and TASK-1 channels ($n = 9$, $p = 0.003$) with no effects on HEK293 cells transfected with TASK-1 alone ($n = 5$, $p = 0.39$). Top left, holding current recorded at -55 mV from a HEK293 cell cotransfected with TASK-1 and 5-HT$_{1A}$ before (thick) and during (thin) the application of 5-HT. Top right, holding current recorded at -55 mV from a HEK293 cell transfected with TASK-1 channels alone before (thick) and during (thin) the application of 5-HT (1 μM). D, application of 5-HT (1 μM) induced an inward holding current in HEK293 cells cotransfected with 5-HT$_{1A}$ receptors and TASK-3 channels ($n = 10$, $p < 0.001$) with no effects on HEK293 cells transfected with TASK-3 alone ($n = 5$, $p = 0.67$). The figure was arranged in the same way as C.

therefore combined anandamide (10 μM) and ruthenium red (10 μM) to inhibit both TASK-1 and TASK-3. Nonetheless, this treatment did not change the effect of 5-HT, either (Fig. 5B).

We further tested whether activation of 5-HT$_{1A}$ receptors modulates TASK channels by expressing 5-HT$_{1A}$ receptors together with TASK-1 or TASK-3 channels in HEK293 cells. Application of 5-HT (1 μM) generated an inward instead of an outward holding current in HEK293 cells cotransfected with 5-HT$_{1A}$ receptors with TASK-1 (Fig. 5C) and TASK-3 (Fig. 5D) channels, whereas application of the same concentration of 5-HT failed to significantly change the holding current in HEK293 cells transfected with TASK-1 (Fig. 5C) or TASK-3 (Fig. 5D) alone. To test whether the effects of 5-HT on the holding current in the transfected cells were mediated by K$^+$ channels, we applied a ramp protocol (from -140 to -40 mV at a rate of 0.1 mV/ms) before and during the application of 5-HT. The reversal potential for the 5-HT-induced current was at -92.9 ± 2.1 mV in cells cotransfected with 5-HT$_{1A}$ and TASK-1 ($n = 5$) and -93.4 ± 1.9 mV in cells cotransfected with 5-HT$_{1A}$ and TASK-3 ($n = 5$). The measured reversal potentials were close to the calculated K$^+$ reversal potential (-96.1 mV) in our recording condition, suggesting that the effects of 5-HT$_{1A}$ receptors were mediated by inhibition of TASK-1 and TASK-3 channels in the transfected cells. These results clearly indicate that 5-HT-mediated hyperpolarization in stellate neurons is unlikely to be mediated via activation of TASK channels because activation of 5-HT$_{1A}$ receptors inhibits not activates TASK channels, consistent with the effects of 5-HT$_{1A}$ receptor activation in spinal motoneurons (Perrier et al., 2003).

We then tested the roles of TREK-1 and TREK-2 in 5-HT-induced hyperpolarization. One important property of these channels is that they are activated by lipids such as arachidonic acid (for review, see Kim, 2003). If TREK channels are expressed in stellate neurons, application of arachidonic acid should activate these channels to generate an outward holding current. However, application of arachidonic acid (10 μM) instead produced an inward holding current (-38.8 ± 8.9 pA, $n = 6$, $p = 0.008$; Fig. 6A), suggesting that these neurons do not express TREK channels. Inhibition of TASK channels by arachidonic acid (Han et al., 2002) might be an explanation for the inward holding current induced by this compound, because TASK channels are expressed in these neurons as suggested by the result that lowering the extracellular pH generated an inward holding current (Fig. 5A). In the presence of arachidonic acid, application of 5-HT (1 μM) still generated a comparable outward holding current (67.1 $\pm$ 9.4 pA, $n = 6$, $p < 0.001$; Fig. 6A). Furthermore, the currents generated by TREK channels are outwardly rectifying (Fink et al., 1996; Lesage et al., 2000), which is different from the net currents produced by 5-HT (inwardly rectifying; Fig. 4A). Together, these results suggest that TREK channels are unlikely to be involved in 5-HT-mediated hyperpolarization.

TWIK-1 Was Involved in 5-HT-Induced Hyperpolarization. We next tested the roles of TWIK-1 by applying quinidine, a TWIK-1 inhibitor (IC$_{50}$ = 95 μM, Lesage et al., 1996). Slices were pretreated with 1 mM quinidine for ~20 min, and the same concentration of quinidine was bath-applied. In the presence of quinidine, application of 5-HT (1 μM) failed to induce an increase in outward holding current (5.9 $\pm$ 3.6 pA, $n = 5$, $p = 0.17$, Fig. 6B), suggesting that 5-HT generates hyperpolarization by activating TWIK-1 channels.

To further confirm the role of TWIK-1 in 5-HT-induced hyperpolarization, we cotransfected 5-HT$_{1A}$ receptors with TWIK-1 channels in HEK293 cells. We used whole-cell, patch-clamp recordings to record the changes of holding current at -55 mV in response to 5-HT from the transfected
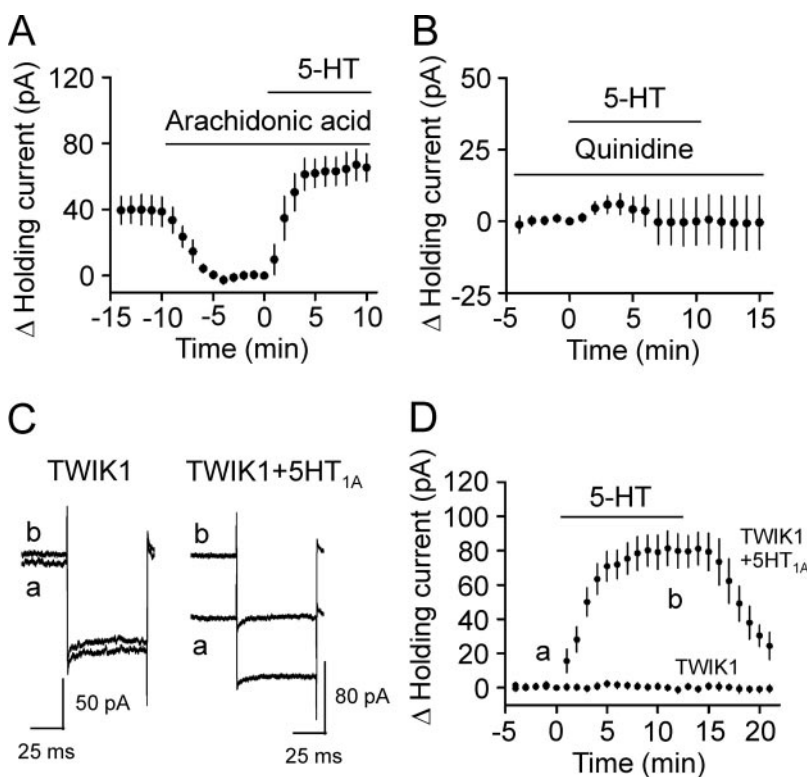

Fig. 6. 5-HT-induced hyperpolarization is independent of TREK channels but mediated by TWIK-1 channels. A, application of arachidonic acid (10 μM) generated an inward holding current but failed to block 5-HT-induced increase in outward holding current ($n = 6$) suggesting that TREK channels are not expressed in stellate neurons. B, application of quinidine (1 mM) blocked 5-HT-induced increase in outward holding current ($n = 5$), suggesting the involvement of TWIK-1 channels. C, left, holding current recorded at -55 mV from a HEK293 cell transfected with TWIK-1 alone before (a) and during (b) the application of 5-HT. Right, holding current recorded at -55 mV from a HEK293 cell cotransfected with 5-HT$_{1A}$ receptors and TWIK-1 channels before (a) and during (b) the application of 5-HT (1 μM). Note that application of 5-HT induced an outward holding current in HEK293 cell cotransfected with 5-HT$_{1A}$ receptors and TWIK-1 channels. D, summarized data from six HEK293 cells transfected with TWIK-1 channels alone and six HEK293 cells cotransfected with 5-HT$_{1A}$ receptors and TWIK-1 channels.

cells. Application of 5-HT (1 μM) failed to significantly change the holding current in cells transfected with TWIK-1 alone (1.0 $\pm$ 1.7 pA, $n = 6$, $p = 0.59$, Fig. 6, C and D), but it induced an outward holding current in cells cotransfected with TWIK-1 and 5-HT$_{1A}$ receptors (79.8 $\pm$ 9.9 pA, $n = 6$, $p < 0.001$, Fig. 6, C and D). We also measured the reversal potential of 5-HT-induced current using the ramp protocol. The reversal potential for 5-HT-induced current in cells cotransfected with TWIK-1 channels and 5-HT$_{1A}$ receptors was -92.5 ± 1.3 mV ($n = 6$), close to the theoretical calculated K$^+$ reversal potential (-96.1 mV) in our recording condition. Together, these results demonstrate that activation of 5-HT$_{1A}$ receptors generates membrane hyperpolarization by activating TWIK-1 channels in stellate neurons of the EC.

5-HT-Induced Hyperpolarization Was G Protein-Dependent and Required Protein Kinase A Activity. Our results have already demonstrated that 5-HT induces membrane hyperpolarization via activation of 5-HT$_{1A}$ receptors (Figs. 1H and 2E). 5-HT$_{1A}$ receptors are G protein-coupled (Pucadyil et al., 2005). We next examined whether the function of G proteins is required for 5-HT-induced hyperpolarization. We replaced the intracellular GTP with GDP-β-S (4 mM), a G protein inactivator, and recorded 5-HT-induced change in holding current at -55 mV. In the presence of GDP-β-S, 5-HT did not significantly change the holding currents (5.8 $\pm$ 5.0 pA, $n = 7$, $p = 0.28$; Fig. 7A) suggesting that 5-HT-induced hyperpolarization is mediated by G-proteins.

We then identified the type of the G-proteins involved in 5-HT-mediated membrane hyperpolarization. 5-HT$_{1A}$ receptors interact with Gα_{i1}, Gα_{i2}, Gα_{i3}, Gα_o, and Gα_z (Pucadyil et al., 2005). We next probed the roles of these Gα subunits in 5-HT-mediated increase in outward holding current by applying the specific antibodies to those subunits in the recording pipettes. We have shown previously that intracellular application of G$\alpha_{q/11}$ antibody via the recording pipettes successfully blocked thyrotropin-releasing hormone-induced depolarization (Deng et al., 2006). We included antibodies for the above Gα subunits in the recording pipettes and waited for ~20 min after the formation of whole-cell recordings. Intracellular application of Gα_{i3} antibody (Anti-Gα_{i3}, 20 μg/ml) via the recording pipettes blocked 5-HT-induced increase in outward holding current (6.5 $\pm$ 3.0 pA, $n = 7$, $p = 0.07$; Fig. 7B). However, application of 5-HT (1 μM) still significantly increased the outward holding current in the presence of anti-Gα_{i1} (20 μg/ml, 69.2 $\pm$ 7.6 pA, $n = 6$, $p < 0.001$; Fig. 7C), anti-Gα_{i2} (20 μg/ml, 61.0 $\pm$ 12.1 pA, $n = 6$, $p = 0.004$; Fig. 7C), anti-Gα_o (20 μg/ml, 70.2 $\pm$ 12.6 pA, $n = 5$, $p = 0.005$; Fig. 7C), anti-Gα_z (20 μg/ml, 69.4 $\pm$ 10.8 pA, $n = 6$, $p = 0.001$; Fig. 7C), or anti-Gβ (20 μg/ml, 65.0 $\pm$ 9.7 pA, $n = 5$, $p = 0.003$; Fig. 7C). Together, these data suggest that the effect of 5-HT was mediated by the Gα_{i3} subunit.

The primary function of 5-HT$_{1A}$ receptors is to inhibit adenylyl cyclase and to reduce the levels of cAMP, thereby resulting in an inhibition of protein kinase A (PKA) (Pucadyil et al., 2005). We next explored the function of PKA in 5-HT-induced hyperpolarization in stellate neurons. We included Rp-cAMPs (1 mM), a specific PKA inhibitor in the recording pipettes, and waited for ~20 min after the formation of whole-cell recordings. In this condition, application of 5-HT (1 μM) did not significantly increase the outward holding current (4.6 $\pm$ 3.8 pA, $n = 9$, $p = 0.26$; Fig. 7D), suggesting

that the activity of PKA is required for 5-HT-induced hyperpolarization.

The above experiments suggest that activation of 5-HT$_{1A}$ activates Gα_{i3}, leading to an inhibition of PKA. Inhibition of PKA results in an activation of TWIK-1 that generates hyperpolarization. If so, activation of PKA should inhibit TWIK-1 to generate depolarization. We therefore bath applied 8-CPT-cAMP (500 μM), a membrane-permeant cAMP analog that could activate PKA. Application of 8-CPT-cAMP generated an inward holding current (-21.0 ± 3.5 pA, $n = 5$, $p = 0.009$; Fig. 7E). Likewise, bath application of forskolin (10 μM) and 3-isobutyl-1-methylxanthine (1 mM) produced an inward holding current (-31.1 ± 8.8 pA, $n = 6$, $p = 0.01$; Fig. 7F). The effect of forskolin and 3-isobutyl-1-methylxanthine was not changed when the extracellular solution contained Cs$^+$ (3 mM) to block Ih or other K$^+$ channels (-29.2 ± 10.2 pA, $n = 6$, $p = 0.69$; Fig. 7F) suggesting that activation of PKA has a tonic inhibition on K$_{2P}$ channels, consistent
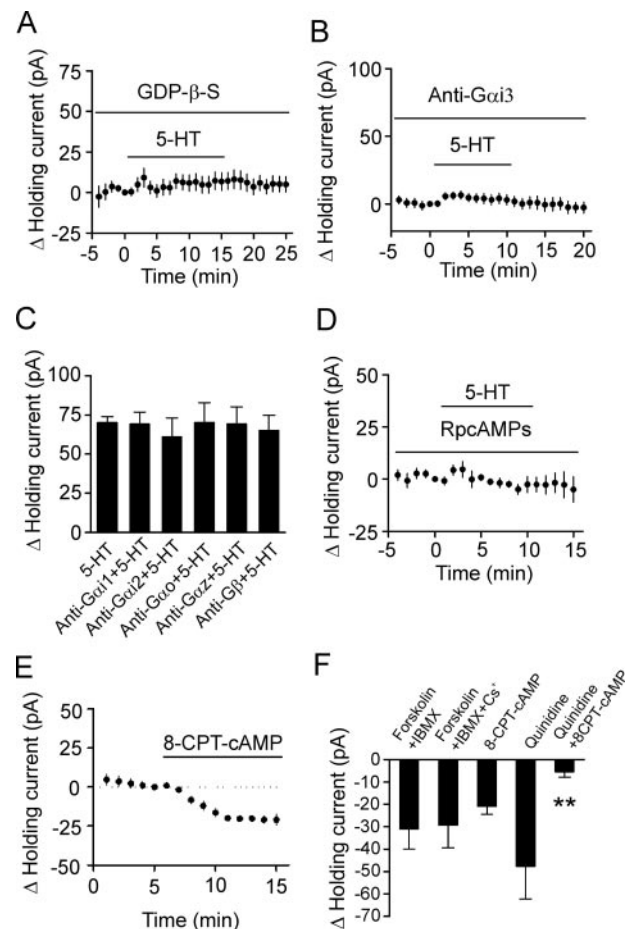

Fig. 7. 5-HT-mediated hyperpolarization requires the function of Gα_{i3} and PKA. A, intracellular application of GDP-β-S (4 mM) via the recording pipettes blocked 5-HT-induced increase in outward holding current ($n = 7$). B, intracellular application of Gα_{i3} antibody (Anti-Gα_{i3}, 20 μg/ml) via the recording pipettes blocked 5-HT-induced increase in outward holding current ($n = 7$). C, 5-HT still increased the outward holding current when antibodies to Gα_{i1} ($n = 6$), Gα_{i2} ($n = 6$), Gα_o ($n = 5$), Gα_z ($n = 6$), or Gβ ($n = 5$) were applied via the recording pipettes. The concentration for each antibody was 20 μg/ml. D, application of Rp-cAMPs (1 mM) in the recording pipettes completely blocked 5-HT-induced increase in outward holding current ($n = 9$). E, bath application of 8-CPT-cAMP (500 μM, $n = 5$) induced an inward holding current. F, 8-CPT-cAMP-induced inward holding current occluded that induced by quinidine suggesting that they interact with the same channels.

with previous results (Fink et al., 1996; Lesage et al., 2000; Murbartián et al., 2005). We also tested the effects of quinidine on 8-CPT-cAMP-induced inward holding current. Whereas application of 8-CPT-cAMP (500 μM) or quinidine (1 mM) separately generated comparable inward holding currents (Fig. 7F), coapplication of these two compounds produced a much smaller inward holding current ($n = 5$, $p < 0.01$; Fig. 7F) suggesting that PKA and quinidine interact with the same channels. Together, these results indicate that 5-HT generates hyperpolarization by disinhibiting PKA-mediated tonic inhibition of K_{2P} (TWIK-1) channels in the EC.

5-HT Inhibited Spontaneous EPSCs with No Effects on Miniature EPSCs in Dentate Gyrus Granule Cells. The axons of stellate neurons form the perforant path that makes glutamatergic synapses onto dentate gyrus granule cells (Steward and Scoville, 1976). We next examined the outcome of 5-HT-induced reduction in the excitability of stellate neurons by recording spontaneous and miniature EPSCs from dentate gyrus granule cells. Application of 5-HT (1 μM) reduced the frequency of spontaneous EPSCs to 26.3 ± 4.9% of control ($n = 6$, $p < 0.0001$, Fig. 8,A and B) without significantly changing the amplitude of spontaneous EPSCs (102.2 ± 3.3% of control, $n = 6$, $p = 0.67$; Fig. 8C). However, application of 5-HT (1 μM) failed to change either the frequency (101.8 ± 7.1% of control, $n = 7$, $p = 0.81$; Fig. 8, D and E) or the amplitude (107.5 ± 9.7% of control, $n = 7$, $p = 0.49$; Fig. 8F) of miniature EPSCs recorded in the presence of TTX (0.5 μM). These results suggest that the effects of 5-HT are dependent on presynaptic action potential firing and that inhibition of neuronal excitability in the EC inhibits the excitation of the hippocampus.

Discussion

Our results demonstrate that 5-HT inhibits the excitability of stellate and pyramidal neurons in the superficial layers of the EC by activating K_{2P} channels. 5-HT inhibited action potential firing frequency and induced membrane hyperpolarization via activation of $5-HT_{1A}$ receptors in these neurons. 5-HT-induced inhibition was irrelevant to the function of H-channels or other cation channels but sensitive to extracellular Ba^{2+}, bupivacaine, and quinidine. Our results excluded the involvement of TASK and TREK but suggested a role for TWIK-1 in 5-HT-induced increase in outward holding current. We further demonstrated that $G\alpha_{i3}$ subunits and PKA were required for the effect of 5-HT. 5-HT-mediated hyperpolarization of stellate neurons in the EC resulted in a reduction in the excitation of the hippocampus.

Ionic Mechanisms of 5-HT-Induced Hyperpolarization. Whereas it is generally recognized that 5-HT reduces neuronal excitability in the superficial layers of the EC via activation of K^+ channels (Grünschlag et al., 1997; Ma et al., 2006), the characteristics of the K^+ channels have not been determined. Our results demonstrate that 5-HT-induced increase in outward holding current was insensitive to the classic K^+ channel blockers (TEA, 4-AP, Cs^+) but sensitive to Ba^{2+} and bupivacaine. Although Ba^{2+} blocks the inward rectifier K^+ channels that are involved in controlling resting membrane potential, the inward rectifier K^+ channels are unlikely to be involved in 5-HT-mediated hyperpolarization in the EC because these channels are also sensitive to TEA and Cs^+. Whereas bupivacaine blocks Na^+ channels and

stellate neurons express a low-threshold persistent Na^+ conductance (Magistretti et al., 1999), it is unlikely that the persistent Na^+ channels are responsible for 5-HT-mediated inhibition in the EC because replacing extracellular Na^+ with NMDG failed to change 5-HT-induced outward holding current (Fig. 3C). The only realistic explanation for these results is the involvement of K_{2P} channels. The Ba^{2+}-sensitivity to different K_{2P} channels has been well characterized. TASK-1 (Han et al., 2002), TASK-3 (Kim et al., 2000; Han et al., 2002), TREK-1 (Fink et al., 1996), TREK-2 (Han et al., 2002), TWIK-1 (Lesage et al., 1996), and TRESK (Sano et al., 2003; Kang et al., 2004) are sensitive to Ba^{2+}. Bupivacaine also inhibits TASK, TREK-1, and TWIK-1 channels (Kindler et al., 1999). We therefore examined the roles of these K_{2P} channels in 5-HT-induced reduction of neuronal excitability. Because TRESK channels are not expressed in the brain and
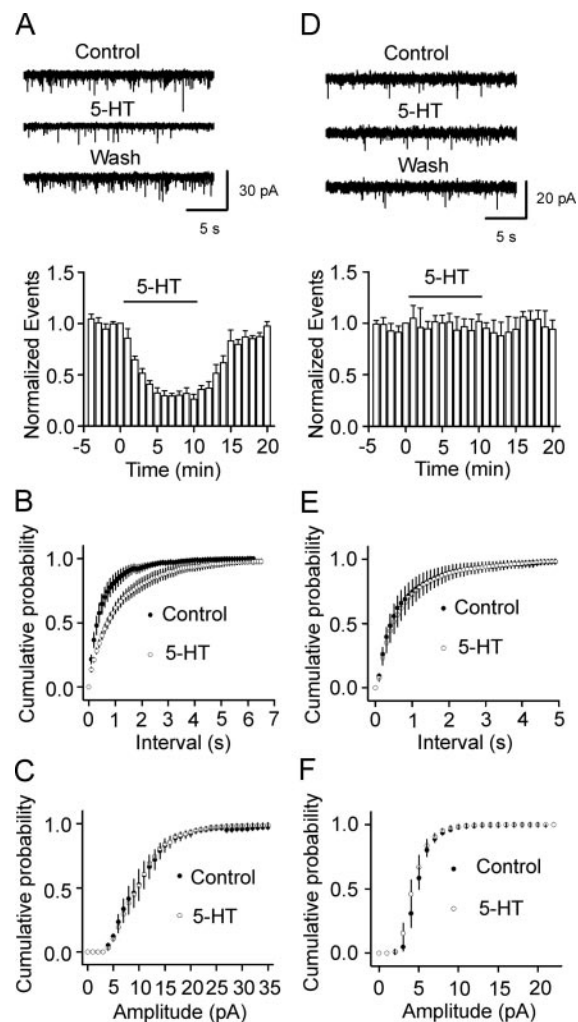

Fig. 8. Application of 5-HT (1 μM) inhibits spontaneous (A–C) with no effects on miniature EPSCs (D–F) recorded from dentate gyrus granule cells. A, bath application of 5-HT (1 μM) reversibly reduced the frequency of the AMPA receptor-mediated sEPSCs recorded in the absence TTX from dentate gyrus granule cells ($n = 6$). Top, sEPSCs recorded before, during, and after the application of 5-HT. B, cumulative probability before and during the application of 5-HT ($n = 6$). Note that 5-HT reduced the frequency of sEPSCs (increase in sEPSC interval). C, 5-HT did not change sEPSC amplitude ($n = 6$). D, bath application of 5-HT (1 μM) had no effects on the frequency of mEPSCs recorded in the presence of TTX (0.5 μM) from dentate gyrus granule cells ($n = 7$). Top, mEPSCs recorded before, during, and after the application of 5-HT. E and F, 5-HT had no effects on the cumulative probability of frequency (E) or amplitude (F).

the voltage-current relationship of TRESK channels is outwardly rectifying (Sano et al., 2003; Kang et al., 2004), which is different from the voltage-current relationship of 5-HT-induced net current (inwardly rectifying), they are not likely to be involved in 5-HT-induced hyperpolarization in stellate neurons. The following lines of evidence clearly excluded the involvement of TASK channels. First, TASK channels are highly acid-sensitive, whereas lowering the extracellular pH failed to block 5-HT-induced increase in outward holding current. Second, application of inhibitors to TASK-1 (anandamide) and/or TASK-3 (Zn^{2+}, ruthenium red) did not change the effect of 5-HT. Third, TASK channels are Goldman-Hodgkin-Katz–rectifier K^+ conductance (Lesage and Lazdunski, 2000), whereas 5-HT-induced currents are inwardly rectifying conductance. Finally, we coexpressed 5-HT$_{1A}$ receptors with TASK-1 or TASK-3 in HEK293 cells and application of 5-HT induced depolarization instead of hyperpolarization in the transfected cells, demonstrating that activation of 5-HT$_{1A}$ receptors inhibits TASK channels consistent with the effects of 5-HT in native neurons (Talley et al., 2000; Perrier et al., 2003). Together, our results indicate that 5-HT inhibits neuronal excitability in the superficial layers of the EC via activating K$_{2P}$ other than TASK channels.

Our results do not support a role for TREK channels in 5-HT-mediated hyperpolarization. First, the currents mediated by TREK were inhibited when the extracellular Na^+ was replaced by NMDG (Fink et al., 1996), whereas in our experiments, replacing the extracellular Na^+ by NMDG did not alter 5-HT-induced increase in outward holding current. Second, both TREK-1 and TREK-2 channels are dramatically activated by arachidonic acid (for review, see Kim, 2003). If TREK channels are expressed in stellate neurons, application of arachidonic acid should activate these channels to generate a hyperpolarization. However, application of arachidonic acid, instead, generated membrane depolarization. The depolarization produced by arachidonic acid may be due to the inhibition of TASK channels by arachidonic acid (Han et al., 2002). Third, the currents generated by TREK channels are outwardly rectifying (Fink et al., 1996; Lesage et al., 2000) in contrast to the net currents produced by 5-HT (inwardly rectifying; Fig. 4A). Together, these data suggest that TREK channels are unlikely to be the target activated by 5-HT in stellate neurons.

The following lines of evidence indicate that 5-HT inhibits neuronal excitability in the EC via activating TWIK-1 channels. First, TWIK-1 channels are sensitive to Ba^{2+}, bupivacaine, and quinidine (Lesage et al., 1996; Kindler et al., 1999), consistent with our results. Second, TWIK-1 channels are highly expressed in the superficial layers of the cortex, including the EC (Talley et al., 2001). Third, the voltage-current relationship of the net current generated by 5-HT was slightly inwardly rectifying (Fig. 4A), consistent with the voltage-current relationship of the TWIK-1 current (Lesage et al., 1996). Finally, application of 5-HT generated an outward holding current in HEK293 cells cotransfected with 5-HT$_{1A}$ receptors and TWIK-1 channels.

Signal Transduction Mechanisms. K$_{2P}$ channels are modulated by a variety of G protein-coupled receptors (Lesage et al., 2000; Talley et al., 2000; Chemin et al., 2003; Chen et al., 2006; Deng et al., 2006). The α subunits of the G proteins are responsible for the modulation of K$_{2P}$ channels (Lesage et al., 2000; Chen et al., 2006; Deng et al., 2006).

However, Gα subunits modulate K$_{2P}$ channels in distinct ways; stimulation of the G$_s$- or G$_q$-coupled receptors inhibits (Lesage et al., 2000; Chemin et al., 2003; Chen et al., 2006; Deng et al., 2006), whereas activation of Gα_i-coupled receptors (Lesage et al., 2000) activates K$_{2P}$ channels. Gα_q-mediated inhibition of K$_{2P}$ channels is generally believed to be mediated via direct G protein-coupling (Chen et al., 2006; Deng et al., 2006), whereas Gα_i-induced activation may require intracellular signals because Gα_i does not directly interact with K$_{2P}$ channels (Chen et al., 2006). Our results are consistent with this mode because we have shown that 5-HT-mediated increase in outward holding current was blocked by intracellular application of GDP-β-S. Because the Gα subunits activated by 5-HT$_{1A}$ include Gα_{i1}, Gα_{i2}, Gα_{i3}, Gα_o, and Gα_z (Pucadyil et al., 2005), we further identified the role of these subunits in 5-HT-mediated hyperpolarization. Our results indicate that the Gα_{i3} subunit is responsible for the effect of 5-HT in the EC. Consistent with our results, Gα_{i3} subunit has been found to have the highest affinity for 5-HT$_{1A}$ receptors (Pucadyil et al., 2005).

PKA is the major intracellular messenger downstream of Gα_i (Pucadyil et al., 2005). Our results indicate that PKA is necessary for 5-HT-induced increase in outward holding current. In fact, K$_{2P}$ channels, including TREK-1 (Fink et al., 1996; Murbartián et al., 2005) and TREK-2 (Lesage et al., 2000), are inhibited by PKA-mediated phosphorylation. Consistent with this scenario, our results indicate that 5-HT$_{1A}$-mediated activation of Gα_i leads to down-regulation of PKA activity and disinhibition of K$_{2P}$ channels mediated by PKA. The final result is the increase in K$_{2P}$ channel activity and hyperpolarization.

Physiological Significance. K$_{2P}$ channels are the major determinants of neuronal membrane potential, and their functional modulation provides a principal mode for neurotransmitters to regulate neuronal excitability. Serotonin modulates different K$_{2P}$ channels via distinct 5-HT receptors. 5-HT inhibits TASK channels via activation of 5-HT$_2$ receptors that are coupled to G$\alpha_{q/11}$ in rat hypoglossal motoneurons (Talley et al., 2000). Activation of 5-HT$_{1A}$ receptors inhibits TASK-1-like channels in turtle spinal motoneurons (Perrier et al., 2003). Consistent with this observation, we also demonstrate that activation of 5-HT$_{1A}$ receptors inhibits TASK channels in HEK293 cells cotransfected with 5-HT$_{1A}$ receptors and TASK-1 (Fig. 5C) or TASK-3 (Fig. 5D) channels. We further showed that application of 5-HT inhibited neuronal excitability by activating TWIK-1 channels in the EC. 5-HT-mediated inhibition of neuronal excitability in the EC is likely to have profound effects in the limbic structures. At the least, 5-HT-mediated inhibition of the neuronal excitability may contribute to its inhibitory effects on epileptiform activity (Schmitz et al., 1997; Gentsch et al., 2000) and memory (Meneses, 1999). In conclusion, our study provides a cellular and molecular mechanism that might explain, at least in part, the roles of serotonin in many physiological functions and neurological diseases.

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