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Serotonin Increases GABA Release in Rat Entorhinal Cortex by Inhibiting Interneuron TASK-3 K⁺ Channels

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Abstract

Whereas the entorhinal cortex (EC) receives profuse serotonergic innervations from the raphe nuclei in the brain stem and is critically involved in the generation of temporal lobe epilepsy, the function of serotonin (5-hydroxytryptamine, 5-HT) in the EC and particularly its roles in temporal lobe epilepsy are still elusive. Here we explored the cellular and molecular mechanisms underlying 5-HT-mediated facilitation of GABAergic transmission and depression of epileptic activity in the superficial layers of the EC. Application of 5-HT increased sIPSC frequency and amplitude recorded from the principal neurons in the EC with no effects on mIPSCs recorded in the presence of TTX. However, 5-HT reduced the amplitude of IPSCs evoked by extracellular field stimulation and in synaptically connected interneuron and pyramidal neuron pairs. Application of 5-HT generated membrane depolarization, increased action potential firing frequency but reduced the amplitude of action potentials in presynaptic interneurons suggesting that 5-HT still increases GABA release whereas the depressant effects of 5-HT on evoked IPSCs could be explained by 5-HT-induced reduction in action potential amplitude. The depolarizing effect of 5-HT was mediated by inhibition of TASK-3 K⁺ channels in interneurons and required the functions of 5-HT_{2A} receptors and G_{αq/11} but was independent of phospholipase C activity. Application of 5-HT inhibited low-Mg²⁺-induced seizure activity in slices via 5-HT_{1A} and 5-HT_{2A} receptors suggesting that 5-HT-mediated depression of neuronal excitability and increase in GABA release contribute to its antiepileptic effects in the EC.

Keywords

synapse; transmission; inhibition; channel; G protein; receptor

Introduction

The entorhinal cortex (EC) provides the majority of connections between the hippocampus and other cortical areas (Witter et al., 1989; Witter et al., 2000). Principal (stellate and pyramidal) neurons in the superficial layers (layer II/III) of the EC receive excitatory glutamatergic afferents from olfactory structures, parasubiculum, perirhinal cortex, claustrum, amygdale and neurons in the deep layers of the EC (layers IV–VI) (Burwell, 2000; Witter et al., 1989) as well as inhibitory GABAergic inputs from local interneurons (de Curtis and Pare, 2004; Woodhall

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et al., 2005). The axons of stellate neurons in layer II of the EC form the major component of perforant path that innervates the dentate gyrus and CA3 (Steward and Scoville, 1976) whereas pyramidal neurons in layer III provide the primary inputs to CA1 regions (Steward and Scoville, 1976; Witter et al., 2000). The output from the hippocampus is then projected to the deep layers of the EC that relay information back to the superficial layers (Dolorfo and Amaral, 1998a, b; Kohler, 1986; van Haeften et al., 2003) and to other cortical areas (Witter et al., 1989). The EC is an essential structure in the limbic system that is closely related to emotional control (Majak and Pitkanen, 2003), consolidation and recall of memories (Dolcos et al., 2005; Steffenach et al., 2005), Alzheimer's disease (Hyman et al., 1984; Kotzbauer et al., 2001), schizophrenia (Joyal et al., 2002; Prasad et al., 2004) and temporal lobe epilepsy (Spencer and Spencer, 1994).

The raphe nuclei in the brain stem send strong serotonergic projections to the EC (Bobillier et al., 1975) and the EC expresses high densities of serotonin (5-hydroxytryptamine, 5-HT) receptors (Pazos and Palacios, 1985). To date, the EC has been found to express 5-HT₁ (Pazos and Palacios, 1985; Sim et al., 1997; Wright et al., 1995), 5-HT₂ (Pompeiano et al., 1994; Wright et al., 1995) and 5-HT₃ (Kilpatrick et al., 1987) receptors although the neuronal types that express these receptors have not been defined. Furthermore, application of 5-HT in the EC inhibits excitatory transmission (Schmitz et al., 1998) and reduces neuronal excitability via activation of K⁺ channels (Grünschlag et al., 1997; Ma et al., 2007) belonging to the two-pore domain family (Deng et al., 2007). However, the effects of 5-HT on GABAergic transmission in the EC remain unexplored. Here, we examined the role of 5-HT on GABAergic transmission onto the principal neurons in layer II/III of the EC. Our results demonstrate that 5-HT facilitates GABA release via activation of 5-HT_{2A} receptors on GABAergic interneurons. Activation of 5-HT_{2A} receptors increases the excitability of interneurons via inhibition of TASK-3, a two-pore domain K⁺ channel and 5-HT-mediated inhibition in the EC contributes to its antiepileptic effects.

Results

5-HT increases the frequency and amplitude of sIPSCs

We initially recorded sIPSCs from the principal (stellate and pyramidal) neurons in layer II/III of the EC and tested the roles of 5-HT on GABAergic transmission. We identified stellate and pyramidal neurons by their morphology and location (Deng and Lei, 2007; Deng et al., 2007; Lei et al., 2007). Stellate neurons are usually located in layer II or the border of layer II and III and they have larger and polygonal soma with variable numbers of main dendrites radiating out from the cell body, but are devoid of a clearly dominant dendrite. Pyramidal neurons have a pyramidal or elongated soma with dendrites orientated 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 towards the deeper layers. Application of 5-HT (100 μ M) significantly increased the frequency ($244 \pm 37\%$ of control, $p=0.004$, Fig. 1A₁, A₂, A₃) and amplitude ($135 \pm 9\%$ of control, $p=0.004$, Fig. 1A₄) of sIPSCs in 10 out of 10 stellate neurons examined. Similarly, application of 5-HT (100 μ M) significantly increased the frequency ($208 \pm 23\%$ of control, $p=0.001$, Fig. 1B₁, B₂, B₃) and amplitude ($134 \pm 8\%$ of control, $p=0.002$, Fig. 1B₄) of sIPSCs in 10 out of 10 pyramidal neurons examined. Because there were indistinguishable differences for 5-HT-mediated increases in sIPSC frequency ($p=0.42$) and amplitude ($p=0.94$) between stellate and pyramidal neurons (Fig. 1C) suggesting that 5-HT-induced up-regulation of sIPSCs is not cell-specific, we performed the rest of the experiments in both stellate and pyramidal neurons. The EC₅₀ value for 5-HT was measured to be 2.4 μ M (Fig. 1D).

Involvement of 5-HT₂ receptors

The EC has been reported to express 5-HT₁ (Pazos and Palacios, 1985; Sim et al., 1997; Wright et al., 1995), 5-HT₂ (Pompeiano et al., 1994; Wright et al., 1995) and 5-HT₃ (Kilpatrick et al., 1987) receptors although the identities of the neurons expressing these receptors have not been determined. We have previously shown that 5-HT generates neuronal hyperpolarization in the EC via activation of 5-HT_{1A} receptors although the EC₅₀ for 5-HT-induced hyperpolarization (0.48 μ M, Deng et al., 2007) is lower than that of 5-HT-mediated increases in sIPSCs (2.4 μ M). Pretreatment of slices with and bath application of S(-)-UH-301 (10 μ M), a specific antagonist for 5-HT_{1A} receptors, failed to change 5-HT-mediated increases in sIPSC frequency (241 $\pm$ 31% of control, n=6, p=0.006, Fig. 2A) and amplitude (141 $\pm$ 10% of control, n=6, p<0.001). Furthermore, application of 8-hydroxy-DPAT·HBr (8-OH-DPAT, 5 μ M), a specific agonist for 5-HT_{1A} receptors did not significantly change sIPSC frequency (93 $\pm$ 13% of control, n=5, p=0.59) or amplitude (94 $\pm$ 4% of control, n=5, p=0.25). These results suggest that 5-HT_{1A} receptors are not involved in 5-HT-mediated up-regulation of sIPSCs in the EC.

We then tested a potential role of 5-HT₃ receptors because 5-HT₃ receptors are Ca²⁺-permeable cation channels and activation of 5-HT₃ receptors could theoretically increase GABA release resulting in increases in sIPSC frequency and amplitude. However, application of tropisetron (10 μ M), a selective 5-HT₃ receptor inhibitor, failed to block 5-HT-induced increases in sIPSC frequency (255 $\pm$ 8% of control, n=6, p<0.001, Fig. 2B) and amplitude (141 $\pm$ 13% of control, n=6, p=0.03). Furthermore, application of *m*-chlorophenylbiguanide (100 μ M), a selective 5-HT₃ receptor agonist, did not change sIPSC frequency (101 $\pm$ 4% of control, n=6, p=0.77) and amplitude (102 $\pm$ 6% of control, n=6, p=0.72). Because our results below showed that 5-HT increased GABA release by facilitating the excitability of GABAergic interneurons and interneurons expressing 5-HT₃ receptors frequently express cannabinoid CB1 receptors (Morales et al., 2004), we tested a possibility that endocannabinoids potentially released from the recorded cells due to the depolarization produced by the positive holding potential (+30 mV) could have interacted with the CB1 receptors on the interneurons and overwhelmed the effects of 5-HT₃ receptor activation on sIPSCs. We employed two approaches to test this possibility. First, application of AM251 (10 μ M), a CB1 receptor antagonist failed to block 5-HT-induced increases in sIPSC frequency (221 $\pm$ 26% of control, n=6, p=0.01) and amplitude (137 $\pm$ 13% of control, n=6, p=0.02, data not shown). Second, we recorded sIPSCs at -60 mV with intracellular solution containing CsCl. In this condition, application of tropisetron (10 μ M) still failed to block 5-HT-induced increases in sIPSC frequency (323 $\pm$ 63% of control, n=7, p=0.006) and amplitude (125 $\pm$ 8% of control, n=7, p=0.01, data not shown). Together, these results indicate that the effects of 5-HT are not mediated by 5-HT₃ receptors.

We next tested the roles of 5-HT₂ receptors. Pretreatment of slices with and bath application of kentanaserin (10 μ M), an antagonist for 5-HT_{2A} and 5-HT_{2C} receptors, completely blocked 5-HT-induced increases in sIPSC frequency (103 $\pm$ 9% of control, n=6, p=0.73, Fig. 2C) and amplitude (111 $\pm$ 13% of control, n=6, p=0.42) whereas application of SB204741 (50 μ M), a potent and selective 5-HT_{2B} receptor antagonist, failed to alter 5-HT-induced increases in sIPSC frequency (175 $\pm$ 15% of control, n=5, p=0.008, Fig. 2D) and amplitude (130 $\pm$ 5% of control, n=5, p=0.003) excluding the involvement of 5-HT_{2B} receptors. We then differentiated whether the effects of 5-HT were mediated via 5-HT_{2A} and/or 5-HT_{2C} receptors. Pretreatment of slices with and bath application of R-96544 (10 μ M), a 5-HT_{2A} receptor antagonist, completely blocked 5-HT-induced increases in sIPSC frequency (95 $\pm$ 7% of control, n=8, p=0.54, Fig. 2E) and amplitude (96 $\pm$ 5% of control, n=8, p=0.46) whereas application of RS 102221 (25 μ M), an inhibitor of 5-HT_{2C}, did not significantly change 5-HT-induced increases in sIPSC frequency (217 $\pm$ 18% of control, n=6, p=0.01, Fig. 2F) and amplitude (138 $\pm$ 12% of control, n=6, p=0.03) suggesting that the effects of 5-HT on sIPSCs are mediated via activation of 5-HT_{2A} receptors.

5-HT does not modulate mIPSCs but inhibits the amplitudes of evoked IPSCs

We next examined the effects of 5-HT on mIPSCs recorded in the presence of TTX (1 μ M). Application of 5-HT (100 μ M) failed to change either the frequency ($97\pm 4\%$ of control, $n=5$, $p=0.65$, Fig. 3A, 3B and 3C) or the amplitude ($99\pm 1\%$ of control, $n=5$, $p=0.95$, Fig. 3D) of mIPSCs suggesting that 5-HT facilitates GABAergic transmission by increasing GABA release with no effects on postsynaptic GABA_A receptors. Because sIPSCs are action potential-dependent whereas mIPSCs are not, these results also suggest that 5-HT-mediated increases in GABA release are action potential-dependent.

We then studied the effects of 5-HT on the evoked IPSCs (eIPSCs). We initially recorded eIPSCs by placing a stimulation electrode in layer III (~ 100 μ m from the recorded neuron) to stimulate GABAergic inputs. Application of 5-HT (100 μ M) did not increase but reduced the amplitude of eIPSCs ($61\pm 8\%$ of control, $n=4$, $p=0.02$, Fig. 3E). This phenomenon (increases in sIPSCs but reduction in eIPSCs) has also been observed for norepinephrine in the hippocampus (Madison and Nicoll, 1988) and EC (Lei et al., 2007) and nicotine in the striatum (Liu et al., 2007).

One explanation for the opposite effects of 5-HT on sIPSCs and eIPSCs is that 5-HT-induced depolarization of presynaptic interneurons (see below) could have inactivated Na⁺ channels and decreased the amplitude of action potentials resulting in smaller eIPSCs. To test this possibility, we used paired recordings between a synaptically connected interneuron and a pyramidal neuron in layer III of the EC to monitor simultaneously presynaptic action potentials and postsynaptic IPSCs. Pre- and postsynaptic neurons were filled with biocytin during recording and labeled with fluorescein-conjugated streptavidin (Fig. 3F). The electrode sealed to presynaptic interneuron contained K⁺-gluconate whereas that sealed to postsynaptic pyramidal neuron contained CsCl. Postsynaptic pyramidal neurons were held at -60 mV. Interneurons were identified by the criteria described below. Under these conditions, action potentials generated by brief depolarization of presynaptic interneuron generated IPSCs that were sensitive to bicuculline (10 μ M, data not shown) indicating that the recorded IPSCs were mediated by GABA_A receptors. Application of 5-HT (100 μ M) significantly reduced the amplitudes of IPSCs ($44.8\pm 10.0\%$ of control, $n=6$, $p=0.003$, Fig. 3G, 3H). We also observed a significant reduction of the amplitude of action potentials simultaneously recorded from the presynaptic interneurons (control: 84.4 ± 8.0 mV, 5-HT: 74.6 ± 9.3 mV, $n=6$, $p=0.03$, Fig. 3G). Together, these results suggest that a reduction of presynaptic action potential amplitudes at least contributes to 5-HT-mediated depression of eIPSCs in the EC.

5-HT increases the excitability of GABAergic interneurons in the EC

We then tested the effects of 5-HT on the excitability of interneurons by recording from layer III interneurons holding currents at -55 mV, a potential close to the resting membrane potentials. Interneurons were initially differentiated from pyramidal neurons in layer III by their smaller size (Fig. 4A₁, B₁) and fast firing spikes and pronounced afterhyperpolarization (Kumar and Buckmaster, 2006; Lei et al., 2007; Fig. 4A₂, B₂). There are two major types of interneurons in layer III of the EC that can be classified by their distinct morphologies and electrophysiological properties (Kumar and Buckmaster, 2006). Type I interneurons showed little voltage sag in response to hyperpolarizing current injection and no rebound burst firing (Fig. 4A₂) whereas type II interneurons displayed prominent voltage sag in response to hyperpolarizing current injection and rebound burst firing (Fig. 4B₂). Type I interneurons had dendrites that extend into layer II or layer I (Fig. 4A₃) whereas type II interneurons had dendrites that ramify locally (Fig. 4B₃). However, application of 5-HT (100 μ M) induced an inward holding current in both type I (-16.3 ± 2.4 pA, $n=10$, $p<0.001$, Fig. 4A₄) and type II (-17.3 ± 1.4 pA, $n=8$, $p<0.001$, Fig. 4B₄) interneurons indicating that 5-HT depolarizes both types of GABAergic interneurons in the EC. Because there was no distinguished difference

for the effects of 5-HT between these two types of interneurons ($p=0.73$), we performed the rest experiments on both types of interneurons. The pooled holding currents from these two types of interneurons were -16.7 ± 1.4 pA ($n=18$, $p<0.001$).

Application of 5-HT (100 μ M) significantly increased the frequency of action potentials recorded from layer III interneurons ($461 \pm 107\%$ of control, $n=8$, $p=0.012$, Fig. 5A, 5B) suggesting that 5-HT increases the excitability of GABAergic interneurons. This effect was mediated by 5-HT_{2A} receptors because pretreatment of slices with and co-application of R-96544 (10 μ M) blocked 5-HT-induced increases in action potential firing ($105 \pm 5\%$ of control, $n=5$, $p=0.33$, Fig. 5C). For some of the recorded interneurons, we examined the immunoreactivity of GABA and they indeed expressed GABA (Fig. 5D). Collectively, these results indicate that 5-HT increases GABA release by facilitating the excitability of GABAergic interneurons in the EC.

5-HT induces membrane depolarization by inhibiting two-pore domain K⁺ channels in interneurons

Whereas our results demonstrated that 5-HT did not increase GABA release by interaction with 5-HT₃ receptors which are cation channels, we still tested the possibility that activation of 5-HT_{2A} receptors opens other cation channels to generate membrane depolarization. If this is the case, the extracellular Na⁺ and Ca²⁺ should be the major cations to mediate membrane depolarization. We initially replaced the extracellular Na⁺ with the same concentration of NMDG. Under these conditions, application of 5-HT (100 μ M) still induced a comparable inward holding current (control: -16.7 ± 1.4 pA, $n=18$; NMDG: -17.8 ± 4.2 pA, $n=9$, $p=0.76$, Student's unpaired *t* test, Fig. 6A). Furthermore, omission of extracellular Ca²⁺ failed to change 5-HT-induced increases in inward holding currents (control: -16.7 ± 1.4 pA, $n=18$; 0 Ca²⁺: -17.4 ± 2.8 pA, $n=8$, $p=0.81$, Student's unpaired *t* test, Fig. 6B). Together, these results demonstrate that it is unlikely that 5-HT increases the excitability of interneurons by activating a cationic conductance.

We then tested the hypothesis that 5-HT inhibits background K⁺ channels to generate membrane depolarization. If this is the case, the 5-HT-induced currents should have a reversal potential close to the K⁺ reversal potential. We used a ramp protocol (from -130 mV to -60 mV, at a speed of 0.1 mV/ms) to construct the voltage-current curve before and during the application of 5-HT (100 μ M). The intracellular solution contained 100 mM K⁺-gluconate and the extracellular solution was supplemented with (in μ M) 1 TTX, 100 Cd²⁺, 10 DNQX, 50 dl-APV and 10 bicuculline to block synaptic currents and other voltage-gated ion channels. Under these conditions, 5-HT induced a current which had a reversal potential (-82.8 ± 1.9 mV, $n=5$) close to the calculated K⁺ reversal potential (-85.4 mV) when the extracellular K⁺ concentration was 3.5 mM (Fig. 6C) suggesting that 5-HT induces membrane depolarization by inhibiting a resting membrane K⁺ conductance.

We next characterized the properties of the involved K⁺ channels. 5-HT-induced increases in inward holding currents recorded from interneurons in the EC were not blocked by application of TEA (10 mM, -18.2 ± 1.8 pA, $n=9$, $p<0.001$, Fig. 6D), Cs⁺ (3 mM, -16.3 ± 1.9 pA, $n=8$, $p<0.001$, Fig. 6D), 4-aminopyridine (4-AP, 2 mM, -15.9 ± 1.4 pA, $n=9$, $p<0.001$, Fig. 6D) suggesting that 5-HT-mediated inhibition of K⁺ channels is insensitive to the classic K⁺ channel blockers. Moreover, application of tertiapin (50 nM), an inward rectifier K⁺ channel inhibitor, failed to block 5-HT-induced increases in inward holding currents (-18.1 ± 2.6 pA, $n=8$, $p<0.001$, Fig. 6D) suggesting that 5-HT-induced membrane depolarization is unlikely to be mediated by inward rectifier K⁺ channels although this type of K⁺ channels is involved in modulation of resting membrane potentials.

Because 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 depolarization in GABAergic interneurons. The family of K_{2p} channels includes TWIK, THIK, TREK, TASK, TALK and TRESK (Bayliss et al., 2003; Lesage, 2003), some of which are sensitive to Ba²⁺ and bupivacaine. We therefore tested the role of Ba²⁺ and bupivacaine in 5-HT-induced membrane depolarization. Application of Ba²⁺ (3 mM) alone induced an inward holding current (-27.1 ± 8.8 pA, $n=6$, $p=0.02$, Fig. 6E) suggesting that Ba²⁺-sensitive K⁺ channels have a substantial role in controlling resting membrane potential. In the presence of Ba²⁺, 5-HT-induced increases in inward holding currents were significantly reduced (control: -16.7 ± 1.4 pA, $n=18$, Ba²⁺: -4.2 ± 0.7 pA, $n=6$, $p<0.001$, Fig. 6E) suggesting that 5-HT increases GABA release in the EC by inhibiting a type of K_{2p} channels that are sensitive to Ba²⁺. Similarly, application of bupivacaine (400 μ M) *per se* induced an inward holding current (-21.4 ± 7.6 pA, $n=5$, $p=0.04$, Fig. 6F) and subsequent application of 5-HT (100 μ M) induced a significantly smaller inward holding current (control: -16.7 ± 1.4 pA, $n=18$, bupivacaine: -3.9 ± 1.6 pA, $n=5$, $p<0.001$, Fig. 6F) suggesting that the K_{2p} channels underlying 5-HT-induced increases in GABA release are sensitive to bupivacaine as well.

Among the K_{2p} channels, TASK-1 (Han et al., 2002), TASK-3 (Han et al., 2002; Kim et al., 2000), TREK-1 (Fink et al., 1996), TREK-2 (Han et al., 2002), TWIK-1 (Lesage et al., 1996) and TRESK (Kang et al., 2004; Sano et al., 2003) are sensitive to Ba²⁺. Bupivacaine also inhibits TASK, TREK-1, TWIK-1 and the K_{2p} channels isolated from yeast (*TOK1*) or *Drosophila* (*ORK1*) (Kindler et al., 1999). We then tested the roles of these K_{2p} channels in 5-HT-induced increases in inward holding currents in interneurons by including in the recording pipettes the specific antibodies against each type of these K_{2p} channels. We took the advantage of the fact that these antibodies were raised against the epitopes of the intracellular C- or N-terminal domain and they should bind to the intracellular domain of the K_{2p} channels and interfere with the functions of the K_{2p} channels. To ensure a complete dialysis of the antibodies into the cells, we waited for ~50 min after the formation of whole-cell recording configuration. Under these conditions, 5-HT-induced increases in inward holding currents were not significantly changed by application of control Ig-G (-15.9 ± 4.7 pA, $n=5$, $p=0.81$ vs. control, Fig. 7A–F) and antibodies to TWIK-1 (-13.6 ± 1.9 pA, $n=6$, $p=0.64$ vs. Ig-G, Fig. 7A), TREK-1 (-16.1 ± 2.9 pA, $n=6$, $p=0.97$ vs. Ig-G, Fig. 7B), TREK-2 (-18.4 ± 2.8 pA, $n=6$, $p=0.64$ vs. Ig-G, Fig. 7C), TRESK (-16.6 ± 1.8 pA, $n=7$, $p=0.87$ vs. Ig-G, Fig. 7D) and TASK-1 (-21.5 ± 4.6 pA, $n=5$, $p=0.43$ vs. Ig-G, Fig. 7E) whereas application of antibody to TASK-3 significantly reduced 5-HT-induced increases in inward holding currents (-1.7 ± 3.1 pA, $n=5$, $p=0.03$ vs. Ig-G, Fig. 7F) suggesting that 5-HT facilitates GABA release by inhibiting TASK-3 channels in GABAergic interneurons. Because TASK channels are sensitive to acid, anandamide (TASK-1), ruthenium red (TASK-3) and zinc (TASK-3, Bayliss et al., 2003), we next examined the effects of these inhibitors on 5-HT-induced increases in inward holding currents. Lowering the extracellular pH from 7.4 to 6.4 induced an inward holding current (-12.7 ± 3.9 pA, $n=8$, $p=0.03$, Fig. 7G) and significantly reduced 5-HT-induced increases in inward holding currents (-3.9 ± 1.7 pA, $n=8$, $p<0.001$ vs. control, Fig. 7G) suggesting that the K_{2p} channels inhibited by 5-HT are acid-sensitive. Application of methanandamide (MA, 10 μ M), a nonhydrolysable anandamide analog, did not significantly alter 5-HT-induced increases in inward holding currents ($n=6$, $p=0.71$, Fig. 7H) whereas the effects of 5-HT were significantly attenuated in the presence of ruthenium red (RR, 10 μ M, $n=6$, $p<0.01$, Fig. 7H) and Zn²⁺ (100 μ M, $n=6$, $p<0.01$, Fig. 7H). Together, these results indicate that 5-HT facilitates GABA release in the EC via inhibition of TASK-3 channels in GABAergic interneurons. Consistent with the electrophysiological data, TASK-3 channels were expressed on GABAergic interneurons in the EC (Fig. 7I).

Signal transduction mechanism

Our results demonstrate that 5-HT increases GABA release by activating 5-HT_{2A} receptors on GABAergic interneurons. Activation of 5-HT_{2A} receptors is coupled to Gα_{q/11} resulting in activation of phospholipase C (PLC). We initially tested whether G-proteins were involved in 5-HT-induced increases in GABA release. We replaced GTP in the intracellular solution with GDP-β-S (2 mM), a G-protein inactivator, and recorded the holding currents at −55 mV from interneurons in layer III. Application of 5-HT (100 μM) in the presence of GDP-β-S failed to change the holding currents significantly (−0.8±2.6 pA, n=5, p=0.78, Fig. 8A). The involvement of G-proteins was further tested by applying another G-protein inhibitor, suramin. Slices were pretreated with suramin (100 μM) and the same concentration of suramin was bath-applied. Under these conditions, application of 5-HT (100 μM) did not significantly increase the inward holding currents (−1.3±2.5 pA, n=5, p=0.64, Fig. 8B). These results demonstrate that G-proteins are required for 5-HT-induced increases in GABA release. We then tested the roles of Gα and Gβγ by applying antibodies to Gα_{q/11} and Gβ via the recording pipettes. In the presence of anti-Gα_{q/11} (20 μg/ml), application of 5-HT (100 μM) did not significantly alter the holding currents (−3.4±1.9 pA, n=6, p=0.15, Fig. 8C) whereas application of 5-HT in the presence of anti-Gβ (20 μg/ml) still induced a comparable inward holding current (−26.1±6.3 pA, n=5, p=0.014, Fig. 8D). These results suggest that 5-HT-mediated membrane depolarization is mediated via Gα_{q/11}.

Because activation of 5-HT_{2A} receptors increases PLC activity, we next tested whether PLC was required for 5-HT-induced membrane depolarization. Pretreatment of slices with and bath application of a PLC inhibitor, U73122 (20 μM), failed to block 5-HT-induced increases in inward holding currents (−17.7±1.7 pA, n=6, p<0.001, Fig. 8E). Similar treatment of slices with edelfosine (20 μM), another PLC inhibitor, did not alter 5-HT-induced increases in holding currents (−23.0±2.9 pA, n=6, p<0.001, Fig. 8F). To ensure that U73122 and edelfosine were effective in inhibiting PLC activity, we performed a positive control experiment. Because brain-derived neurotrophic factor (BDNF) has been reported to inhibit GABAergic transmission in CA1 region of the hippocampus via activation of Trk-B and PLC-γ (Tanaka et al., 1997), we recorded eIPSCs from CA1 pyramidal neurons by placing a stimulation electrode in the stratum pyramidale. Bath application of BDNF (100 ng/ml) significantly inhibited the amplitude of eIPSCs (56±9% of control, n=6, p=0.005). However, application of the same concentration of BDNF failed to significantly inhibit the amplitude of eIPSCs in slices pretreated with U73122 (20 μM, 93±5% of control, n=6, p=0.21) or edelfosine (20 μM, 90±5% of control, n=6, p=0.12). These results indicate that the activity of PLC is not required for 5-HT-induced increases in GABA release in the EC.

5-HT depresses low-Mg²⁺-induced seizure activity in vitro via activation of both 5-HT_{1A} and 5-HT_{2A} receptors

We then probed the function of 5-HT-induced increases in GABA release in the EC. The EC is one of the principal structures involved in temporal lobe epilepsy (Spencer and Spencer, 1994) and application of 5-HT inhibited low-Mg²⁺-induced seizure activity in slices (Schmitz et al., 1997) although the underlying mechanisms are unknown. Our results demonstrated that, in the EC, 5-HT inhibits the excitability of principal neurons via activation of 5-HT_{1A} receptors (Deng et al., 2007) but increased GABA release via activation of 5-HT_{2A} receptors. Both effects can potentially depress epilepsy. We thus probed the roles of 5-HT_{1A} and 5-HT_{2A} receptors in 5-HT-induced depression of seizure activity in low-Mg²⁺-induced seizure model. Application of 5-HT (100 μM) significantly inhibited low-Mg²⁺-induced seizure activity to 11.5±5.7% of control (control: 5.3±1.3/min, 5-HT: 0.7±0.5/min, n=8 slices, p=0.003, Fig. 9A). 5-HT-induced depression of seizure activity was reversible after washing in 5-HT-free solution for ~25 min (85.8±16.8% of control, n=8 slices, p=0.24, Fig. 9A). These results suggest that 5-HT depresses low-Mg²⁺-induced seizure activity, consistent with previous results (Schmitz

et al., 1997). Application of S(-)-UH-301 (2 μ M) did not significantly change low-Mg²⁺-induced seizure activity (95 $\pm$ 8% of control, n=6 slices, p=0.55, Fig. 9B) but significantly reduced 5-HT-mediated inhibition of seizure activity (55 $\pm$ 5% of control, n=6 slices, p=0.004, Fig. 9B) suggesting that the activity of 5-HT_{1A} partially contributes to 5-HT-mediated depression of seizure activity. Similarly, application of R-96544 (10 μ M), a 5-HT_{2A} receptor antagonist, did not significantly alter low-Mg²⁺-induced seizure activity (109 $\pm$ 18% of control, n=8 slices, p=0.62, Fig. 9C) but significantly reduced 5-HT-mediated inhibition of seizure activity (74 $\pm$ 10% of control, n=8 slices, p=0.04, Fig. 9C) suggesting that the activity of 5-HT_{2A} receptors partially contributes to 5-HT-mediated depression of seizure activity as well. Moreover, co-application of S(-)-UH-301 and R-96544 failed to change significantly the seizure activity (103 $\pm$ 8% of control, n=7 slices, p=0.88, Fig. 9D) but completely blocked 5-HT-induced depression of seizure activity (92 $\pm$ 3% of control, n=7 slices, p=0.06, Fig. 9D). Together, these results demonstrate that 5-HT-induced depression of epilepsy is mediated via activation of both 5-HT_{1A} and 5-HT_{2A} receptors.

Discussion

Our results demonstrate for the first time that 5-HT increases GABA release and inhibits seizure activity in low-Mg²⁺-induced seizure model in the EC. 5-HT facilitates GABA release by inhibiting TASK-3, a type of K_{2P} channels, resulting in membrane depolarization and increases in action potential firing of GABAergic interneurons in the EC. 5-HT-induced increases in GABA release require the functions of 5-HT_{2A} receptors and G $\alpha_{q/11}$, but are independent of PLC suggesting a direct coupling of G proteins and TASK-3 channels. Our results also demonstrate that both 5-HT_{1A} and 5-HT_{2A} receptors are involved in 5-HT-induced depression of seizures suggesting that 5-HT-induced inhibition of neuronal excitability and enhancement of GABA release in the EC contribute to its antiepileptic effects.

Our results demonstrate that 5-HT-mediated increases in GABA release are action potential-dependent because application of 5-HT increased sIPSC frequency and amplitude with no effects on mIPSC frequency and amplitude. However, application of 5-HT depressed the amplitudes of IPSCs evoked by extracellular field stimulation and in pairs of synaptically connected interneurons and pyramidal neurons. Because we have observed a reduction in action potential amplitude recorded in presynaptic interneurons in response to 5-HT application, one explanation is that 5-HT-mediated depolarization in GABAergic interneurons could induce inactivation of Na⁺ channels resulting in a reduction in action potential amplitude when presynaptic terminals were stimulated. Reduction in action potential amplitude is likely to generate smaller amplitudes of eIPSCs. Alternatively, 5-HT-induced robust increases in sIPSCs could deplete the readily releasable pool of GABA. If sIPSCs and eIPSCs share the same pool of GABA, increases in sIPSCs could lead to an occlusion of eIPSCs induced by exogenous stimulation. Analogously, we (Lei et al., 2007) and others (Bennett et al., 1998; Braga et al., 2004; Hirono and Obata, 2006; Madison and Nicoll, 1988) have found that norepinephrine increases sIPSCs but has heterogeneous effects on eIPSCs and activation of nicotinic acetylcholine receptors increases mIPSC frequency but attenuates eIPSC amplitude in striatal medium spiny projection neurons (Liu et al., 2007). Because sIPSCs represent a more natural process of transmission than eIPSCs, these results suggest that the authentic effect of 5-HT is to increase GABA release *in vivo*.

Our results demonstrate that 5-HT facilitates GABA release by generating membrane depolarization resulting in an increase in the excitability of GABAergic interneurons in the EC. 5-HT-induced depolarization is not mediated by opening of a cationic conductance because replacement of extracellular Na⁺ with NMDG or deprivation of extracellular Ca²⁺ failed to alter 5-HT-induced increases in inward holding currents. The result that 5-HT-induced net current in interneurons had a reversal potential close to the K⁺ reversal potential suggests that

the effects of 5-HT are mediated by inhibition of background K^+ channels. However, 5-HT-induced depolarization was insensitive to the classic K^+ channel blockers (TEA, Cs^+ , 4-AP) but sensitive to Ba^{2+} and bupivacaine suggesting the involvement of K_{2P} channels. We took the advantage of the fact that the sensitivities of K_{2P} channels to Ba^{2+} have been well-documented to further identify the subtype(s) of the K_{2P} channels involved in 5-HT-induced facilitation of GABA release. Among the K_{2P} channels, TASK-1 (Han et al., 2002), TASK-3 (Han et al., 2002; Kim et al., 2000), TREK-1 (Fink et al., 1996), TREK-2 (Han et al., 2002), TWIK-1 (Lesage et al., 1996) and TRESK (Kang et al., 2004; Sano et al., 2003) are sensitive to Ba^{2+} . We further identified the roles of these K_{2P} channels in 5-HT-induced depolarization in entorhinal interneurons by intracellular infusion of their specific antibodies via the recording pipettes because these antibodies specifically interact with the intracellular N- or C-terminals of the K_{2P} channels. The result that application of the antibody to TASK-3 blocked 5-HT-induced depolarization in interneurons suggests that 5-HT increases GABA release via inhibition of TASK-3 channels. Consistent with our results, TASK-3 channels are expressed in interneurons (Berg and Bayliss, 2007; Torborg et al., 2006) and inhibited by Gq-coupled receptors (Chen et al., 2006; Veale et al., 2007; Mathie, 2007) including 5-HT_{2A} receptors (Albert et al., 1996; Browning and Travagli, 1999; Hopwood and Trapp, 2005).

We have shown that 5-HT-mediated facilitation of GABA release in the EC is mediated via activation of 5-HT_{2A} receptors, consistent with the effects of 5-HT on GABA release in the prefrontal cortex (Abi-Saab et al., 1999) and hippocampus (Shen and Andrade, 1998). 5-HT_{2A} receptors are coupled to $G_{q/11}$ resulting in activation of PLC. We have shown that 5-HT-induced membrane depolarization in interneurons is dependent on $G_{q/11}$ but independent of PLC suggesting that $G_{q/11}$ directly interacts with TASK-3 channels to facilitate GABA release. Consistent with our results, $G_{q/11}$ is essential but PLC is not necessary for the inhibition of TASK-3 channels mediated by other G-protein-coupled receptors (Chen et al., 2006; Veale et al., 2007).

The importance of the EC in temporal lobe epilepsy has been highlighted by recent clinical data. Sclerotic lesions observed in temporal lobe epilepsy occur in both the hippocampus (Babb, 1991; Meenchke and Veith, 1991; Swanson, 1995) and the EC (Gloor, 1991; Levesque et al., 1991; Du et al., 1993). Field potentials recorded in the hippocampus generated by stimulation of the EC resemble spontaneous interictal discharge (Rutecki et al., 1989), and limbic seizures recorded with depth or subdural strip electrodes demonstrate focal seizure generation in the EC (Spencer and Spencer, 1994). Moreover, surgical removal of the EC has been used to treat intractable temporal lobe seizures (Goldring et al., 1992, 1993; Fried, 1993; Feindel et al., 1996). Therefore, functional changes in the EC induced by neurotransmitters likely form an important approach to control the status of epilepsy.

5-HT exerts powerful inhibition in the EC via at least three avenues. First, as we have shown previously that 5-HT decreases the excitability of principal neurons via activation of 5-HT_{1A} receptors (Deng et al., 2007). Second, 5-HT has been reported to depress excitatory synaptic transmission in the EC via 5-HT_{1A} receptors (Schmitz et al., 1998; Schmitz et al., 1999). Third, the present study demonstrates that 5-HT increases GABA release via activation of 5-HT_{2A} receptors. Whereas application of 5-HT has been shown to inhibit epileptiform activity (Schmitz et al., 1997), the underlying mechanisms have not been defined previously. Here, we employed the low- Mg^{2+} -induced *in vitro* seizure model and tested the anti-epileptic effects of 5-HT. Our results demonstrate that 5-HT exerted powerful inhibition of epilepsy and 5-HT-mediated anti-epileptic effects are dependent on both 5-HT_{1A} and 5-HT_{2A} receptors. Consistent with our results, Schmitz et al. (1997) showed that blockade of 5-HT_{1A} receptors alone failed to prevent 5-HT-induced depression of seizure activity suggesting the involvement of other types of 5-HT receptors. Our results filled this gap demonstrating that 5-HT_{2A}-mediated inhibition of GABA release participates in the antiepileptic actions of 5-HT.

Furthermore, the gene for 5-HT_{2A} receptors was down-regulated in the EC of patients suffering from mesial temporal lobe epilepsies (Jamali et al., 2006) highlighting an indispensable role of 5-HT_{2A} receptors in 5-HT-mediated inhibition of epilepsy. Together, our findings provide a cellular and molecular mechanism that could explain the powerful anti-epileptic actions of 5-HT and potentially lead to the development of novel therapeutic strategies for temporal lobe epilepsies.

Methods

Slice preparation

Horizontal brain slices (400 μ m) including the EC, subiculum and hippocampus were cut using a vibrating blade microtome (VT1000S; Leica, Wetzlar, Germany) usually from 13- to 20-day-old Sprague Dawley rats as described previously (Deng and Lei, 2006; Deng et al., 2006). After being deeply anesthetized with isoflurane, rats were decapitated and their brains were dissected out in ice-cold saline solution that contained (in mM) 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, 5.0 MgCl₂, and 10 glucose, saturated with 95% O₂ and 5% CO₂, 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 (~24°C) until use. All animal procedures conformed to the guidelines approved by the University of North Dakota Animal Care and Use Committee.

Recordings of spontaneous, miniature and evoked GABA_A receptor-mediated IPSCs

Whole-cell patch-clamp recordings using two Multiclamp 700B amplifiers (Molecular Devices, Sunnyvale, CA) in voltage-clamp mode were made from the principal neurons in layer II/III of the EC visually identified with infrared video microscopy (BX51WI; Olympus, Tokyo, Japan) and differential interference contrast optics (Deng and Lei, 2007; Deng et al., 2007). The recording electrodes were filled with the following solution (in mM): 100 caesium gluconate, 0.6 EGTA, 5 MgCl₂, 8 NaCl, 2 ATP₂Na, 0.3 GTPNa, 40 HEPES and 1 QX-314, pH 7.3. The extracellular solution contained (in mM) 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 1.5 MgCl₂, 2.5 CaCl₂ and 10 glucose, saturated with 95% O₂ and 5% CO₂, pH 7.4. To record GABA_A receptor-mediated spontaneous IPSCs (sIPSCs), the external solution was supplemented with dL-2-amino-5-phosphonovaleric acid (DL-APV) (100 μ M) and 6,7-dinitroquinoxaline-2,3(1H, 4H)-dione (DNQX) (10 μ M) to block NMDA and AMPA receptor-mediated responses, respectively. sIPSCs were recorded at a holding potential of +30 mV (Deng and Lei, 2006; Deng et al., 2006). Under these conditions, the recorded inhibitory currents were completely blocked by bicuculline methobromide (10 μ M), confirming that they were mediated by GABA_A receptors. Miniature IPSCs (mIPSCs) were recorded by including TTX (1 μ M) in the above external solution to block action potential-dependent responses. Evoked IPSCs were recorded from the principal neurons in layer II/III of the EC using the preceding internal and external solutions by placing a stimulation electrode in layer III to stimulate GABAergic inputs. Synaptic responses were evoked at 0.2 Hz by low-intensity stimulation (80–100 μ sec duration; 10–40 μ A intensity) via a constant-current isolation unit (A360; World Precision Instrument, Sarasota, FL) connected to a patch electrode filled with extracellular solution. Series resistance was rigorously monitored by delivery of 5 mV voltage steps after each evoked current. Experiments were discontinued if the series resistance changed by >10%. Data were filtered at 2 kHz, digitized at 10 kHz and acquired on-line using pCLAMP 9 (Clampex) software (Molecular Devices). The recorded sIPSCs and mIPSCs were subsequently analyzed by Mini Analysis 6.0.1 (Synaptosoft Inc., Decatur, GA, USA). Each event was inspected visually to exclude obvious artifacts before analysis. The threshold for detection was set to 3 times the standard deviation of the noise as recorded in an event-free stretch of data (Clements and Bekkers, 1997). Mean amplitude, frequency, cumulative amplitude and frequency histograms were calculated by this program. The recorded evoked IPSCs were analyzed by pClamp 9 (Clampfit). For the experiments involving application of

inhibitors for 5-HT receptors or other intracellular molecules, slices were pretreated with these inhibitors for 15–30 min and the same concentrations of the inhibitors were bath applied continually to ensure a complete inhibition. To avoid potential desensitization induced by repeated applications of 5-HT, one slice was limited to only one application of 5-HT. For the experiment involving N-methyl-D-glucamine (NMDG), the extracellular NaCl concentration was replaced by the same concentration of NMDG and HCl was used to adjust pH to 7.4.

Recording of action potentials

Action potential firing was recorded from interneurons in layer III of the EC with the intracellular solution containing (in mM) 100 potassium gluconate, 0.6 EGTA, 5 MgCl₂, 8 NaCl, 2 ATP₂Na, 0.3 GTPNa and 40 HEPES, pH 7.3. Biocytin (0.2%) was added to the above intracellular solution for *post hoc* histological staining. Because dialysis of K⁺-containing internal solution into cells can change the resting membrane potential and influence action potential firing, we waited for ~15 min after the formation of whole-cell recordings to allow the resting membrane potential to stabilize unless stated otherwise. Usually for most of the cells a positive current injection 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. The frequency of the action potentials was calculated by Mini Analysis 6.0.1.

Recording of holding currents

Holding currents at -55 mV were recorded from interneurons in layer III of the EC in the extracellular solution containing TTX (1 μM) to block action potential firing. The intracellular solution was the preceding K⁺-containing solution. Because gradual dialysis of K⁺ into cells changed the holding currents, 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 currents recorded for the last minute prior to the application of 5-HT from those recorded at different time points to zero the basal level of the holding currents for better comparison.

Construction of voltage-current curves

Voltage-current curves were constructed from interneurons in layer III of the EC. K⁺-gluconate internal solution was used and the external solution was supplemented with (in μM) 1 TTX, 100 CdCl₂, 10 DNQX, 50 dl-APV and 10 bicuculline. Voltage-current relationship was obtained by using a ramp protocol from -130 mV to -60 mV at a rate of 0.1 mV/ms. We compared the voltage-current curves recorded prior to and during the application of 5-HT for 5–10 min.

Paired recordings from synaptically connected interneuron and pyramidal neuron in the EC

The electrode sealed to the presynaptic interneuron contained the above K⁺-gluconate intracellular solution and the electrode sealed to the postsynaptic pyramidal neuron was filled with the preceding Cs⁺-containing intracellular solution except that Cs⁺-gluconate was replaced with the same concentration of CsCl. Biocytin (0.2%) was included in both electrodes for *post hoc* identification of recorded interneurons and pyramidal neurons. Presynaptic interneuron was held in current-clamp mode and stimulated at a frequency of 0.3 Hz by brief current pulses (duration 10 msec, amplitude 0.2–0.25 nA) to initiate action potentials. Postsynaptic pyramidal neuron was held in voltage-clamp mode (holding potential -60 mV). The recorded currents were completely blocked by application of bicuculline (10 μM) indicating that they were mediated by GABA_A receptors.

Immunocytochemistry

For immunocytochemical identification of GABAergic interneurons, slices were fixed in 4% paraformaldehyde for 24 h after recordings and then washed extensively with 0.1 M phosphate buffer saline (PBS). Slices were incubated initially with PBS containing 5% normal goat serum and 1% Triton X-100 for 2 h followed by incubation with rabbit anti-GABA antibody (Chemicon, CA) at a dilution of 1:500 for 48 h at 4°C. For detection, slices were incubated for 2 h with goat anti-rabbit IgG-Texas red (1:200) and Fluorescein-conjugated streptavidin (1:200) at room temperature. Fluorescein-streptavidin was also used to label neurons after paired recordings and type I interneurons whereas Texas red-streptavidin was used to label the recorded type II interneurons (Fig. 4 A3 and B3). Each incubation was followed by thorough washing with PBS. Slices were mounted on slides, cover-slipped, visualized and photographed with an Olympus Fluoview 300 confocal microscope.

For immunocytochemical detection of TASK-3 channels in GABAergic interneurons, rats were anaesthetized with pentobarbital sodium (60 mg/kg, ip) and perfused transcardially with 0.9% normal saline followed by 4% paraformaldehyde in 0.1 M PBS. Brains were rapidly removed, postfixed in the same fixative for an additional 2 h and then cryoprotected with 30% sucrose in PBS for 12 h. Brain sections of 25 µm in thickness were cut horizontally in a Leica cryostat (CM 3050 S) at -21°C. Free-floating sections were washed in 0.1 M PBS and incubated for 1 h at room temperature in blocking buffer (0.1 M PBS, 10% normal donkey serum, and 0.3% Triton X-100). Primary antibodies were diluted in a dilution buffer containing 0.1 M PBS, 1% normal donkey serum, and 0.3% Triton X-100. Tissue sections were incubated with polyclonal rabbit anti-GAD67 (1:200, Santa Cruz) and goat anti-TASK-3 (1:200, Santa Cruz) at 4°C for 48 h on a shaker. After primary incubation, the sections were washed 5 times for 10 min with 0.1 M PBS followed by incubation for 2 h at room temperature with the secondary antibodies (donkey anti-rabbit IgG-rhodamine and donkey anti-goat IgG-FITC, 1:200 dilution in 0.1 M PBS). After secondary incubation, the sections were washed 5 times for 10 min with 0.1 M PBS and mounted on glass slides and coverslipped. Slides were visualized and photographed with an Olympus Fluoview 300 confocal system coupled to an Olympus IX70 inverted fluorescence microscope.

Low-Mg²⁺-induced seizure model in vitro slices

Extracellular field recordings with a glass electrode containing 2 M NaCl were conducted in layer III of the EC from horizontal brain slices. The slice was placed in the recording chamber and perfused with the preceding extracellular solution except that no Mg²⁺ was added and Ca²⁺ concentration was reduced to 1.6 mM. Seizure activity began to occur usually in 30 min and was stabilized in 60–90 min. After recording basal stable seizure activity for 15 min, we applied 5-HT (100 µM) in the above Mg²⁺-free extracellular solution for 15 min and continued to record in the absence of 5-HT for another 30 min. When 5-HT_{1A} receptor antagonist, S(-)-UH-301 and 5-HT_{2A} receptor antagonist, R-96544, were used, they were applied 10 min prior to the application of 5-HT. For analysis, we counted the spontaneous discharges during a time period of 4 min for each treatment after the seizure activity was stabilized. The number of epileptiform events is expressed as events per minutes in the results.

Data analysis

Data are presented as the means ± S.E.M. Concentration-response curve of 5-HT was fit by Hill equation: $I = I_{\max} \times \{1/[1 + (EC_{50}/[ligand])^n]\}$, where $I_{\max}$ is the maximum response, EC_{50} is the concentration of ligand producing a half-maximal response, and n is the Hill coefficient. Student's paired or unpaired *t* test or analysis of variance (ANOVA) was used for statistical analysis as appropriate; P values are reported throughout the text and significance was set as $P < 0.05$. For sIPSC or mIPSC cumulative probability plots, events recorded 5 min prior to and 5 min after reaching the maximal effect of 5-HT were selected. Same bin size (25

ms for frequency and 2 pA for amplitude) was used to analyze data from control and 5-HT treatment. Kolmogorov-Smirnov test was used to assess the significance of the cumulative probability plots. N number in the text represents the cells examined unless stated otherwise.

Chemicals

Ketanserin, SB 204741, R-96544, RS 102221, ruthenium red, methanandamide, GDP- β -S, suramin, and U73122 were purchased from TOCRIS (Ellisville, MO). Anti-G $\alpha_{q/11}$ and anti-G β were from BIOMOL (Plymouth Meeting, PA) and other antibodies were products of Santa Cruz Biotechnology, Inc. 1-O-Octadecyl-2-O-methyl-*rac*-glycero-3-phosphorylcholine (edelfosine) was purchased from Calbiochem (Darmstadt, Germany). 5-HT and other chemicals were products of Sigma-Aldrich (St. Louis, MO).

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Fig. 1. 5-HT increases the frequency and amplitude of sIPSCs recorded from stellate (A₁–A₄) and pyramidal (B₁–B₄) neurons

A₁, sIPSCs recorded from a stellate neuron before and during the application of 5-HT (100 μM). **A₂**, Pooled time course of sIPSC frequency from 10 stellate neurons. **A₃**, Cumulative probability of sIPSC frequency prior to and during the application of 5-HT (n=10). Note that 5-HT increased sIPSC frequency (reduction in the intervals of events). **A₄**, Cumulative probability of sIPSC amplitude before and during the application of 5-HT (n=10). **B₁–B₄**, Data were from pyramidal neurons. The figures are arranged in the same fashion. **C**, There were no significant differences for 5-HT-induced increases in sIPSC frequency and amplitude between stellate (n=10) and pyramidal (n=10) neurons. **D**, Concentration-response curve by plotting

the percentage of increase in frequency versus the concentrations of 5-HT. Numbers in the parenthesis are number of cells recoded.

Fig. 2. 5-HT up-regulates sIPSCs via activation of 5-HT_{2A} receptors

A, Pretreatment of slices with and bath application of S(-)-UH-301 (10 μ M), a 5-HT_{1A} receptor blocker, did not block 5-HT-induced increase in sIPSC frequency (n=6). **B**, Pretreatment of slices with and bath application of tropisetron (10 μ M), a 5-HT₃ receptor blocker, did not block 5-HT-induced increase in sIPSC frequency (n=6). **C**, Pretreatment of slices with and bath application of ketanserin (10 μ M), an antagonist for 5-HT_{2A} and 5-HT_{2C} receptors, completely blocked 5-HT-induced increases in sIPSC frequency (n=6). **D**, Application of SB204741 (50 μ M), a potent and selective 5-HT_{2B} receptor antagonist, failed to change 5-HT-induced increases in sIPSC frequency (n=5). **E**, Application of R-96544 (10 μ M), a 5-HT_{2A} receptor antagonist, completely blocked 5-HT-induced increases in sIPSC frequency (n=8). **F**,

Application of RS 102221 (25 μ M), an inhibitor of 5-HT_{2C}, did not significantly change 5-HT-induced increases in sIPSC frequency (n=6).

Fig. 3. 5-HT did not modulate mIPSCs but reduced the amplitudes of eIPSCs recorded by extracellular field stimulation and in synaptically connected interneuron and pyramidal neuron pairs

A, mIPSCs recorded from a neuron before and during the application of 5-HT (100 μ M) in the presence of TTX (1 μ M). Note that 5-HT had no effects on mIPSCs. **B**, Pooled time course of mIPSC frequency (n=5). **C**, Cumulative probability of mIPSC frequency before and during the application of 5-HT (n=5). **D**, Cumulative probability of mIPSC amplitude before and during the application of 5-HT (n=5). **E**, Application of 5-HT (100 μ M) decreased the amplitude of eIPSCs (n=4). A stimulation electrode filled with the extracellular solution was placed in a position in layer III that was $\sim$ 100 μ m from the recorded cell. Inset shows the

currents averaged from 10 traces before (a) and during (b) the application of 5-HT. **F**, Visualization of a pair of an interneuron (IN) and a pyramidal cell (PC) in layer III after recording. Scale bar represents 20 μm . **G**, Superimposed IPSCs (black) and their average (red) evoked by single presynaptic action potentials (upper panels) in an interneuron-pyramidal cell pair in layer III before (left) and after (right) application of 5-HT (100 μM). Note that the amplitude of action potential became smaller in the presence of 5-HT. **H**, Summarized time course of 5-HT-mediated inhibition of IPSC amplitudes from 6 interneuron-pyramidal cell pairs.

Fig. 4. 5-HT induces depolarization in both Type I and Type II GABAergic interneurons in the EC
A₁–A₄, Morphology and electrophysiological properties of Type I interneurons. **A₁,** A Type I interneuron identified under an infrared video microscopy. **A₂,** Voltage response of a Type I interneuron when currents ($\pm 250\text{ pA}$) were injected via the recording pipette. Note that there is no apparent voltage sag and rebound burst firing. **A₃,** *Post hoc* labeling of a Type I interneuron after electrophysiological recordings. Note that a main dendrite goes to layer II and layer I. **A₄,** Application of 5-HT (100 μM) induced an inward holding current in Type I interneurons ($n=10$). **B₁–B₄,** Morphology and electrophysiological properties of Type II interneurons. **B₁,** A Type II interneuron identified under an infrared video microscopy. **B₂,** Voltage response of a Type II interneuron when currents ($\pm 250\text{ pA}$) were injected via the

recording pipette. Note that there is pronounced voltage sag and rebound burst firing indicated by the arrows. **B₃**, *Post hoc* labeling of a Type II interneuron after electrophysiological recordings. Note that dendrites ramify locally. **B₄**, Application of 5-HT (100 μ M) induced an inward holding current in Type II interneurons (n=8).

Fig. 5. 5-HT increases action potential firing frequency in interneurons of the EC via 5-HT_{2A} receptors

A, Action potentials recorded from an interneuron prior to and during the application of 5-HT (100 μ M). **B**, Pooled time course of action potential firing frequency before, during and after the application of 5-HT (100 μ M, $n=8$). Note that 5-HT significantly increased the firing frequency of action potentials in interneurons. **C**, Pretreatment of slices with and co-application of R-96544 (10 μ M), a 5-HT_{2A} blocker, blocked 5-HT-induced increases in action potential firing frequency in interneurons ($n=5$). **D**, Histological staining of GABA expression in a recorded interneuron filled with biocytin. *Left*: A biocytin-filled interneuron detected by fluorescein-conjugated streptavidin. *Middle*: The same interneuron was stained with anti-GABA antibody. *Right*: The two stainings were merged to show their colocalization in one neuron.

Fig. 6. 5-HT inhibits K⁺ channels of the interneurons in layer III to enhance GABA release

A, Replacement of extracellular Na⁺ with the same concentration of NMDG failed to change 5-HT-induced increases in inward holding currents (n=9). **B**, Omission of extracellular Ca²⁺ did not alter 5-HT-induced increases in inward holding currents (n=8). **C**, Voltage-current relationship recorded by a ramp protocol (from -130 mV to -60 mV, at a speed of 0.1 mV/ms) before and during the application of 5-HT (100 μ M) when the extracellular K⁺ concentration was 3.5 mM. Traces in the figure were averaged traces from 5 cells. The 5-HT-induced net current reversed at $\sim$ -83 mV close to the calculated K⁺ reversal potential ($\sim$ -85.4 mV). **D**, 5-HT-induced increases in holding currents were insensitive to TEA (n=9), Cs⁺ (n=8), 4-AP (n=9) and tertiapin (n=8). **E**, Bath application of Ba²⁺ (3 mM) induced an inward holding current and significantly reduced 5-HT-induced increases in holding currents (n=6). **F**, Bath

application of bupivacaine (400 μ M) induced an inward holding current and significantly attenuated 5-HT-induced augmentation of inward holding currents (n=5).

Fig. 7. 5-HT facilitates GABA release via inhibition of TASK-3 channels in entorhinal interneurons
A–F, Intracellular application of control Ig-G (40 μ g/ml, n=5) and the antibodies to TWIK-1 (n=6, **A**), TREK-1 (n=6, **B**), TREK-2 (n=6, **C**), TRESK (n=7, **D**) and TASK-1 (n=5, **E**) in the same concentration did not significantly alter 5-HT-mediated increases in inward holding currents whereas application of antibody to TASK-3 (40 μ g/ml) significantly reduced 5-HT-induced increases in inward holding currents (n=5, **F**). **G**, Lowering extracellular pH from 7.4 to 6.4 induced an inward holding current *per se* and significantly reduced 5-HT-induced increases in inward holding currents (n=8). **H**, 5-HT-induced increases in inward holding currents were not significantly changed in the presence of methanandamide (MA, 10 μ M, n=6) whereas the effect of 5-HT was significantly reduced in the presence of ruthenium red (RR,

10 μ M, n=6) and zinc (100 μ M, n=6, ** $p<0.01$). **I**, Confocal microscopic images of double immunofluorescent staining showing that GABAergic interneurons (red) express TASK-3 channels (green) in layer III of the EC. *Left*: staining for GAD67; *Middle*: staining for TASK-3; *Right*: merged image.

Fig. 8. Signaling mechanism underlying 5-HT-induced membrane depolarization

A, Application of GDP- β -S (2 mM) via the recording pipettes blocked 5-HT-induced increases in inward holding currents ($n=5$). **B**, Pretreatment of slices with and continuous bath application of suramin (100 μ M) blocked 5-HT-induced increases in inward holding currents ($n=5$). **C**, Application of anti- $G_{\alpha_{q/11}}$ (20 μ g/ml) via the recording pipettes significantly reduced 5-HT-induced increases in inward holding currents ($n=6$). **D**, Application of anti- G_{β} (20 μ g/ml) via the recording pipettes failed to block 5-HT-induced enhancement of inward holding currents ($n=5$). **E**, Pretreatment of slices with and continuous bath application of U73122 (20 μ M) did not block 5-HT-induced increases in inward holding currents ($n=6$). **F**, Pretreatment of slices with and continuous bath application of edelfosine (20 μ M) did not block 5-HT-induced increases in inward holding currents ($n=6$).

Fig. 9. 5-HT depresses low-Mg²⁺-induced seizure activity in slices

A, Application of 5-HT (100 μ M) drastically inhibited low-Mg²⁺-induced seizure activity. *Upper*: Seizure activity induced by low-Mg²⁺ prior to, during and after application of 5-HT. Arrows indicate the enlarged scale of the events. *Lower*: Pooled data from 8 slices. **B**, Application of the 5-HT_{1A} receptor antagonist, S(-)UH-301 (2 μ M), did not change low-Mg²⁺-induced seizure activity but significantly reduced 5-HT-induced inhibition of seizures (n=6 slices). **C**, Application of the 5-HT_{2A} receptor antagonist, R-96544 (10 μ M), did not change low-Mg²⁺-induced seizure activity but significantly reduced 5-HT-induced inhibition of seizure events (n=8 slices). **D**, Application of both S(-)UH-301 (2 μ M) and R-96544 (10 μ M), did not change low-Mg²⁺-induced seizure events but prevented 5-HT-induced inhibition of seizures (n=7 slices).