Serotonergic afferents from the dorsal raphe decrease the excitability of pyramidal neurons in the anterior piriform cortex

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The olfactory system receives extensive serotonergic inputs from the dorsal raphe, a nucleus involved in control of behavior, regulation of mood, and modulation of sensory processing. Although many studies have investigated how serotonin modulates the olfactory bulb, few have focused on the anterior piriform cortex (aPC), a region important for olfactory learning and encoding of odor identity and intensity. Specifically, the mechanism and functional significance of serotonergic modulation of the aPC remain largely unknown. Here we used pharmacologic, optogenetic, and fiber photometry techniques to examine the serotonergic modulation of neural activity in the aPC in vitro and in vivo. We found that serotonin (5-HT) reduces the excitability of pyramidal neurons directly via 5-HT$_{2C}$ receptors, phospholipase C, and calcium-activated potassium (BK) channels. Furthermore, endogenous serotonin attenuates odor-evoked calcium responses in aPC pyramidal neurons. These findings identify the mechanism underlying serotonergic modulation of the aPC and shed light on its potential role.

anterior piriform cortex | serotonergic modulation | electrophysiology | optogenetics

Serotonin (5-hydroxytryptamine; 5-HT) is a critically important neuromodulator of brain function. It originates predominantly from serotonergic neurons located in the raphe nuclei of the brainstem, which project extensively to multiple brain regions. The serotonergic system is implicated in a variety of functions, including anxiety and depression, the sleep–wake cycle, reward, and patience in decision making (1–5). Although less studied, serotonin also modulates sensory processing in the auditory, visual, and olfactory systems (6–9).

The two key brain centers in the olfactory system, the olfactory bulb (OB) and the piriform cortex, are robustly innervated by serotonergic neurons from the dorsal raphe nucleus (DRN). These inputs are important for odor preference learning in neonatal rats and regulation of odor input (10–12). Many studies have investigated how serotonergic inputs regulate neural activity and olfactory information processing in the OB, and the mechanism underlying the effects of serotonin in the OB has been identified (13–17). However, how serotonergic inputs modulate neural activity in the piriform cortex remains largely unknown.

The anterior piriform cortex (aPC) is a three-layered cortical region. Layer 2 contains densely packed pyramidal neurons that receive direct input from mitral/tufted cells of the OB. The aPC is critically involved in olfactory learning (18, 19), odor pattern separation (20, 21), and coding of odor identity and intensity (22–24). Expression of 5-HT$_{2C}$ receptors in the aPC has been reported (25, 26), suggesting potential serotonergic modulation of the aPC. Although a recent study demonstrated that optogenetic activation of serotonergic neurons in the DRN rapidly inhibits spontaneous, but not odor-evoked, activity in the aPC in anesthetized mice (3), the mechanism underlying this effect remains elusive. Furthermore, given the crucial role of serotonin in awake behaving animals (1, 15, 27, 28), it is important to study how optogenetic activation of serotonergic neurons regulates the odor-evoked response in awake animals.

In the present study, we investigated how 5-HT affects the activity of pyramidal neurons in the aPC. We identified the mechanism underlying this action through pharmacologic manipulations and optogenetic activation of serotonergic inputs in the aPC in vitro. Furthermore, we combined calcium fiber photometry and optogenetics to investigate how serotonergic inputs modulate the odor-evoked neural responses of pyramidal neurons at the cell population level in awake mice.

Results

Serotonin Reduces the Excitability of Pyramidal Neurons in the aPC. To determine whether 5-HT regulates the excitability of pyramidal neurons in the aPC, we recorded current-evoked action potentials (APs) from pyramidal neurons in layer 2, the main input layer of the piriform cortex, in response to current injections at different intensities (70 pA to 220 pA; Fig. 1*A*). The frequency of APs increased with the size of the current step (Fig. 1*A*). Bath application of 50 μM 5-HT significantly decreased the frequency of current-evoked APs (two-way ANOVA, $P = 0.0001$, $F_{(1,168)} = 15.60$,

Significance

Serotonergic neurons in the dorsal raphe nucleus have been implicated in olfactory learning and processing of olfactory information. While the piriform cortex is an important olfactory center critically involved in olfactory learning and coding of odor information and receives intensive serotonergic innervation, how the piriform cortex is modulated by the serotonergic inputs is largely unknown. We investigate this issue in vitro and in vivo. Our findings provide direct evidence for serotonergic modulation of neural activity in the anterior piriform cortex, identify the mechanism for this modulation, and shed light on its potential role. This study is important to understanding how centrifugal inputs modulate processing of olfactory information and the role of the serotonergic system in the brain as a whole.

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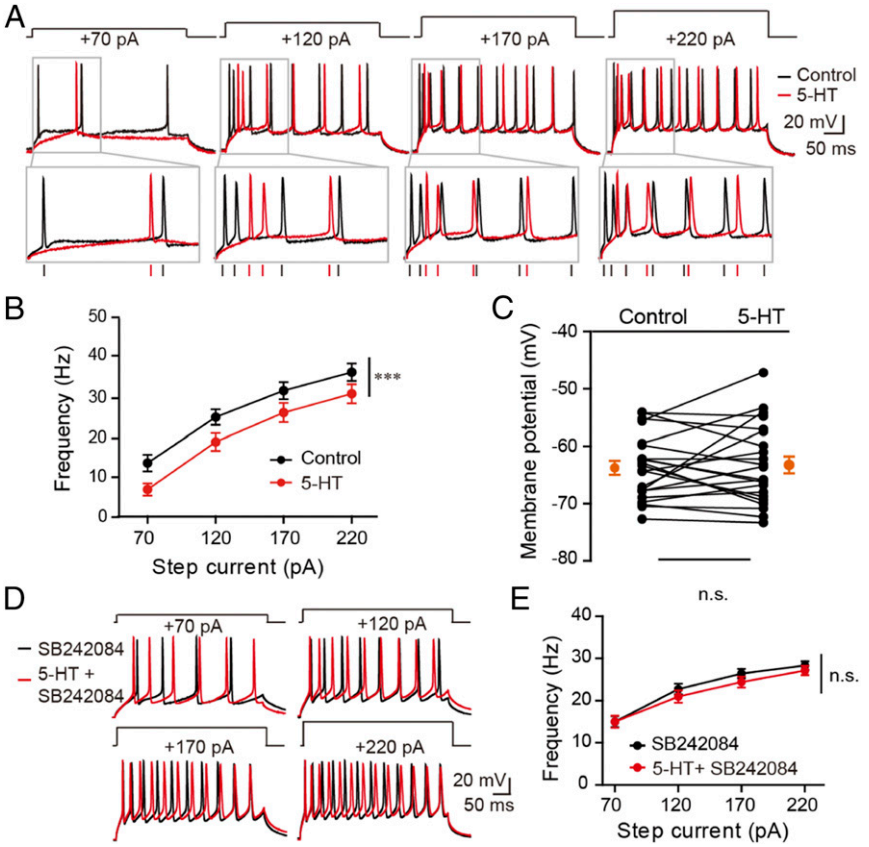

Fig. 1. Serotonin decreases the frequency of current-evoked APs in aPC pyramidal neurons. (*A*) Typical current-evoked APs in response to gradually increasing current injections in a pyramidal neuron before (black) and after (red) bath application of 5-HT. For clarity, the early phase of each response (outlined by a gray box) is shown at higher resolution beneath the full trace. Each tick mark represents a spike. (*B*) Mean frequency of current-evoked APs in response to different step currents before (black) and after (red) 5-HT application. Two-way ANOVA, ***$P = 0.0001$, $F_{(1,168)} = 15.60$. (*C*) Comparison of the membrane potential of aPC pyramidal neurons before and after 5-HT application, across all neurons recorded. Orange dots indicate the mean membrane potential. Paired *t* test, *n.s.*, $P = 0.67$, $t_{(21)} = 0.44$. (*D* and *E*) Representative traces (*D*) and mean firing rates (*E*) in response to different step currents before and after 5-HT application in the presence of SB242084. Two-way ANOVA, $P = 0.16$, $F_{(1,160)} = 2.00$.

$n = 22$ from 11 mice; Fig. 1 *A* and *B*). 5-HT had no effect on the resting membrane potential of pyramidal neurons (paired *t* test, $P = 0.67$, $t_{(21)} = 0.44$, $n = 22$ from 11 mice; Fig. 1*C*). These results were from slices obtained from mice aged P17 to P23; however, similar results were observed in pyramidal neurons isolated from adult mice (*SI Appendix*, Fig. S1). In the following in vitro pharmacologic experiments (all except the optogenetic experiments), the recordings were performed in mice aged P17 to P23. These results suggest that 5-HT reduces the excitability of pyramidal neurons without changing the resting membrane potential.

Since there are two classes of pyramidal neurons in layer 2 of the aPC, superficial pyramidal (SP) and semilunar (SL) cells (29, 30), we next asked whether the effects of 5-HT differ between these neurons. As described in previous studies, SP and SL cells can be distinguished by cell morphology and firing pattern (29, 30). Current evoked firing rates were decreased by 5-HT in both SP and SL cells (SP: two-way ANOVA, $P = 0.01$, $F_{(1,56)} = 6.80$, $n = 8$ from 8 mice; SL: two-way ANOVA, $P = 0.001$, $F_{(1,104)} = 11.00$, $n = 14$ from 8 mice; *SI Appendix*, Fig. S2 *A* and *B*). Therefore, 5-HT reduces the excitability of both SP and SL pyramidal neurons in the aPC.

Previous studies have reported the expression of 5-HT_{2C} receptors in pyramidal neurons of the aPC (26). We thus performed a pharmacologic experiment to test whether the inhibitory effect of 5-HT on pyramidal neurons was mediated by 5-HT_{2C} receptors. Current-evoked APs were recorded in the presence of SB242084 (10 μM), a 5-HT_{2C} receptor blocker (Fig. 1*D*). Bath application of 5-HT failed to decrease the frequency of APs in the presence of SB242084 (two-way ANOVA, $P = 0.16$, $F_{(1,166)} = 2.00$, $n = 21$ from 13 mice; Fig. 1*E*). This suggests that the inhibitory effect of 5-HT on pyramidal neurons is mediated by 5-HT_{2C} receptors.

5-HT Reduces the Excitability of Pyramidal Neurons in a GABA-Independent Manner. Although we observed inhibitory effects of 5-HT on pyramidal neurons via 5-HT_{2C} receptors (Fig. 1), previous studies have reported that activation of 5-HT_{2C} receptors exerts an excitatory effect (13, 31, 32). This raises the possibility that the inhibitory effect of 5-HT on pyramidal neurons is mediated indirectly via an excitatory effect on GABAergic inhibitory interneurons. To test this possibility, aPC slices were treated with bicuculline, a $GABA_A$ receptor antagonist. Interestingly, we found that 10 μM bicuculline did not block the inhibitory effect of 5-HT on pyramidal neurons (two-way ANOVA, $P = 0.0007$, $F_{(1,144)} = 11.90$, $n = 19$ from 7 mice; Fig. 2 *A* and *B*). This result indicates that $GABA_A$ receptors do not contribute to the inhibitory effect of 5-HT on pyramidal neurons.

To confirm this conclusion, we recorded miniature inhibitory postsynaptic currents (mIPSCs) in pyramidal neurons (Fig. 2*C*). As shown in Fig. 2 *D* and *E*, neither the amplitude nor the frequency of mIPSCs was altered by 5-HT (amplitude: paired *t* test, $P = 0.16$, $t_{(11)} = 1.50$, $n = 12$ from 5 mice; frequency: paired *t* test, $P = 0.32$, $t_{(11)} = 1.04$, $n = 12$ from 5 mice). These results suggest that 5-HT reduces the excitability of pyramidal neurons directly rather than through effects on presynaptic activity.

To directly test the effect of 5-HT on GABAergic neurons, we recorded current-evoked APs from GABAergic neurons. Virus

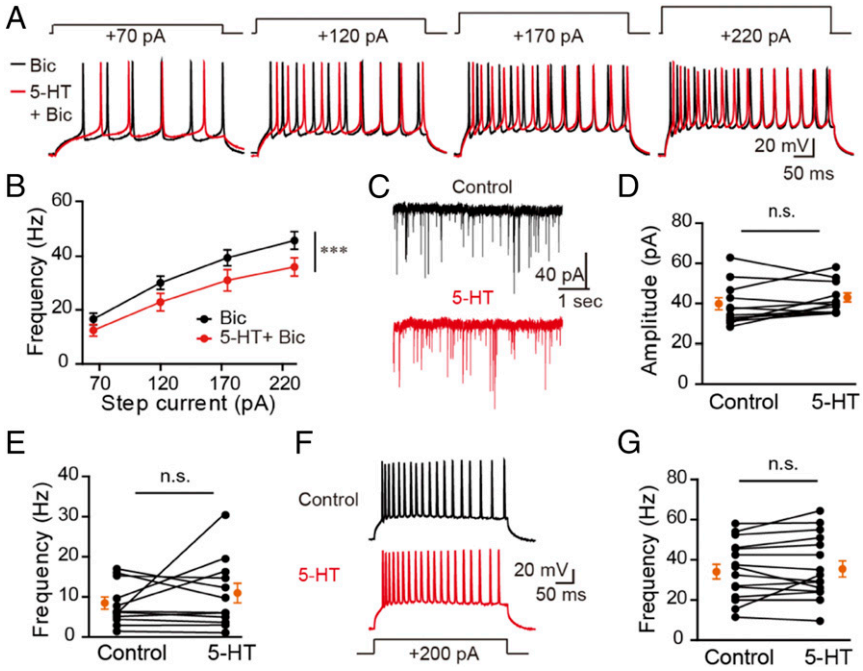

Fig. 2. Inhibitory modulation by 5-HT does not depend on GABAergic interneurons. (*A* and *B*) Representative traces (*A*) and comparison of the frequency (*B*) of current-evoked APs in response to different step currents before and after bath application of 5-HT in the presence of bicuculline. Two-way ANOVA, ***P = 0.0007, $F_{(1,144)} = 11.90$. (*C*) Voltage-clamp recording of mIPSCs in a representative pyramidal neuron in control and 5-HT conditions. (*D* and *E*) Comparison of amplitude (*D*) and frequency (*E*) of mIPSCs before and after 5-HT application. *D*: paired *t* test, $P = 0.16$, $t_{(11)} = 1.50$; *E*: paired *t* test, $P = 0.32$, $t_{(11)} = 1.04$. (*F*) Raw traces of APs fired in response to current injection (200 pA) recorded in a representative GABAergic neuron before and after 5-HT application. (*G*) Comparison of the frequency of current-evoked APs before and after 5-HT application. Paired *t* test, $P = 0.39$, $t_{(15)} = 0.88$.

(AAV-VGAT1-mCherry) was injected into the aPC to label GABAergic neurons. We found that 5-HT had no effect on the firing rate of APs recorded from GABAergic neurons (paired *t* test, $P = 0.39$, $t_{(15)} = 0.88$, $n = 16$ from 6 mice; Fig. 2 *F* and *G*). Taken together, the data recorded from pyramidal neurons and GABAergic neurons consistently indicate that 5-HT reduces the excitability of pyramidal neurons directly in a GABA-independent manner.

The pyramidal neurons in layer 2 of the aPC receive direct, dense input from the OB via the lateral olfactory tract (LOT) (33). We next asked whether 5-HT has an effect on LOT-evoked excitatory postsynaptic currents (EPSCs). We positioned stimulation electrodes on the LOT and recorded the EPSCs evoked in response to pairs of stimulation pulses separated by an 85-ms interpulse interval. As shown in *SI Appendix*, Fig. S3*A*, electrical stimulation of the LOT induced strong EPSCs in pyramidal neurons. Bath application of 5-HT produced no significant changes in EPSC amplitude or paired-pulse ratio (PPR) (amplitude: two-way ANOVA, $P = 0.37$, $F_{(1,110)} = 0.81$, $n = 12$ from 5 mice; PPR: two-way ANOVA, $P = 0.44$, $F_{(1,110)} = 0.60$, $n = 12$ from 5 mice; *SI Appendix*, Fig. S3 *B* and *C*). This result suggests that 5-HT does not alter synaptic transmission between the LOT and the pyramidal neurons.

Phospholipase C and Calcium-Activated Potassium (BK) Channels Are Necessary for the Inhibitory Effect of 5-HT. After identifying the inhibitory effect of 5-HT on pyramidal neurons, we set out to investigate the signaling pathways involved. Type 2 5-HT receptors are primarily coupled to G_q protein, which activates the phospholipase C (PLC) pathway to release Ca^{2+} from intracellular stores (32). We evaluated the contribution of the PLC pathway to the inhibitory effect of 5-HT by applying the selective PLC antagonist U73122. In the presence of 10 μM U73122, 5-HT failed to significantly decrease the frequency of current-evoked APs (two-way ANOVA, $P = 0.11$, $F_{(1,64)} = 2.57$, $n = 9$ from 4 mice; Fig. 3 *A* and *B*). When the U73122 was applied in the internal solution of the pipette, a similar result was observed (*SI Appendix*, Fig. S4 *A* and *B*). Therefore, 5-HT binds to G_q-protein-coupled 5-HT receptors to regulate the excitability of pyramidal neurons via the PLC pathway.

Previous studies have implicated the involvement of potassium channels in serotonergic modulation (34, 35). Another serotonin receptor subtype, 5-HT_4, inhibits neural activity via BK channels, which are activated by Ca^{2+} signals (36). Therefore, it is likely that BK channels are the downstream effector of the PLC pathway and may be responsible for the inhibitory effect of 5-HT on pyramidal neurons. Bath application of paxilline (10 μM), a BK channel blocker, eliminated the inhibitory effect of 5-HT (two-way ANOVA, $P = 0.29$, $F_{(1,64)} = 1.13$, $n = 9$ from 5 mice; Fig. 3 *A* and *C*), indicating the involvement of BK channels. A similar result was observed when paxilline was applied in the internal solution of the pipette (*SI Appendix*, Fig. S4 *A* and *C*). These results suggest that both PLC and BK channels are necessary for the regulatory effect of 5-HT on pyramidal neurons.

To further investigate this, we recorded K^+ channel currents from aPC pyramidal neurons in slices in the presence of 5-HT and paxilline. Fig. 4*A* shows representative current traces from K^+ channels before and after administration of 5-HT. The current–voltage curve was shifted to the left after 5-HT application. Total K^+ channel current density was significantly increased after application of 5-HT (two-way ANOVA, $P = 0.001$, $F_{(1,378)} = 10.89$, $n = 12$ from 5 mice; Fig. 4*A*).

To test whether BK channels are required for the 5-HT modulation of K^+ channel currents, the BK channel inhibitor was applied and K^+ channel currents were compared before and after 5-HT application. Paxilline significantly decreased the current density, and 5-HT failed to increase K^+ channel currents in the presence of paxilline (two-way ANOVA, control vs. paxilline: $P = 0.02$, $F_{(1,306)} = 4.95$, paxilline vs. paxilline + 5-HT: $P = 0.60$, $F_{(1,306)} = 0.27$, $n = 8$ from 3 mice; Fig. 4*B*).

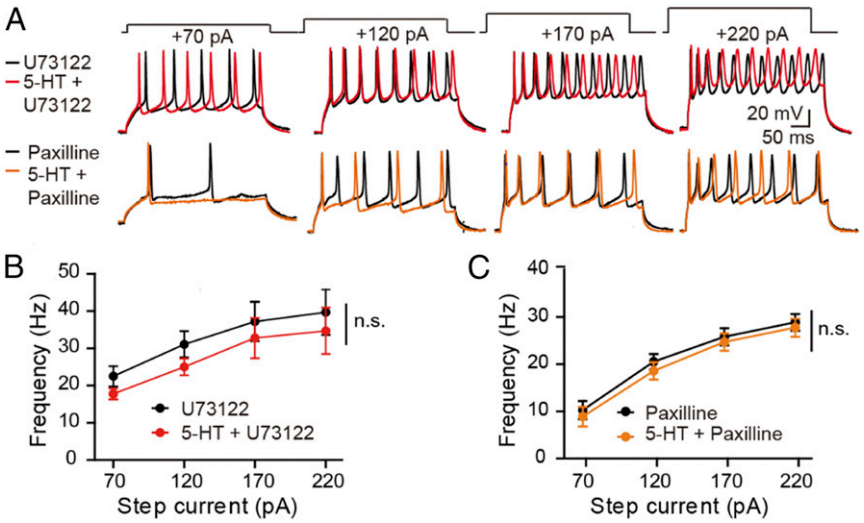

Fig. 3. The inhibitory effect of 5-HT requires the PLC-BK channels pathway. (*A*) Representative current-evoked APs in response to different step currents in a pyramidal neuron before and after bath application of 5-HT in the presence of U73122 (a PLC antagonist) and paxilline (a BK channel blocker). (*B* and *C*) Mean frequency of current-evoked APs before and after 5-HT application in the presence of U73122 (*B*) and paxilline (*C*). *B*: two-way ANOVA, $P = 0.11$, $F_{(1,64)} = 2.57$; C: two-way ANOVA, $P = 0.29$, $F_{(1,64)} = 1.13$.

To examine the effect of 5-HT on BK channels is mediated via 5-HT_{2C} receptors, the 5-HT_{2C} receptor blocker SB242084 was applied and K^+ channel currents were compared. We found that 5-HT had no effect on K^+ channel current density in the presence of SB242084 (two-way ANOVA, $P = 0.90$, $F_{(1,170)} = 0.0002$, $n = 6$ from 2 mice, Fig. 4*C*). These results demonstrate that the 5-HT–5-HT_{2C} receptor–BK channel pathway is necessary for the regulatory effect of 5-HT on pyramidal neurons.

Serotonergic Inputs from the DRN Drive Inhibition of Pyramidal Neurons in the aPC. Having examined serotonergic modulation and the underlying mechanism through exogenous application of 5-HT, we next asked whether direct release of 5-HT from raphe axons has a similar effect. To address this question, we expressed channelrhodopsin-2 (ChR2) in serotonergic neurons in the DRN by injecting an adeno-associated virus (AAV-DIO-ChR2-eYFP) into the DRN of SERT-Cre mice (*SI Appendix*, Fig. S5*A*). Four weeks after viral injection, extensive expression of eYFP was found in serotonergic neurons of the DRN (*SI Appendix*, Fig. S5*A*). ChR2-eYFP expression was restricted to serotonergic neurons, as shown by colocalization with Tph2 (tryptophan hydroxylase 2), a marker of central serotonergic neurons. To evaluate the properties of the photocurrents mediated by expression of ChR2 in neurons, we performed whole-cell recordings from serotonergic neurons in acute slices of the DRN (*SI Appendix*, Fig. S5*B*). In serotonergic neurons, long illumination (500 ms) with 473-nm light induced large, rapid, and continuous current (*SI Appendix*, Fig. S5*C*).

To test whether ChR2 photocurrents were capable of producing APs, we recorded light-evoked responses in current clamp mode. Brief, 5-ms light pulses that generated a rapid current in voltage clamp mode produced an AP in current clamp mode (*SI Appendix*, Fig. S5*D*). Trains of light flashes produced precisely timed, highly reliable APs in current clamp mode and stimulus-locked inward currents in voltage clamp mode (*SI Appendix*, Fig. S5*E*). Therefore, ChR2 can be used to tightly control the activation of serotonergic neurons in the DRN.

We examined serotonergic modulation of aPC pyramidal neurons by activating ChR2-expressing fibers in aPC slices. Dense eYFP-expressing fibers were observed in the aPC (Fig. 5*A*). These represent expression of ChR2 in axonal projections within the aPC that arise from serotonergic neurons in the DRN. We recorded from pyramidal neurons in voltage clamp mode to determine the effects of serotonergic inputs on neuron excitability (Fig. 5*B*). As shown in Fig. 5*C*, activation of serotonergic fibers in an aPC slice by a brief (10-ms) flash of light did not result in conventional postsynaptic currents.

Next, we compared the frequency of current-evoked APs in pyramidal neurons before and after optogenetic activation of serotonergic fibers (a 3-s train of 10-ms light pulses at 20 Hz, 5 mW). The frequency of current-evoked APs was significantly lower after optogenetic activation of serotonergic fibers (paired *t* test, $P = 0.002$, $t_{(14)} = 3.79$, $n = 15$ from 7 mice; Fig. 5*D*). When other frequencies of light pulse (10 Hz and 40 Hz) were applied, only the 40-Hz pulses had a significant effect on the frequency of current-evoked APs in aPC pyramidal neurons (*SI Appendix*, Fig. S6). In the subsequent studies, 20-Hz light pulses were applied. SERT-Cre mice injected with virus that lacked ChR2 (AAV-DIO-eYFP) served as controls; pyramidal neurons in the control experiment showed no change in firing rate after the train of light pulses (paired *t* test, $P = 0.15$, $t_{(13)} = 1.53$, $n = 14$ from 3 mice; Fig. 5*E*). These results suggest that optogenetic activation of serotonergic fibers is sufficient to reduce the excitability of pyramidal neurons. It is likely that serotonergic fibers inhibit pyramidal neurons via release of 5-HT. As predicted, in the presence of a 5-HT_{2C} receptor antagonist, optogenetic activation of serotonergic fibers did not change the frequency of current-evoked APs (paired *t* test, $P = 0.18$, $t_{(8)} = 1.49$, $n = 9$ from 4 mice; Fig. 5*F*).

In conjunction with the pharmacologic experiments described above, these results demonstrate that in the aPC, 5-HT released by serotonergic neurons from the DRN binds to G_q-coupled 5-HT_{2C} receptors in the aPC, activating the PLC pathway. PLC hydrolyzes phosphatidylinositol 1,4,5-bisphosphate into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which then induces the release of intracellular Ca^{2+}. Increased intracellular Ca^{2+} levels activate BK channels, decreasing the excitability of pyramidal neurons (Fig. 5*G*).

Optogenetic Activation of Serotonergic Neurons in the DRN Decreases Odor-Evoked Activity of Pyramidal Neurons. We tested whether serotonergic neurons in the DRN modulate the activity of pyramidal neurons in awake mice. We used fiber photometry to record calcium signals from pyramidal neurons specifically. By

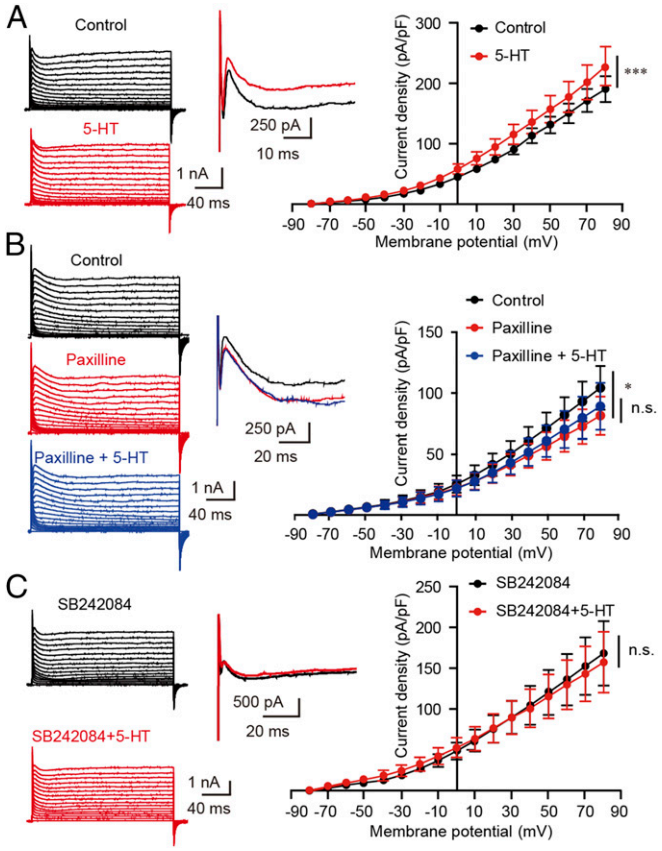

Fig. 4. BK channels are required for the 5-HT and 5-HT$_{2C}$ receptor-mediated increase in K^+ current. (*A*) Representative K^+ current traces and average current intensity before and after 5-HT application. (*Inset*) Current traces in response to a +80-mV voltage step in each condition. Two-way ANOVA, $***P = 0.001$, $F_{(1,378)} = 10.89$. (*B*) Representative K^+ current traces and averaged current intensity in the presence and absence (control) of paxilline (a BK channel blocker) with and without 5-HT. Two-way ANOVA, control vs. paxilline: $*P = 0.02$, $F_{(1,306)} = 4.95$; paxilline vs. paxilline + 5-HT: $P = 0.60$, $F_{(1,306)} = 0.27$. (*C*) Representative K^+ current traces and average current intensity before and after 5-HT application in the presence of SB242084 (a 5-HT$_{2C}$ receptor blocker). Two-way ANOVA, $P = 0.90$, $F_{(1,170)} = 0.0002$.

combining optogenetics and fiber photometry, we were able to specifically activate serotonergic neurons in the DRN while simultaneously recording the calcium signal from the population of pyramidal neurons in the aPC (Fig. 6*A*). We used a SERT-Cre mouse line injected with AAV-DIO-ChR2-eYFP into the DRN and AAV-CaMKIIα-GCaMP6s into the aPC, and implanted optical fibers above both the DRN and the aPC. Consistent with our previous study (37), at 3 wk after the viral injections, we observed extensive expression of GCaMP6s in layer 2/3 of the aPC (Fig. 6*B*). We first examined whether serotonergic neurons in the DRN modulate spontaneous activity in pyramidal neurons. Light stimulation (a 2-s train of 10-ms light pulses at 20 Hz) had no effect on the population calcium signal (paired *t* test, $P = 0.38$, $t_{(5)} = -0.97$, $n = 6$ mice; *SI Appendix*, Fig. S7), indicating that optogenetic activation of serotonergic neurons does not affect the spontaneous activity of pyramidal neurons in awake mice. This finding is inconsistent with the in vitro studies showing a significant effect of 5-HT on the excitability of pyramidal neurons (Fig. 1).

It is likely that the inconsistent results from these two experiments are due to the differences between in vitro slice recording and in vivo calcium recording in awake mice. Whereas aPC pyramidal neurons in slices have weak inputs, the aPC pyramidal neurons in awake animals receive dense active inputs, including the afferent sensory input relayed from the OB, recurrent collaterals from local principal neurons, and top-down innervation from other brain regions. In addition, the calcium signals were recorded from a population of pyramidal neurons, whereas the voltage signals in slice recordings were obtained from individual neurons.

Then we asked whether serotonergic neurons modulate the odor-evoked activity of pyramidal neurons. Fig. 6*C* shows the heatmaps and corresponding trial-averaged traces induced by four odorants with and without light stimulation. Consistent with our previous studies (38), odors evoked an increase in the population calcium signal in pyramidal neurons. In general, light stimulation significantly decreased the odor-evoked response across all of the animal-odor pairs (paired *t* test, $P = 0.001$, $t_{(23)} = 3.62$, $n = 24$ animal-odor pairs from 6 mice; Fig. 6 *D*–*F*). Furthermore, we found a significant negative linear correlation between the odor-evoked response without light stimulation and the change in response caused by the light stimulation (linear regression, $P = 0.0006$, $r = -0.65$; Fig. 6*G*). This suggests that light stimulation tends to decrease the responses of pyramidal neurons with higher odor-evoked responses. These results suggest that optogenetic activation of serotonergic neurons in the DRN decreases odor-evoked responses but does not alter the spontaneous activity of pyramidal neurons in the aPC.

To further characterize the effect of serotonergic modulation on odor-evoked response in the pyramidal cell population, a support vector machine (SVM) was trained to classify the responses to the four different odors with and without photostimulation. During the light-off trials, classification performance increased from chance (50%) before odor stimulation to 85% during the odor delivery period. Interestingly, during the light-on trials, performance of the classifier increased more slowly and never exceeded 75% (Fig. 6*H*). These results indicate that the performance to discriminate odors by the pyramidal neurons population decreased on optogenetic activation of the serotonergic neurons in the DRN.

SERT-Cre mice injected in the DRN with virus that lacked ChR2 (AAV-DIO-eYFP) but injected with AAV-CaMKIIα-GCaMP6s in the aPC served as controls. Although odor-evoked calcium responses were observed in these mice, these responses were unaffected by light stimulation (paired *t* test, $P = 0.29$, $t_{(15)} = 1.09$, $n = 16$ animal-odor pairs from 4 mice; *SI Appendix*, Fig. S8 *A*–*D*). No correlation was observed between the odor-evoked response without light stimulation and the change in response caused by the light stimulation (*SI Appendix*, Fig. S8*E*). In both light-off and light-on trials, classification performance improved from chance (50%) before odor stimulation to approximately 90% during the odor delivery period (*SI Appendix*, Fig. S8*F*).

Serotonergic Neurons Are Activated during a Go/No Go Odor Discrimination Task. Finally, if serotonergic modulation plays an important role in odor responses, the serotonergic neurons should be activated during odor discrimination. To test this hypothesis, we performed a go/no go task experiment. We recorded the responses of the serotonergic neuron population using fiber photometry of GCaMP6s fluorescence in a go/no go odor discrimination task (*SI Appendix*, Fig. S9*A*). The performance of mice improved from near chance levels (50% correct) in block one to well above the learning threshold (80% correct) in block five (*SI Appendix*, Fig. S9*B*). Odors evoked strong responses in the population of serotonergic neurons during both the learning and proficient states (*SI Appendix*, Fig. S9 *C* and *D*). Interestingly, the response difference between the two odors was small before the mice had learned to discriminate them; however, the difference increased once the mice had learned to discriminate the odors (*SI Appendix*, Fig. S9*E*). These data demonstrate that serotonergic neurons are activated by the odors during an odor discrimination task, and that

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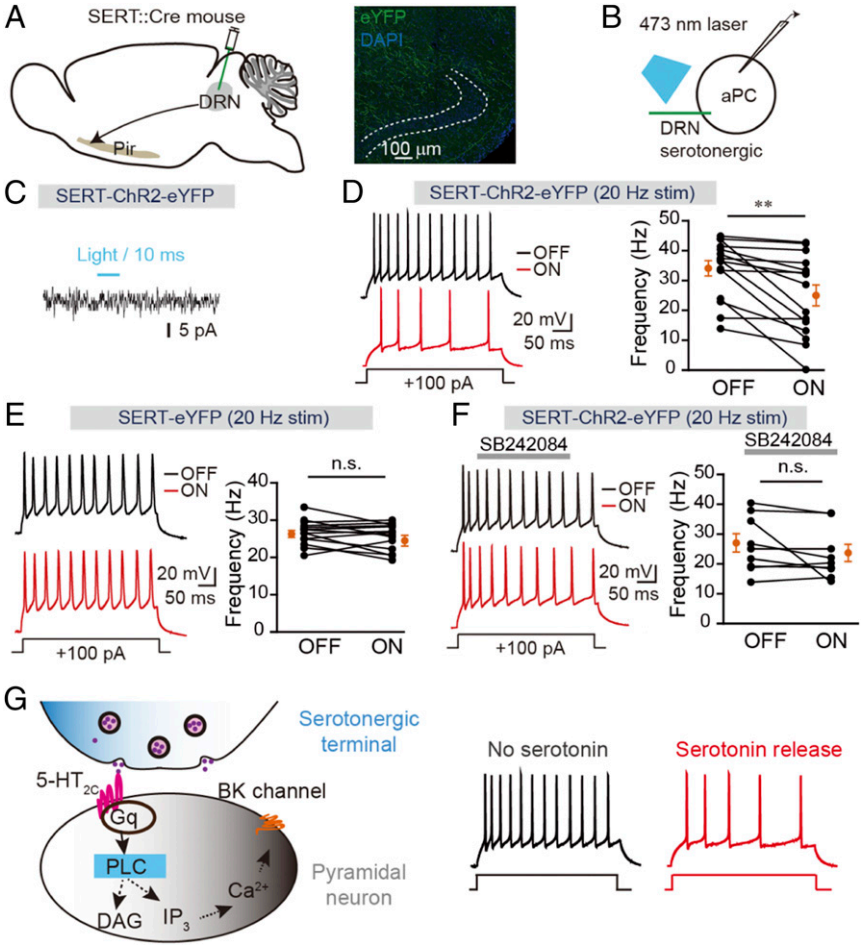

Fig. 5. ChR2 activation of serotonergic fibers reduces the excitability of pyramidal neurons in the aPC. (*A*) Fluorescence image of an aPC slice indicating ChR2 expression in the aPC. (*B*) Schematic of the electrophysiological recording experiment. Light illumination was applied to aPC slices and activity was recorded from pyramidal neurons. (*C*) Whole-cell voltage-clamp recording from a pyramidal neuron at −70 mV showing a lack of postsynaptic currents with optogenetic activation of serotonergic fibers. Blue bars depict the timing of the light stimulation. (*D*) Typical raw traces and average frequency of current-evoked APs recorded from a pyramidal neuron before and after optogenetic activation of serotonergic fibers. Paired *t* test, $^{**}P = 0.002$, $t_{(14)} = 3.79$. (*E*) Typical raw traces and averaged frequency of current-evoked APs before and after light stimulation in SERT-Cre mice injected with AAV-DIO-eYFP (control, lacking ChR2). Paired *t* test, $P = 0.15$, $t_{(13)} = 1.53$. (*F*) Typical raw traces and average frequency of current-evoked APs before and after optogenetic activation of serotonergic fibers in the presence of SB242084 (a 5-HT_{2C} receptor blocker) in SERT-Cre mice injected with AAV-DIO-ChR2-eYFP. Paired *t* test, $P = 0.18$, $t_{(8)} = 1.49$. (*G*) Schematic summary of the mechanism underlying inhibitory serotonergic regulation of pyramidal neurons in the aPC.

the odor-evoked response of the serotonergic neuron population is plastic during the learning process.

Discussion

In this study, we investigated how serotonergic neurons modulate the activity of pyramidal neurons in the aPC. Our data indicate that 5-HT reduces the excitability of pyramidal neurons via 5-HT_{2C} receptors, and that this inhibition is likely a direct effect independent of GABAergic interneurons. Further experiments demonstrated that the inhibitory effect of 5-HT requires the PLC signaling pathway and BK channels. The inhibitory effect of 5-HT on pyramidal neurons was further confirmed by optogenetic activation of serotonergic fibers in the aPC. Moreover, in vivo fiber photometry studies in awake mice demonstrated that optogenetic activation of serotonergic neurons decreases odor-evoked responses but has no effect on the spontaneous activity of pyramidal neurons at the cell population level. These findings provide direct evidence for serotonergic modulation of neural activity in the aPC both in vitro and in vivo and identify the mechanism responsible for this modulation.

Several previous studies have shown that direct application of 5-HT inhibits the activity of pyramidal neurons in the piriform cortex, but that this inhibition is an indirect effect mediated by GABAergic interneurons (37–39). However, a recent study demonstrated that activation of 5-$HT_{2A/C}$ receptors inhibits pyramidal neurons in the piriform cortex but has no effect on interneurons (40). This is consistent with our present findings indicating a direct inhibitory effect of 5-HT on aPC pyramidal neurons via 5-HT_{2C} receptors (Fig. 1), which we further confirmed by optogenetic activation of serotonergic fibers in the aPC (Fig. 5). The direct effect of 5-HT on pyramidal neurons is supported by several lines of evidence (Fig. 2), including the suppression of current-evoked APs in pyramidal neurons was not blocked by previous $GABA_A$ receptor inhibition; neither the amplitude nor the frequency of mIPSC in pyramidal neurons was changed by 5-HT; and 5-HT had no effect on current-evoked APs in GABAergic interneurons.

Both the piriform cortex and the OB are important olfactory centers and receive extensive serotonergic input (16, 41–43). Interestingly, contrary to our finding of a direct, inhibitory effect of 5-HT on pyramidal neurons in the aPC, serotonergic input to the

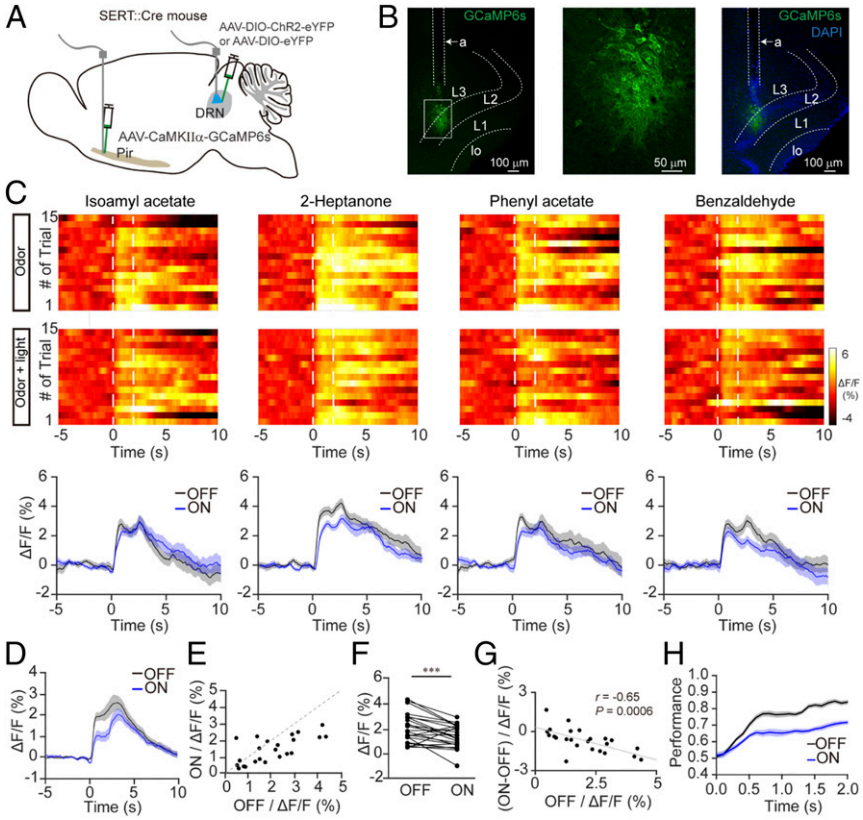

Fig. 6. Optogenetic activation of DRN serotonergic neurons decreases odor-evoked responses in aPC pyramidal neurons. (*A*) Schematic of virus injection and fiber implantation. In SERT-Cre mice, AAV-DIO-ChR2-eYFP was injected into the DRN, and AAV-CaMKIIα-GCaMP6s was injected into the aPC. Optical fibers were implanted in both regions. (*B*) Expression of GCaMP6s in the aPC. The location of fiber implantation is indicated by "a". (*C*) Heatmaps and trial-averaged traces of ΔF/F induced by four odors with and without light stimulation from a representative mouse. Dashed lines in heatmaps represent the 2-s odor and light stimulation. (*D*) Averaged traces of odor responses across all animal-odor pairs ($n = 24$ animal-odor pairs from 6 mice). (*E* and *F*) Comparison of averaged ΔF/F (0 to 2 s) between light-on trials and light-off trials. (*E*) Each dot represents one animal-odor pair. (*F*) Paired *t* test, $^*P = 0.001$, $t_{(23)} = 3.62$. (*G*) Relationship between the change in ΔF/F with photostimulation and ΔF/F in the light-off condition. Linear regression, $^{***}P = 0.0006$, $r = -0.65$. (*H*) Classification performance for odor identity with and without photostimulation for four odor presentations.

OB exerts an excitatory effect on mitral/tufted cells both directly and indirectly, with the indirect effects mediated by GABAergic interneurons (13, 31). Therefore, although serotonergic neurons innervate both the OB and piriform cortex and dramatically modulate activity in both of these olfactory centers, the effects and the underlying neural mechanisms are strikingly different. This suggests that serotonergic modulation of the olfactory system is complex and may have different effects on different olfactory functions. This is supported by our recent study showing that the OB and the aPC use different strategies to represent odor information in awake behaving mice (44).

What is the signaling pathway underlying the effect of 5-HT on the piriform cortex? Many 5-HT receptors are coupled to stimulatory G proteins and signal through the downstream PLC pathway (32). PLC selectively catalyzes the hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to form DAG and IP_3, inducing Ca^{2+} release from intracellular stores. Thus, the PLC signaling pathway downstream of 5-HT receptors is well established; however, the final target of this pathway is largely unknown. Previous studies have shown that $5\text{-}HT_{2C}$ receptors exert excitatory effects on neural activity through their eventual influence on several ion channels, including activation of Ca^{2+}-activated chloride channels and TRPC (putative transient receptor potential C) channels or inhibition of K^+ channels (45–48). Elevated K^+ channel activity has also been observed after application of 5-HT (35). Since our present study found that the inhibitory effect of 5-HT on pyramidal neurons was independent of GABAergic transmission, we speculated that the inhibitory effect may be mediated by BK channels, activated by Ca^{2+} release induced by the PLC signaling pathway. This speculation was confirmed by our pharmacologic experiments indicating that PLC and BK channels are required for the inhibitory effects of 5-HT in pyramidal neurons (Fig. 3).

To further test this, we recorded K^+ currents and found that K^+ channels are activated on application of 5-HT, and that this activation is blocked by an inhibitor of BK channels (Fig. 4). Therefore, the mechanism underlying 5-HT modulation of neural activity in aPC pyramidal neurons is well identified in our study. In brief, 5-HT binds to $5\text{-}HT_{2C}$ receptors, which are coupled to a G protein that activates the PLC signaling pathway to release DAG and IP_3, and then IP_3 induces Ca^{2+} release, which ultimately activates BK channels (Fig. 5*G*).

What is the functional significance of serotonergic modulation of the olfactory system? Consistent with our in vitro results demonstrating an inhibitory effect of serotonergic input on pyramidal neurons, our in vivo experiments using fiber photometry indicate that optogenetic activation of serotonergic neurons decreases odor-evoked responses and the ability of pyramidal neurons to discriminate among odors. Previous studies have indicated that serotonergic modulation is important for olfactory learning (10, 11, 49). However, studies targeting serotonin synthesis found that a lack of serotonin had no effect on performance in several olfactory behavioral assays in mice (50). These

conflicting results suggest that the role of 5-HT in olfaction is likely complex.

Interestingly, although we found that optogenetic activation of serotonergic inputs decreased the odor-evoked response of pyramidal neurons in awake mice, a recent study reported the converse in anesthetized mice; serotonergic activation inhibited spontaneous firing but not odor-evoked responses of pyramidal neurons (51). Therefore, it is likely that the effect of serotonergic modulation on the aPC is dependent on the brain state of the animal. This is consistent with the fact that the neural representation of odor information by olfactory centers is largely dependent on behavioral and brain states (52). However, it is also possible that this discrepancy is due to the different neural signals recorded. Lottem et al. (51) recorded electrophysiological activity from single cells in their study, whereas we recorded the calcium signal at the cell population level. Further studies using spike recording or calcium recording in anesthetized and awake states are needed to determine whether the effect of serotonergic activation is truly brain state-dependent.

In summary, the present study reveals the underlying mechanism by which serotonergic input modulates the activity of pyramidal neurons in the aPC, and the functional significance of this modulation in awake mice. These findings are important for understanding how the olfactory system is modulated by centrifugal inputs and the role of the serotonergic system in the brain as a whole.

Materials and Methods

C57BL/6J and SERT-Cre mice were used in this study. The SERT-Cre mouse line (27) was used to express channelrhodopsin-2 (ChR2) selectively in serotonergic neurons. All of the mice were bred in the animal facilities of the Xuzhou Medical University and housed in a vivarium on a 12:12-h light:dark cycle with food and water ad libitum. After surgery, mice were housed individually for at least 1 wk to allow for recovery before further experiments. All experimental procedures were carried out in accordance with protocols submitted to and approved by the Xuzhou Medical University Institutional Animal Care and Use Committee. Experimental details are provided in *SI Appendix, Materials and Methods*.

All data discussed in the paper are available at https://github.com/PenglaiLiu/Data-with-figure-information/tree/master.

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