Systems/Circuits

Serotonergic Regulation of Excitability of Principal Cells of the Dorsal Cochlear Nucleus

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The dorsal cochlear nucleus (DCN) is one of the first stations within the central auditory pathway where the basic computations underlying sound localization are initiated and heightened activity in the DCN may underlie central tinnitus. The neurotransmitter serotonin (5-hydroxytryptamine; 5-HT), is associated with many distinct behavioral or cognitive states, and serotonergic fibers are concentrated in the DCN. However, it remains unclear what is the function of this dense input. Using a combination of *in vitro* electrophysiology and optogenetics in mouse brain slices, we found that 5-HT directly enhances the excitability of fusiform principal cells via activation of two distinct 5-HT receptor subfamilies, 5-HT$_{2A/2C}$R (5-HT$_{2A/2C}$ receptor) and 5-HT$_7$R (5-HT$_7$ receptor). This excitatory effect results from an augmentation of hyperpolarization-activated cyclic nucleotide-gated channels (I_h or HCN channels). The serotonergic regulation of excitability is G-protein-dependent and involves cAMP and Src kinase signaling pathways. Moreover, optogenetic activation of serotonergic axon terminals increased excitability of fusiform cells. Our findings reveal that 5-HT exerts a potent influence on fusiform cells by altering their intrinsic properties, which may enhance the sensitivity of the DCN to sensory input.

Key words: auditory; serotonin; tinnitus

Introduction

The serotonergic system modulates diverse physiological and behavioral functions, such as sleep, feeding, nociception, mood, and emotions (Lucki, 1998). Serotonergic dysfunction has been implicated in a variety of psychiatric disorders, including depression, anxiety, schizophrenia, Parkinson's disease, and Alzheimer disease (Meltzer et al., 1998; Jones and Blackburn, 2002; Huot et al., 2011). The majority of serotonergic neurons are found in the dorsal and medial raphe nuclei, sending widespread projections to many brain regions including the auditory system (Dahlström and Fuxe, 1964; Steinbusch, 1981; Descarries et al., 1982), with dense innervation in the cochlear nucleus (Steinbusch, 1981; Thompson and Thompson, 2001). Although the physiological function of 5-HT in the auditory system is unclear, it may differentially modulate the response to simple and complex sounds such as vocalizations (Ebert and Ostwald, 1992; Revelis et al., 1998; Hurley and Pollak, 1999, 2005; Hurley and Hall, 2011; Wood et al., 2013). Dysfunction of the serotonergic system is implicated in the generation or perception of tinnitus (Marriage and Barnes, 1995; Simpson and Davies, 2000; Salvinelli et al., 2003; Caperton and Thompson, 2010). Moreover, it has been suggested that selective 5-HT reuptake inhibitors (SSRIs) often used in the treatment of depression and anxiety disorders (Stark et al., 1985; Wong et al., 1995), might be also used to treat tinnitus (Shemen, 1998; Folmer and Shi, 2004; Fornaro and Martino, 2010; Oishi et al., 2010; Baldo et al., 2012). Therefore, understanding the normal physiological effects of 5-HT in auditory system may provide insight into normal brain function and suggest new approaches in the treatment of tinnitus.

The role of 5-HT in auditory system may be of particular interest in the dorsal cochlear nucleus (DCN), where auditory and multisensory signals converge at fusiform principal cells. Among proposed functions of the DCN is sound source localization and orientation to sounds of interest (Sutherland et al., 1998; Imig et al., 2000; May, 2000; Oertel and Young, 2004), and is a proposed site of central tinnitus generation and modulation (Levine, 1999; Brozoski et al., 2002, 2012; Kaltenbach et al., 2004; Wang et al., 2009; Middleton et al., 2011; Dehmel et al., 2012; Koehler and Shore, 2013; Li et al., 2013; Luo et al., 2014). Moreover, the DCN receives a dense serotonergic innervation, originating predominantly from the dorsal and medial raphe nuclei (Parent et al., 1981; Steinbusch, 1981; Willard et al., 1984; Klepper and Herbert, 1991; Thompson et al., 1994, 1995; Hurley and Thompson, 2001; Thompson and Thompson, 2001), and contains multiple subtypes of 5-HT receptors, including 5-HT$_{1A}$R, 5-HT$_{2A}$R, and 5-HT$_{2C}$R (Pazos et al., 1985; Thompson et al., 1994; Wright et al., 1995; Cornea-Hébert et al., 1999; Thompson and Wiechmann, 2002), and measureable levels of 5-HT (Cransac et al., 1995). Yet, despite the potential physiological functions of serotonergic raphe-DCN pathway, it is unclear what 5-HT does in the DCN. Here, we found that 5-HT activates

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$5\text{-HT}_{2A/2C}$R and 5-HT_7R, and thereby exerts excitatory control of fusiform cells by altering their intrinsic properties; thus, 5-HT regulates the output of a primary auditory nucleus.

Materials and Methods

Slice preparation. All procedures were approved by the Oregon Health and Science University's IACUC. C57BL/6J wild-type mice of both sexes at P16–P49 were used for the majority of the experiments. For optogenetic experiments, the mice were B6; SJL-Tg (Tph2-COP4*H13R/EYFP)5Gfng/J (Tph2-ChR2-YFP, JAX stock #014555; Zhao et al., 2011). Mice were anesthetized by isoflurane inhalation and then decapitated. Brains were quickly removed and placed in a vibratome (Leica). Coronal slices containing the DCN (260–300 μm) were prepared in an ice-cold cutting solution containing the following (in mM): 87 NaCl, 25 $NaHCO_3$, 25 glucose, 75 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, and bubbled with 95% O_2/5% CO_2, and then the slices were maintained for 1 h in warm (~33°C) ACSF solution containing the following (in mM): 130 NaCl, 2.1 KCl, 1.7 $CaCl_2$, 1.0 $MgSO_4$, 1.2 KH_2PO_4, 20 $NaHCO_3$, 3 Na-HEPES, 11 glucose; bubbled with 95% O_2/5% CO_2, 300–310 mOsm. After 1 h recovery, slices were maintained in the same solution at room temperature (~22°C) until recording.

Electrophysiology. Slices were transferred into a recording chamber, and continuously perfused with ~33°C 95% O_2/5% CO_2 oxygenated ACSF at ~2 ml/min. The DCN neurons were visualized by Dodt contrast optics with a 60× water-immersion objective on the stage of an upright microscope (Olympus, BX51W). Fusiform and cartwheel cells were identified based on their location, morphology and electrophysiological properties (Manis et al., 1994; Zhang and Oertel, 1994; Golding and Oertel, 1997; Tzounopoulos et al., 2004). Synaptic transmission was blocked in most experiments by adding 10 μM NBQX, 5 μM R-CPP, 1 μM strychnine, and 10 μM SR95531. For whole-cell recordings, pipettes were filled with a solution containing the following (in mM): 113 K-gluconate, 9 HEPES, 2.75 $MgCl_2$, 1.75 $MgSO_4$, 0.1 EGTA, 14 $Tris_2$-phosphocreatine, 4 Na_2-ATP, 0.3 Tris-GTP; osmolality adjusted to ~290 mOsm with sucrose, pH adjusted to 7.25 with KOH. The membrane potential values are corrected for a 10 mV junction potential. For loose cell-attached recordings, pipettes were filled with a normal ACSF solution. Patch pipettes (3–5 MΩ) were pulled from borosilicate glass (WPI). For all voltage-clamp experiments, series resistance (<20 MΩ) was compensated by 65–80% and membrane potential was held constant at −70 mV except for experiments shown in Figure 4. Experiments were excluded if series resistance varied >20% over the course of the recording. In current-clamp recordings, the pipette capacitance was canceled and bridge balance was maintained.

For microiontophoresis, methods were similar to a previous study (Perrier and Cotel, 2008): a micropipette (40–80 MΩ) was filled with 50–100 mM serotonin hydrochloride dissolved in 165 mM NaCl, and the pH adjusted to 4.5 using HCl. Retaining current of −20 nA were applied to micropipette to reduce drug leakage between ejection periods. 5-HT was ejected by applying positive currents (+40–80 nA) for 3–5 s. In control experiments, iontophoresis of drug carrier saline (165 mM NaCl) alone failed to evoke any detectable currents using the same iontophoresis current level as using those to eject 5-HT. The micropipette was positioned close to the soma of the recorded neurons.

Optogenetic stimulation was performed as previously described (Apostolides and Trussell, 2013). Briefly, wide-field activation of ChR2 was achieved with blue light from a LED (470 nm) transmitted through the fluorescent light path of the microscope. Photostimulation was delivered at 3 min intervals, and the maximal light intensity reaching the brain slice was ~15 mW/mm^2.

Data acquisition and analysis. Data were collected using a Multiclamp 700B amplifier and pClamp 10 software (Molecular Devices). Signals were digitized at 10–50 kHz with a Digidata 1322A (Molecular Devices) and low-pass filtered at 1–10 kHz for offline analysis.

To assess the effects of 5-HT on activation parameters of I_h, an activation curve was obtained by fitting averaged normalized I_h currents with a Boltzmann equation of the form $I/I_{max} = 1/[1 + \exp(V_m - V_{1/2})/s]$ in which I/I_{max} is the normalized current, V_m is the membrane potential, $V_{1/2}$ is the potential at half-maximal conductance and s is the slope factor. The time constant of I_h activation was determined by fitting the currents evoked by a step to −80 or −90 mV potential with a double-exponential function of the form: $I_t = I_{ss} + I_{fast} \times \exp(-t/\tau_{fast}) + I_{slow} \times \exp(-t/\tau_{slow})$, where I_t is the current amplitude at time t, I_{ss} is the steady-state current at a given potential, I_{fast} and I_{slow} denote the amplitude of the slow and fast current components, and τ_{fast} and τ_{slow} are the corresponding time constants for I_h activation.

All data are presented as mean ± SEM unless specified otherwise, and statistical significance was assessed using Student's t tests as appropriate; $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Immunohistochemistry. Tph2-ChR2 transgenic mice (P20–P30) were deeply anesthetized with isoflurane and were transcardially perfused with warm (~38C°) PBS, pH 7.4, followed by ice-cold 4% paraformaldehyde (PFA) in PBS. After perfusion, mouse brains were dissected out and postfixed in 4% PFA overnight at 4°C. The brains were rinsed thoroughly in PBS, pH 7.4, embedded in 4% agar, and sliced into 30 μm coronal sections using a vibratome (Leica VT1000S). Next, sections were washed in PBS for 30 min and subsequently permeabilized in 1% Triton X-100 in PBS for 1 h. After again being washed in PBS for 30 min, sections were incubated for 30 min in blocking solution consisting 2% fish gelatin in PBS. The sections were then incubated with anti-GFP AlexaFluor 488-conjugated antibody (10 μg/ml; Invitrogen) in blocking solution overnight at 4°C. After being washed in PBS for 30 min, sections were postfixed in 4% PFA for 1 h. After being washed again in PBS, sections were then mounted on slides and coverslipped with mounting medium. Images were acquired using laser-scanning confocal microscopy (Olympus FV1000).

Reagents. 5-HT was applied by either bath application or iontophoresis, and all other drugs were applied either in the bath or through the recording pipette. NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[f]quinoxaline-7-sulfonamide), R-CPP ((R)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid), SR95531 (Gabazine), and TTX (tetrodotoxin) were obtained from Abcam. 5-HT (serotonin hydrochloride), ketanserin, MDL-11939, SB-242084, α-methyl-5-HT, SB-269970, WAY-100135, ZD7288, SQ22536, 8-Br-cAMP, PKC_{19-31}, genistein, PP1 (1-(1,1-dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine), and PP2 (3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine) were purchased from Tocris Bioscience. All other drugs and chemicals were from Sigma-Aldrich.

Results

5-HT regulates excitability of fusiform cells

To explore the effects of 5-HT in DCN, whole-cell and loose cell-attached recordings were obtained from acute slices of mouse brainstem containing DCN. Under current-clamp, bath application of 10 μM 5-HT produced a significant depolarization of the resting membrane potential (control: −68.1 ± 1.5 mV, 5-HT: −63.0 ± 1.4 mV; $p < 0.001$, paired t test, $n = 8$). This depolarization led to enhancement of spontaneous spike activity (control: 0.2 ± 0.1 Hz, 5-HT: 3.9 ± 1.1 Hz; $p < 0.01$, $n = 10$; Fig. 1*A*,*B*). These (and subsequent) experiments were performed in the presence of blockers of ionotropic glutamate receptors, $GABA_A$ receptors, and glycine receptors, except as otherwise noted, and thus the excitatory actions of 5-HT are likely to be postsynaptic in nature. Moreover, the depolarization produced by 5-HT persisted in the presence of 1 μM TTX or blockers of synaptic transmission, again indicating a postsynaptic mechanism of 5-HT on fusiform cells. The 5-HT-elicited increase in firing rate was reversed by returning the membrane potential to control levels with a negative bias current (data not shown), indicating that it was the 5-HT-induced depolarization that initiated spike firing, probably at a region at or close to the cell body. To examine the nature of 5-HT actions without any disturbance to the intracellular environment, loose cell-attached voltage-clamp recordings were performed in fusiform cells. As with whole-cell recording, 5-HT (10 μM) significantly increased the spike rate (baseline: 5.2 ± 1.7 Hz, 5-HT, 10.6 ± 2.4 Hz; $p < 0.01$, $n = 12$; Fig. 1*C*,*D*). To examine how 5-HT affects the sensitivity of fusiform cells to current stimuli, a series of 1 s current steps were injected into fusiform cells, starting with −30 pA and incrementing by 20 pA, and spike rate at each current level was measured with and without 5-HT. These experiments showed that 5-HT shifted the input-output relation to the left in a dose-dependent manner (Fig. 1*E*,*F*). To determine the specificity of this effect we also looked at the action of 5-HT on cartwheel interneurons, which provide potent inhibition to fusiform cells, and found that no detectable effects on spike rate or membrane potential (data not shown).

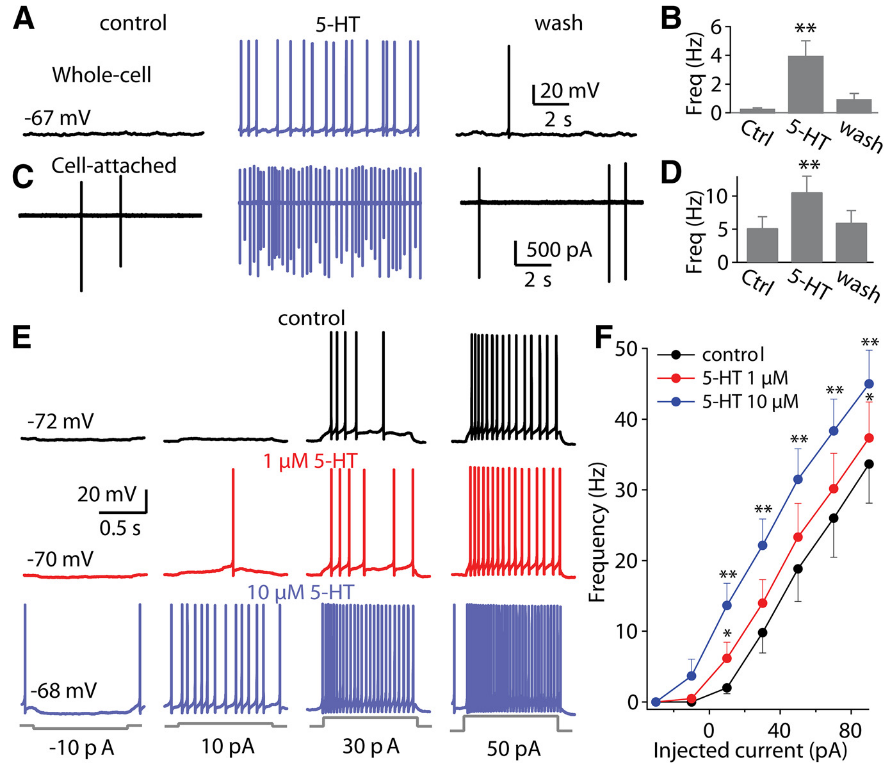

Figure 1. 5-HT directly enhances the excitability of fusiform cells. ***A***, ***C***, 5-HT depolarized the membrane potential and increased spontaneous spike rate under whole-cell (***A***) and cell-attached (***C***) recordings. ***B***, ***D***, 5-HT significantly increased spontaneous spike rate both under whole-cell ($n = 10$) and cell-attached ($n = 12$) conditions. ***E***, ***F***, Current-clamp recordings from a fusiform cell (control: black traces; in the presence of 1 and 10 μM 5-HT: red and blue traces, respectively), showing responses to increasing current injections into soma (starting with −30 pA and increasing by 20 pA, 1 s duration). ***E***, Representative traces of action potential firing elicited by somatic injection of current pulses illustrating the effects of 1 or 10 μM 5-HT. ***F***, Firing frequency as a function of injection current amplitude ($n = 8$). Negative bias current (∼−20 pA under the threshold of action potential) was used to prevent or reduce spontaneous firing. Error bars represent ± SEM; *$p < 0.05$, **$p < 0.01$ (paired *t* test, unless indicated otherwise).

5-HT-evoked response is mediated by 5-$HT_{2A/2C}$R and 5-HT_7R

Consistent with the current-clamp data, fusiform cells voltage-clamped at holding potentials of −70 mV or close to the resting membrane potential, responded to bath application (1, 5, or 10 μM) or iontophoresis (50 mM) of 5-HT with a slow, steady, and reversible inward current (Fig. 2*A*,*B*). The pharmacological identification of 5-HT receptor subtypes mediating the excitatory effects of 5-HT was investigated by using specific agonists and antagonists for 5-HT receptors in voltage-clamped cells. Previous studies have shown that 5-HT can produce a slow depolarization of the resting membrane potential by activation of 5-HT_2R (5-HT_2 receptor) in cortical neurons (Pierce and Peroutka, 1990; Araneda and Andrade, 1991; Tanaka and North, 1993; Zhang, 2003). Moreover, 5-HT_{2A}R and 5-HT_{2C}R are present in cochlear nucleus (Pazos et al., 1985; Thompson et al., 1994; Wright et al., 1995; Cornea-Hébert et al., 1999), We therefore asked whether these receptors mediate the 5-HT response in DCN. Application of the 5-HT_{2A}R antagonist ketanserin at 10 μM largely blocked the inward current elicited by bath application (10 μM) or iontophoresis (50 mM) of 5-HT (control: −60.2 ± 9.5 pA, ketanserin: −2.2 ± 2.6 pA; $p < 0.001$, $n = 9$; Fig. 2*A1*,*A2*,*C*), suggesting that the inward current may be mediated by 5-HT_{2A}R. However, 2 μM ketanserin only partially blocked the response (data not shown), and a similar partial block was achieved with the selective 5-HT_{2A}R antagonist MDL-11939 (2 μM; 45.3 ± 8.6% of control, $p < 0.05$, $n = 11$; Fig. 2*A3*,*C*). Moreover, the 5-HT_{2C}R antagonist SB-242084 (2 μM) slightly suppressed the 5-HT response (86.0 ± 7.9% of control, $p < 0.05$ $n = 6$; Fig. 2*C*). Additionally, application of 25 μM α-methyl-5-HT, a selective agonist of 5-HT_2R, induced an inward current (−37.0 ± 6.8 pA; $n = 4$; Fig. 2*B*) similar to that of 5-HT. These data suggest that a combination of 5-$HT_{2A/2C}$Rs contribute to the 5-HT-evoked response.

However, the inward current was not fully blocked by the 5-HT_{2A}R antagonist MDL-11939 plus 5-HT_{2C}R antagonist SB-242084 (31.5 ± 13.9% of control, $p < 0.05$, $n = 3$). Previous studies have shown that ketanserin also exhibits affinity for 5-HT_7R (5-HT_7 receptor) (Shen et al., 1993; Jasper et al., 1997; Adham et al., 1998). Because ketanserin at high concentration (10 μM) could almost completely block the 5-HT-evoked response, we wondered whether ketanserin was also blocking 5-HT_7R response to 5-HT. Indeed, it is well established that activation of 5-HT_7R can mediate a depolarizing effect on some central neurons (Cardenas et al., 1999; Chapin and Andrade, 2001a, b; Béïque et al., 2004). Therefore, we examined the effects of SB-269970, a potent and selective antagonist for 5-HT_7R, on the 5-HT-evoked current. We found that SB-269970 (1 μM) partially suppressed the 5-HT-evoked current (43.9 ± 11.3% of control; $p < 0.01$, $n = 6$; Fig. 2*A4*,*C*). In addition, coapplication of SB-269970 and MDL-11939 completely blocked the 5-HT current (2.0 ± 9.4% of control; $p < 0.01$, $n = 4$; Fig. 2*C*). These data suggest that the 5-HT response results from the coactivation of 5-$HT_{2A/2C}$R and 5-HT_7R. Immunochemical studies have shown that 5-HT_{1A}R is also expresses in the cochlear nucleus (Pazos et al., 1985; Wright et al., 1995; Thompson and Wiechmann, 2002), although it is unclear in what cell types. However, WAY-100135 (10 μM), a selective antagonist for 5-HT_{1A}R, did not affect the 5-HT current (100.6 ± 15.1% of the control; $p > 0.05$, $n = 3$), suggesting that 5-HT_{1A}R are not involved in the effects of 5-HT on fusiform cells. Overall, these results indicate that activation of both 5-$HT_{2A/2C}$R and 5-HT_7R result in excitation of fusiform cells.

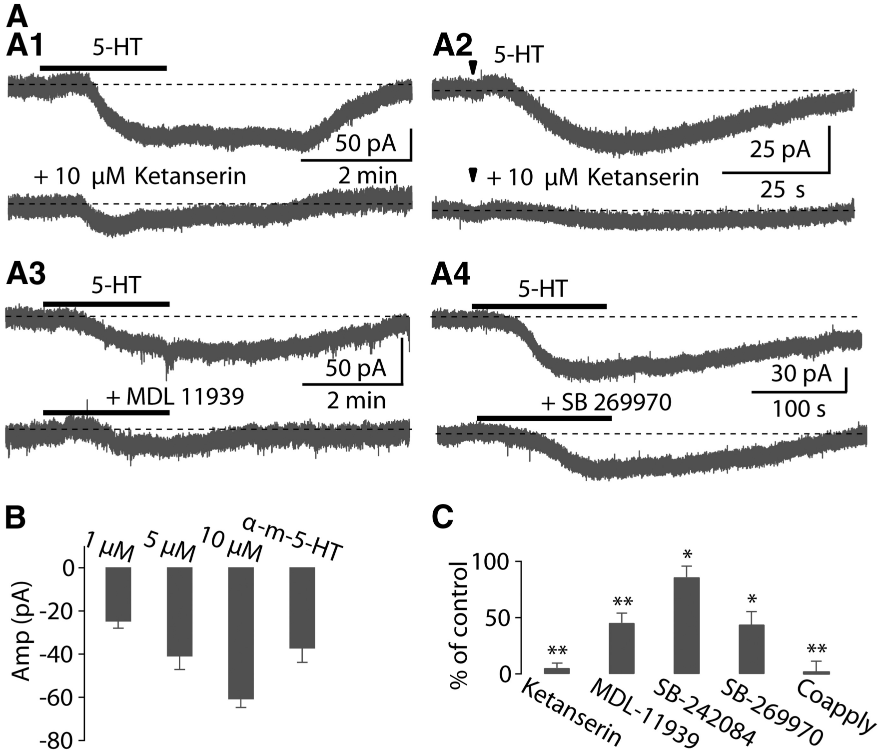

Figure 2. 5-$HT_{2A/2C}$R and 5-HT_7R are largely responsible for the inward current produced by 5-HT. ***A***, Under voltage-clamp, representative traces of slow inward currents elicited by bath application of 10 μM or iontophoresis of 50 mM 5-HT were almost completely abolished by 10 μM ketanserin (***A1***, ***A2***), partially blocked by 2 μM MDL-11939 (***A3***), or 1 μM SB-269970 (***A4***). Iontophoresis of 5-HT indicated by arrowhead. In this figure and following figures, gray dashed lines denote basal holding currents or the resting membrane potential before application of 5-HT. ***B***, Summary of currents evoked by 5-HT (1 μM, $n = 7$; 5 μM, $n = 7$; 10 μM, $n = 35$), and α-methyl-5-HT (25 μM, $n = 4$). ***C***, Summary of effects of ketanserin ($n = 9$), MDL-11939 ($n = 11$), SB-242084 ($n = 6$), SB-269970 ($n = 6$), and coapplication of SB-269970 plus MDL-11939 ($n = 4$) on the 5-HT-induced current. Error bars are ± SEM.

HCN channels are the downstream targets of serotonergic signaling

Fusiform cells express a wide array of ion channels, including Na^+, Ca^{2+}, K_{ir}, K_A, KCNQ, and HCN channels, the latter being the channel subtype that generates the I_h current (Hirsch and Oertel, 1988; Harasztosi et al., 1999; Kanold and Manis, 1999; Molitor and Manis, 2003; Pál et al., 2003; Leao et al., 2012). To determine what ion channels are responsible for the 5-HT response, the effects of blockers of Na^+, Ca^{2+}, K^+, and HCN channels were tested on the 5-HT-induced current. Of these blockers, TTX, Cd^{2+}, Ba^{2+}, and XE991 failed to reduce the 5-HT-induced current (data not shown). However, Cs^+ and ZD7288, selective blockers of HCN channels, completely inhibited the 5-HT response (Fig. 3). Moreover, bath application of either 2 mM Cs^+ or 10 μM ZD7288 alone induced an outward shift of holding current under voltage-clamp (Fig. 3*A*,*B*). Because 5-HTR (5-HT receptor) antagonists by themselves had no effect on holding current, this result indicates that HCN channels are active partially at rest, and indeed contribute to the resting membrane potential and regulate the excitability of fusiform cells, consistent with the previous studies (Pál et al., 2003; Apostolides and Trussell, 2014). We further compared the inward current evoked by 5-HT before and after application of 2 mM Cs^+ or 10 μM ZD7288. The inward current evoked by 5-HT was completely blocked by Cs^+ (control: −68.8 ± 19.5 pA, Cs^+: −5.7 ± 2.3 pA; $p < 0.05$, $n = 5$; Fig. 3*C*,*D*) or ZD7288 (control: −64.3 ± 8.0 pA, 10 min after applying ZD7288: −2.2 ± 3.0 pA; $p < 0.001$, $n = 7$). Similarly, under current-clamp conditions, bath application of either Cs^+ or ZD7288 alone caused a hyperpolarization of the membrane potential, and blocked spontaneous spiking firing, and also abolished the effects of 5-HT on the membrane potential or spike activity (Fig. 3*E*,*F*). These data indicate that HCN channels are the primary downstream targets for these observed excitatory actions of 5-HT on fusiform cells.

We next explored how 5-HT affected the biophysical properties of I_h by measuring the voltage dependence of I_h from the amplitude of tail current relaxations. The experiments were performed in ACSF that contains 1 μM TTX and 200 μM Ba^{2+}, to block Na^+ channels and inward rectifier K^+ channels. Activation curves for I_h were constructed by applying a series of voltage steps from a holding potential at −60 mV to various levels to activate I_h, and then activation voltage steps were followed by a test pulse to −75 mV with minimal contamination of the tail currents. The amplitude of tail current is proportional to the level of I_h conductance for a given prepulse voltage step. Activation curves for I_h were obtained by plotting tail current amplitudes against initial step potential, and then fitting with a Boltzmann function (see Materials and Methods). Application of 10 μM 5-HT resulted in a 5.6 mV positive shift of the activation curve of I_h on the voltage axis (control: −93.9 ± 2.2 mV, 5-HT: −88.3 ± 1.8 mV; $p < 0.05$, *t* test, $n = 7$; Fig. 4*A*–*C*) without significantly affecting the amplitude of maximal tail current (control: −418.1 ± 84.7 pA, 5-HT: −439.8 ± 96.6; $p > 0.05$, $n = 7$), suggesting that the enhancement of I_h resulted from a shift in the activation properties of the I_h, but not from an increase in the maximal level I_h activation. Accompanying this shift in the activation curve of I_h was a reduction in the time constant for I_h activation at any given potential by 5-HT. We characterized this effect of 5-HT by applying a 10 s voltage step to fully activate the I_h at −80 mV and −90 mV, and then comparing the time constant of I_h before and after 5-HT application. The activation time course of I_h was significantly faster after applying 5-HT at holding potentials of −80 mV (τ_{fast} control: 695.7 ± 28.3 ms, 5-HT: 538.8 ± 55.7 ms, $p < 0.01$; τ_{slow} control: 5.7 ± 0.9 s, 5-HT: 4.2 ± 0.5 s, $p < 0.05$; $n = 4$; Fig. 4*D*,*E*), or −90 mV (τ_{fast} control: 544.2 ± 27.6 ms, 5-HT: 421.6 ± 58.6 ms, $p < 0.05$; τ_{slow} control: 4.4 ± 0.3 s, 5-HT: 3.0 ± 0.2 s, $p < 0.01$; $n = 4$). These results indicate that excitation of fusiform cells by 5-HT is accompanied by an acceleration of HCN channel gating and shift in voltage sensitivity.

Serotonergic regulation of excitability is G-protein-dependent and involves cAMP and Src kinase signaling pathways

We next determined what signaling pathways couple 5-HTR to I_h. Both 5-$HT_{2A/2C}$R and 5-HT_7R are G-protein-coupled receptors (GPCRs). To confirm that G-proteins are required for the 5-HT-induced response, we replaced the GTP in the internal recording solution with 1.5 mM GTP-γ-S, a nonhydrolysable

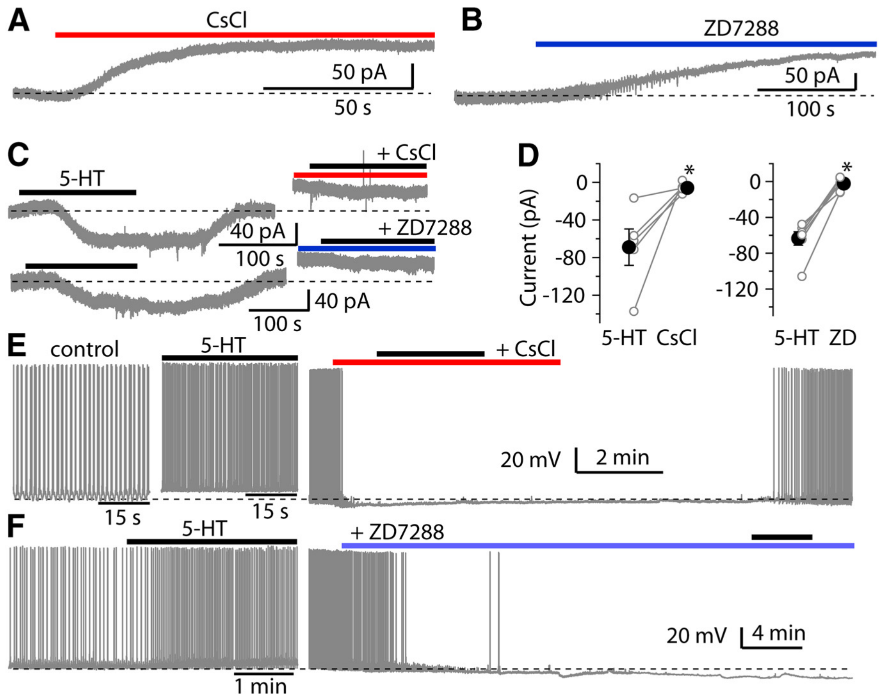

Figure 3. HCN channels are important determinants of intrinsic excitability and the downstream targets of 5-HT-induced response. ***A***, ***B***, Representative traces showing that bath application of 2 mM Cs^+ or 10 μM ZD7288 induced an outward shift of holding current in fusiform cells. ***C***, Response to 10 μM 5-HT was blocked by HCN channel blockers 2 mM Cs^+ or 10 μM ZD7288, (top and bottom traces, respectively). ***D***, Summary of the effects of Cs^+ ($n = 5$) and ZD7288 ($n = 7$) on the 5-HT evoked current. Open symbols represent 5-HT currents of individual neurons, and filled symbols represent the mean of 5-HT currents. ***E***, Under current-clamp, representative traces from a fusiform cell showing 5-HT increased the spike firing (left trace: control; middle trace: 5-HT application), and application of Cs^+ produces a hyperpolarization of membrane potential and also suppressed the increase in the spike firing by 5-HT (right trace). ***F***, Similarly, representative traces from another fusiform cell showing that 5-HT increased the spike firing (left trace), and application of ZD7288 produced a hyperpolarization of membrane potential and also suppressed the increase in the spike firing by 5-HT (right trace). Error bars are ± SEM.

GTP analog that should alter G-proteins signaling and potentially disrupt the ability of 5-$HT_{2A/2C}$R and 5-HT_7R to evoke an inward current. Indeed, in the presence of GTP-γ-S, 5-HT failed to induce an inward current (-4.8 ± 4.0 pA, $n = 7$; Fig. 5*A*,*B*), suggesting that the 5-HT-evoked current is mediated by a G-protein-dependent pathway.

5-$HT_{2A/2C}$R are known to couple to G_q to activate PLC (phospholipase C), leading to the release of IP_3 (inositol-1,4,5-trisphosphate) and DAG (diacylglycerol; Hoyer et al., 1994). PLC-mediated IP_3 might initiate the intracellular Ca^{2+} release from intracellular endoplasmic reticulum stores, and increase Ca^{2+} could enhance the I_h (Lüthi and McCormick, 1999). To test this possibility, we determined the effects of intracellular BAPTA on the 5-HT-induced current. When recorded cells were dialyzed with 10 mM BAPTA, 5-HT still induced a robust inward current (-53.8 ± 15.0 pA, $p > 0.05$, unpaired *t* test, $n = 5$; Fig. 5*A*,*B*) compared with the control conditions (-60.6 ± 4.1 pA, $n = 35$), suggesting a Ca^{2+} independent pathway. In addition, PLC-mediated DAG might activate the protein kinase C (PKC), and PKC activation could affect the I_h (He et al., 2014). To determine the role of PKC, we included a PKC inhibitor peptide PKC_{19-31} in the recording pipette and waited >30 min after an establishing whole-cell recording, a procedure that effectively blocks PKC activity in other DCN neurons (Bender et al., 2010). However, intracellular dialysis with 10 μM PKC_{19-31} failed to block the 5-HT-evoked current (-61.7 ± 9.9 pA, $p > 0.05$, unpaired *t* test, $n = 6$; Fig. 5*A*,*B*), compared with the current recorded with normal internal solutions (-60.6 ± 4.1 pA, $n = 35$), suggesting that PLC/PKC signaling pathway is not involved in 5-$HT_{2A/2C}$R signaling. Altogether, these data suggest that serotonergic signaling in fusiform cells does not require a PLC-mediated pathway.

5-HT_7R is known to couple to Gs, and stimulation of Gs leads to activation of adenylyl cyclase and consequently an increase in intracellular cAMP (Hoyer et al., 1994). In addition, it is well known that I_h is often sensitive to intracellular cAMP (Banks et al., 1993; He et al., 2014). Thus, we asked whether intracellular cAMP signaling via activation of 5-HT_7R is involved in the augmentation of I_h. To test this possibility, we examined the effects of inhibitors and activators of the cAMP pathway on the 5-HT current. First, we included in the recording pipette an inhibitor of adenylyl cyclase SQ22536 (1 mM). Under these conditions, the 5-HT current was partially attenuated (-35.7 ± 8.4 pA, $p < 0.05$, unpaired *t* test, $n = 5$; Fig. 5*A*,*B*), compared with control conditions (-60.6 ± 4.1 pA, $n = 35$), suggesting a role for adenylyl cyclase. To further investigate whether cAMP is responsible for the effects of 5-HT on the fusiform cells, we examined the effects of a membrane-permeable cAMP analog 8-Br-cAMP, an adenylate cyclase activator, on the 5-HT-evoked current. Bath application of 8-Br-cAMP (0.5–1.0 mM) induced an inward current in most cells recorded, as expected for an activator of I_h, and moreover resulted in a large reduction of the 5-HT-induced current (control: -59.8 ± 8.2 pA, 20 min after applying 8-Br-cAMP: -11.8 ± 6.8 pA; $p < 0.01$, $n = 8$; Fig. 6*A*,*D*). These results are consistent with the idea that cAMP signaling pathway is partially involved in the excitatory effects of 5-HT on fusiform cells through activation of 5-HT_7R.

Previous studies have shown that Src tyrosine kinase activity is involved in 5-$HT_{2A/2C}$R signaling (González-Maeso et al., 2007; Lu et al., 2008; Schmid and Bohn, 2010; Bigford et al., 2012; Sung et al., 2013), and that Src tyrosine kinase activity could modulate HCN channels (Zong et al., 2005; Arinsburg et al., 2006; Li et al., 2008). To examine the possible contribution of Src tyrosine activity to 5-HT signaling in DCN, we assessed the effects of genistein, PP1, and PP2, selective inhibitors for Src kinase, on the 5-HT-induced inward current. Bath application of 30 μM genistein, a general inhibitor for Src kinase, partially suppressed the 5-HT-evoked current (74.6 ± 15.5% of control; $p < 0.05$, $n = 5$), suggesting that Src tyrosine kinase activity may be involved in the excitatory effects of 5-HT on fusiform cells. Further evidence consistent with this conclusion comes from the effects of PP1 and PP2 on the 5-HT-induced current. Slices were incubated with either PP1 or PP2 (both 20 μM), the selective inhibitors for Src family kinases, before application of 5-HT. Preincubation of PP1 or PP2 for >1 h reversibly blocked the inward current evoked by 5-HT (PP1 preincubation: -12.4 ± 3.6 pA, 1 h after wash of PP1: -43.0 ± 3.7 pA; $p < 0.05$, $n = 7$; PP2 preincubation: -15.8 ± 3.6 pA, 1 h after wash of PP2: -38.9 ± 9.7 pA; $p < 0.05$, $n = 6$; Fig. 6*B*–*C*,*E*–*F*). Together, these data suggest that serotonergic regu-

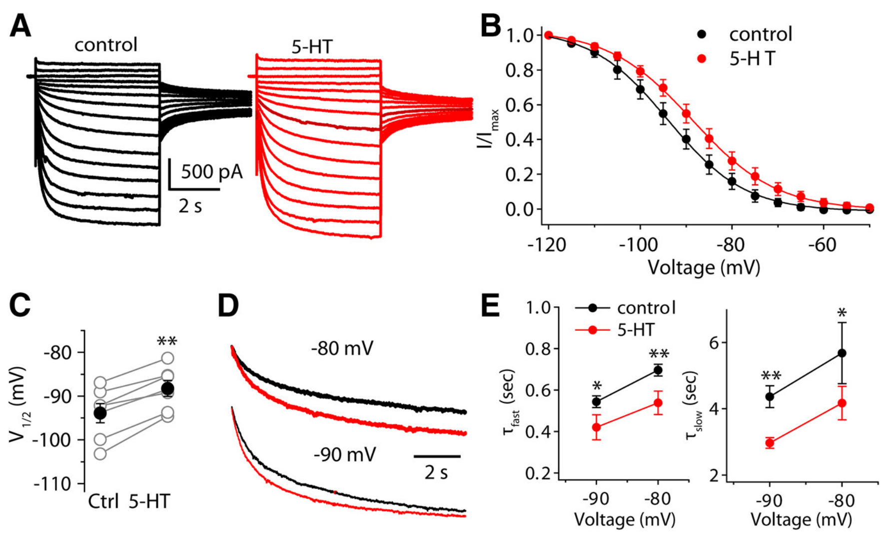

Figure 4. 5-HT positively shifts the activation curve for the I_h and reduces the time constant for I_h activation. ***A***, Sample current traces from a fusiform cell before and after applying 5-HT. Currents were evoked with a series of 5 s voltage steps from −120 to −50 mV, in 5 mV increments, from a holding potential of −60 mV, and followed by a test pulse to −75 mV. ***B***, Mean normalized tail currents are plotted as a function of voltage steps and fit with the Boltzmann equation ($n = 7$). Tail currents were measured at −75 mV in the presence of Ba^{2+} to eliminate the contamination of K_{ir}. ***C***, Pooled data showing the effects of 5-HT on $V_{1/2}$. Open symbols represent $V_{1/2}$ of individual neurons, and filled symbols represent the mean of $V_{1/2}$. ***D***, Superimposition of current traces evoked by 10 s steps to −80 and −90 mV from a holding potential of −60 mV before and after applying 5-HT. ***E***, Pooled data of time constants for I_h activation in the absence and presence of 5-HT ($n = 4$) at two voltages. Error bars are ± SEM.

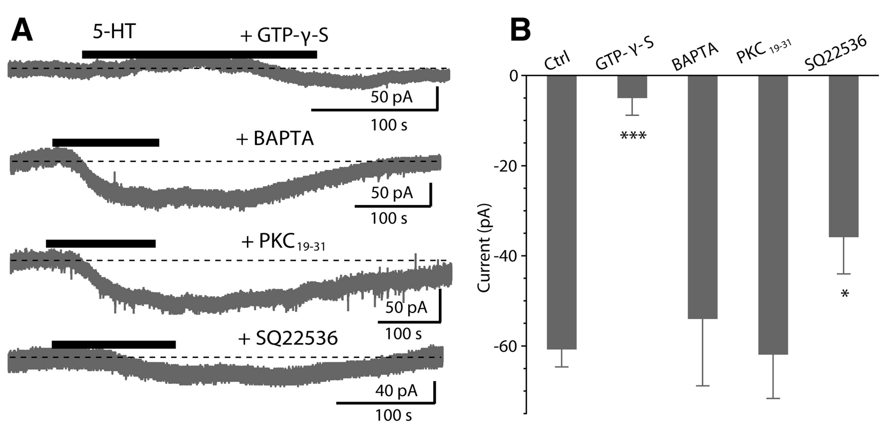

Figure 5. A G-protein-dependent transduction mechanism. ***A***, Thirty minutes after establishing the whole-cell configuration, application of 10 μM 5-HT evoked a response with intracellular perfusions of 1.5 mM GTP-γ-S, 10 mM BAPTA, 10 μM PKC_{19-31} and 1 mM SQ22536. ***B***, Pooled data for the 5-HT-evoked current under normal internal recording solution ($n = 35$) or during intracellular dialysis of GTP-γ-S ($n = 7$), BAPTA ($n = 5$), PKC_{19-31} ($n = 6$), and SQ22536 ($n = 5$). Bars represent mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (unpaired *t* test).

lation of neuronal excitability of fusiform cells is G-protein-dependent, and involves cAMP and Src signaling-dependent pathways through activation of $5\text{-}HT_7R$ and $5\text{-}HT_{2A/2C}R$, thereby enhancing resting activation of I_h.

Stimulation of serotonergic afferents regulates excitability of fusiform cells

While the data so far demonstrate that exogenous 5-HT enhances the excitability of DCN neurons, it is important to ask whether endogenous transmitter released from serotonergic afferent fibers has actions similar to that of exogenous 5-HT. To test this, we took advantage of a Tph2-ChR2-YFP mouse line in which the light activated cation channel ChR2 is expressed selectively in serotonergic neurons (Zhao et al., 2011). We first confirmed that ChR2-EYFP-positive fibers were present in the DCN by labeling sectioned material with a fluorescent antibody which recognizes EYFP (Zhao et al., 2011; see Materials and Methods). Fibers were abundantly present in the cell body and deep layers of the DCN, but nearly absent from the molecular layer (Fig. 7*A*,*B*). A similar pattern of serotonergic innervation of the DCN observed in the Mexican free-tailed bat, labeled fibers are much higher density in fusiform cell layer than in the molecular layer (Hurley and Thompson, 2001). In contrast, fibers are densely concentrated in the molecular layer but less heavily in the fusiform cell layer in rat, cat, guinea pig, and opossum (Willard et al., 1984; Klepper and Herbert, 1991; Thompson et al., 1995; Thompson and Thompson, 2001), suggesting species differences in fiber distribution. Interestingly, label was also absent in the cerebellar molecular layer but apparent in the granule cell region (Fig. 7*A*), which is consistent with the observations in some species (Dieudonné, 2001). We next examined whether optical simulation of serotonergic axonal terminals in brain slices could produce a response in voltage-clamped fusiform cells. Photostimulation with a single 5 ms blue light pulse did not induce detectable responses; however, tetanic photostimulation (20, 50, or 100 Hz, 5 ms light pulses for 10–20 s) resulted in a slow inward current in a significant fraction of fusiform cells tested (33.3%, 18 of 54 cells; 10 μM fluoxetine was added into ACSF solutions to decrease rate of 5-HT clearance in some experiments). This result is consistent with the observation that the 5-HT release by activation of ChR2 depends on the duration of light exposure and light frequency (Dankoski and Wightman, 2013; Dugué et al., 2014; Miyazaki et al., 2014). This inward current was markedly reduced by 10 μM ketanserin (light stimulus: −30.1 ± 5.3 pA, ketanserin: −4.5 ± 3.6 pA; $p < 0.05$, $n = 4$; Fig. 8*A*,*B*), suggesting that the light-evoked slow responses were mediated by serotonergic transmission. These data also suggest that serotonergic transmission is intrinsically slow, consistent with a idea that 5-HT mostly acts via a volume transmission mode in the CNS (Bunin and Wightman, 1998; Ridet and Privat, 2000; Dieudonné, 2001). Consistent with this idea, nonjunctional varicosities have been observed in the DCN (Thompson et al., 1995). More importantly, in some cases, using current-clamp recording, tetanic photostimulation depolarized the cells sufficient to trigger or increases spontaneous spike firing (Fig. 8*C*). To further explore the effect of serotonergic axon stimulation on the excitability of fusiform cells, action potentials were elicited by injecting a series of current steps (−10 to +50 pA, 500–1000 ms, $\Delta I = 20$ pA) before and after light stimulus. Photostimulation increased the firing rate upon 10 or 30 pA depolarizing steps in five of eight cells tested (10 pA baseline: 3.2 ± 1.0 Hz, light stim-

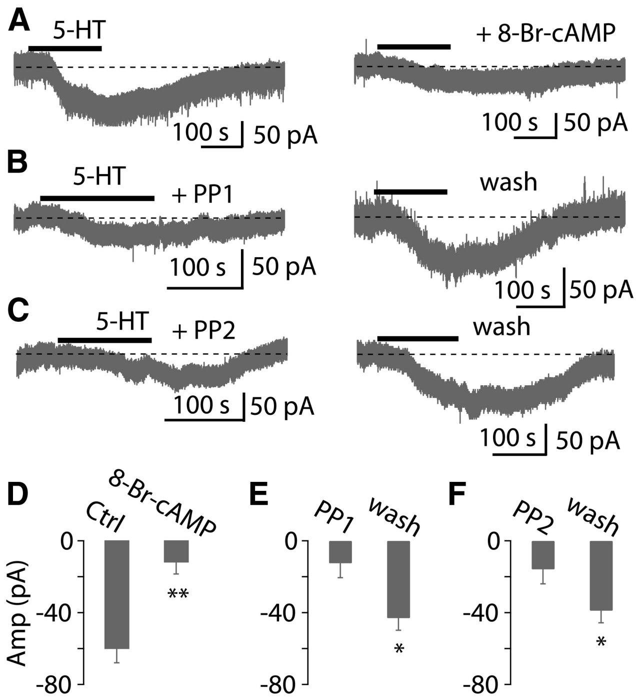

Figure 6. cAMP and Src kinase activities are involved in the serotonergic signaling. ***A***, Left, Application of 10 μM 5-HT induced an inward current. Right, Twenty minute bath application of 1 mM 8-Br-cAMP, a membrane-permeable cAMP analog, largely blocked this inward current. ***B***, ***C***, Left, Preincubation of PP1 or PP2, selective Src kinase inhibitors, for >1 h, application of 10 μM 5-HT induced a small inward current. Right, After 1 h washout of PP1 and PP2 (20 μM), application of 5-HT induced a larger inward current. ***D–F***, Pooled data showing the 5-HT-evoked current in the presence and absence of 8-Br-cAMP ($n = 8$), PP1 ($n = 7$), and PP2 ($n = 6$). Bars are mean ± SEM.

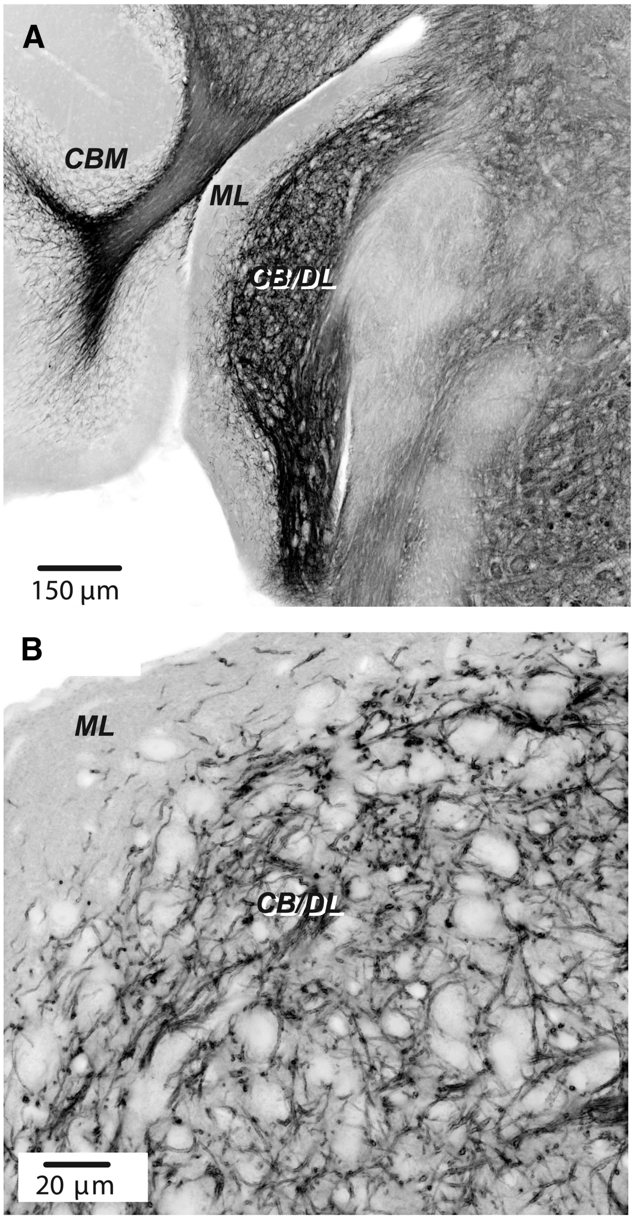

Figure 7. Expression of ChR2 in DCN in a Tph2-ChR2-EYFP mouse line. ***A***, Section showing DCN and adjoining structures. Image printed in negative shows fluorescent labeling using antibody labeling for EYFP. ML, Molecular layer of DCN; CB/DL, fusiform cell body and deep layers; CBM, cerebellar cortex. Confocal image taken with a 10× objective. ***B***, Labeling in DCN imaged using a 60× oil-immersion objective. Confocal image stack of four planes. Note preponderance of labeling up to the cell body layer and relative absence in the molecular layer.

ulus: 5.9 ± 1.0 Hz; 30 pA baseline: 9.6 ± 1.7 Hz, light stimulus: 14.2 ± 2.5 Hz; $p < 0.05$, $n = 5$; Fig. 8*D*,*E*). Thus, these data suggest that 5-HT is released from serotonergic fibers by optical stimulation in the DCN and can regulate the excitability of fusiform cells in a manner similar to that of exogenous 5-HT.

Discussion

Serotonergic regulation of fusiform cells

The DCN is composed of multiple cell types distributed in different sensory processing domains (Oertel and Young, 2004); knowing which cells are affected by 5-HT, and how, may allow the functional role of 5-HT to emerge. Serotonergic fibers densely innervate the cell layer that contains fusiform somata, and the molecular and deep layers that contain the dendrites of fusiform cells (Klepper and Herbert, 1991; Thompson et al., 1995; Hurley and Thompson, 2001; Thompson and Thompson, 2001). We found that after blocking fast synaptic inputs (glutamate, GABA, and glycine), exogenous or endogenous 5-HT depolarized fusiform cells and increased spontaneous spike rate, indicating a direct action on fusiform cell excitability. Therefore, fusiform principal cells in the DCN are a target of serotonergic raphe fibers. Although 5-HT regulates the excitability of fusiform cells by altering their intrinsic properties, it remains to be seen whether 5-HT also modulates synaptic function. Fusiform cells receive excitatory inputs from parallel fibers, auditory nerve and possibly T-stellate cells in the ventral cochlear nucleus (VCN; Oertel et al., 2011). Given that serotonergic fibers also strongly innervate the granule cell domain and VCN (Klepper and Herbert, 1991; Thompson et al., 1995; Thompson and Thompson, 2001), and that T-stellate cells are sensitive to 5-HT (Oertel et al., 2011), 5-HT may also modulate excitatory inputs to fusiform cells. Strong labeling for serotonergic fibers is observed in the DCN region containing inhibitory vertical cells. As serotonergic inputs could potentially contact vertical cells, it will be important to investigate whether 5-HT regulates the activity of vertical cells and their feedforward inhibition to fusiform cells.

Our data indicate that $5\text{-HT}_{2A/2C}$R and 5-HT_7R mediate the effects of 5-HT on fusiform cells. The 5-HT response was partially blocked by the selective 5-HT_{2A}R antagonist MDL-11939, slightly suppressed by 5-HT_{2C}R antagonist SB-242084, and partially mimicked by the 5-HT_2R agonist α-methyl-5-HT, indicating that the 5-HT response was partially mediated by some

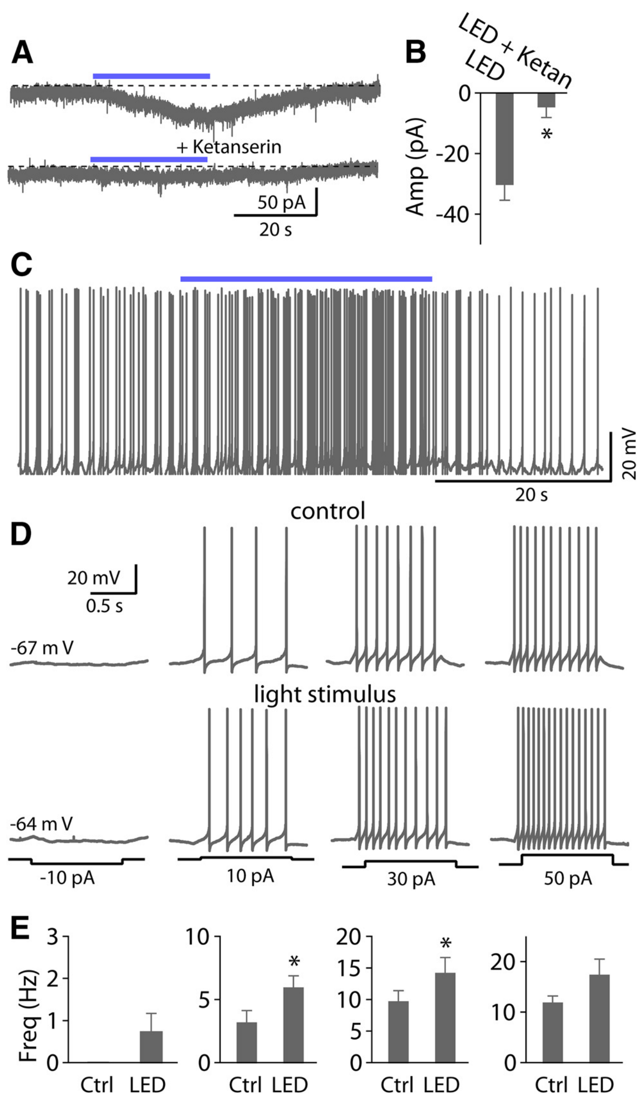

Figure 8. Photostimulation of serotonergic axons expressing ChR2 increases spike firing in fusiform cells. ***A***, Sustained photostimulation (blue light pulse with 5 ms duration) elicited an inward current. Top trace, An inward current evoked by a 30 s train of light pulses at 100 Hz. Bottom trace, This current was blocked by 10 μM ketanserin. ***B***, Pooled data show photostimulation-evoked currents were largely blocked by ketanserin ($n = 4$). ***C***, Example traces from a fusiform cell show that tetanic photostimulation increases the firing rate. ***D***, Representative traces of action potential firing elicited by somatic injection of current pulses (−10, 10, 30, and 50 pA, 1 s duration) illustrating the effects of photostimulation of serotonergic fibers. ***E***, Pooled data show photostimulation of serotonergic fibers significantly increase the firing rate at 10 or 30 pA depolarizing steps ($n = 5$).

combination of 5-HT$_{2A/2C}$R. Although ketanserin at high concentration (10 μM) almost completely blocked the response, it may also block 5-HT$_7$R (Adham et al., 1998), suggesting that 5-HT$_7$R may contribute to the 5-HT response. Indeed, the 5-HT-response was partially blocked by the 5-HT$_7$R antagonist SB-269970, and the remaining currents was suppressed by MDL-11939. In addition, our data show that 8-Br-cAMP partially mimicked and occluded the 5-HT-evoked response, consistent with studies showing that 5-HT$_7$R depolarizes neurons through activation of adenylyl cyclase and increase in cAMP (Hoyer et al., 1994). All these data suggest that the effects of 5-HT on fusiform cells are mediated probably by coactivation of 5-HT$_{2A/2C}$R and 5-HT$_7$R in individual neurons. In support, 5-HT$_{2A}$R and 5-HT$_{2C}$R are present in the DCN (Pazos et al., 1985; Thompson et al., 1994; Wright et al., 1995; Cornea-Hébert et al., 1999), and labeling of large, presumptive fusiform cells is apparent in DCN of Htr7-EGFP mice (www.gensat.org). Similar coexpression of 5-HT$_{2A}$R and 5-HT$_7$R in individual cells has been observed in other central neurons (Béïque et al., 2004; Bonsi et al., 2007).

Ionic mechanism and signal transduction pathways

Despite the multiplicity of receptors involved in the 5-HT response, HCN channels are likely to be entirely responsible for the resulting enhancement of fusiform cell excitability. Our data show that I_h is involved in setting resting potential and ongoing spontaneous firing. Pharmacological block of I_h with Cs^+ or ZD7288 was sufficient to abolish the 5-HT response. 5-HT shifts positively the activation curve for I_h without altering maximal activation, thus activating more channels when the cell is at rest. Finally, 5-HT decreased activation time constants for I_h. Because I_h time constants increase with depolarization (Chen et al., 2001), a uniform decrease in time constants implies a rightward shift in the activation curve. Together, I_h is the primary downstream target of 5-HT in fusiform cells, as seen in several other regions of the CNS (Bobker and Williams, 1989; Pape and McCormick, 1989; Takahashi and Berger, 1990; Larkman and Kelly, 1992; Cardenas et al., 1999).

The actions of 5-HT were G-protein-dependent, probably engaging cAMP and Src kinase signaling pathways. 5-HT responses were abolished by GTP-γ-S, which arrests G-protein function. Furthermore, the adenylyl cyclase inhibitor SQ22536 partially blocked the 5-HT response. 8-Br-cAMP evoked an inward current and occluded the 5-HT response. Moreover, 5-HT's effects on I_h channel gating mirrored the effects of cAMP on these channels (Chen et al., 2001). Accordingly, we speculate that 5-HT$_7$R could stimulate adenylate cyclase to upregulate intracellular cAMP. Increased cAMP may act directly on HCN, although an additional route involving phosphorylation cannot be excluded here. Intracellular dialysis of BAPTA or PKC$_{19-31}$ did not affect the 5-HT current, excluding PLC/IP$_3$/Ca^{2+} and PLC/PKC signaling involvement. Genistein, a tyrosine kinase inhibitor, partially blocked the 5-HT response, and preincubation of the Src kinase inhibitors PP1 or PP2 inhibited the 5-HT response. Thus, activation of 5-HT$_{2A/2C}$R may also lead to increased Src kinase activity, and thereby enhance the I_h. Notably, Src kinase signaling pathways have been shown to modulate I_h in a manner similar to 5-HT and cAMP (Li et al., 2008; He et al., 2014). All these data suggest that serotonergic signaling in fusiform cells may be mediated by both multiple receptors and multiple signaling pathways.

Functional implications

Given the importance of the DCN in auditory function and tinnitus pathophysiology, the serotonergic regulation of neuronal excitability of the primary output neurons may have important outcomes.

Activation of the serotonergic raphe-DCN pathway could increase excitability and reduce acoustic thresholds of fusiform cells. It may be that this pathway functions as a "gain-setter" in controlling DCN output. Serotonergic neurons in the raphe nuclei fire at 3–5 Hz in quiet, awake animals (Trulson and Jacobs, 1979; Trulson and Trulson, 1982a; Rasmussen et al., 1986). In addition, the raphe-DCN pathway may be regulated by sensory inputs and behavioral state (Trulson and Trulson, 1982b). The serotonergic system receives direct input from inferior colliculus (Pollak Dorocic et al., 2014), and acoustic stimuli activate the serotonergic system (Trulson and Trulson, 1982b; Cransac et al., 1998), suggesting that acoustic stimuli might regulate the serotonergic raphe-DCN pathway. Increased activity of serotonergic neurons is associated with behavioral arousal. Thus, 5-HT release in response to sensory stimuli or behavioral events might dynamically regulate the excitability and acoustic threshold of fusiform neurons by modulating their membrane properties.

Aberrant serotonergic transmission at one or more levels in central auditory pathways might play a role in the pathogenesis of tinnitus. Although the mechanisms underlying tinnitus remain unclear, spontaneous hyperactivity in DCN fusiform cells is associated with tinnitus (Kaltenbach et al., 2005; Kaltenbach and Godfrey, 2008; Baizer et al., 2012). Our data show that 5-HT increases excitability of fusiform cells, and may lead to enhanced downstream excitation, consistent with previous *in vivo* studies suggesting that 5-HT_2R and 5-HT_7R play a role in the regulation of auditory network excitability (Bourson et al., 1997; Brennan et al., 1997; Applegate and Tecott, 1998; Holmes et al., 1998; Oliveira and Zatz, 1999). Thus, serotonergic dysfunction (e.g., upregulation of 5-HT levels, 5-HTR density or receptor sensitivity to 5-HT) in DCN might contribute to the generation and maintenance of tinnitus (Marriage and Barnes, 1995; Simpson and Davies, 2000; Caperton and Thompson, 2010; Hurley and Hall, 2011; Baizer et al., 2012). Accordingly, upregulation of extracellular 5-HT levels in DCN increases with noise exposure (Cransac et al., 1998), which can cause tinnitus (Kaltenbach, 2011). Notably, in spinal cord, 5-HT2R density and sensitivity to 5-HT can be upregulated following spinal injury (Murray et al., 2010; Husch et al., 2012). It remains to be investigated whether such modification of serotonergic activity could be induced following cochlear damage. Moreover, SSRIs are commonly used to treat tinnitus in patients with and without depression (Parnes, 1997; Andersson and McKenna, 1998; Folmer and Shi, 2004; Robinson et al., 2007; Fornaro and Martino, 2010; Baldo et al., 2012); however, the success of such treatments are inconsistent, and some patients reported a worsening of tinnitus. Acute treatment of SSRIs should increase extracellular concentrations of 5-HT, which based on our study, may then activate 5-$HT_{2A}/_{2C}$R and 5-HT_7R and enhance DCN output. Responses to pharmaceutical agents may change as transmitter and receptor levels accommodate to the treatment. Thus, activation of 5-HT receptors in fusiform cells may be associated with the onset of a therapeutic response, indeed some patients report tinnitus after beginning SSRIs regiment (Robinson, 2007; Robinson et al., 2007). Perhaps therapeutic action of SSRIs on tinnitus results from secondary plasticity in 5-HT signaling (e.g., downregulation of 5-HT receptor) induced by boosting serotonergic activity after chronic SSRIs treatment. Indeed, it is well established that a decrease in 5-$HT_{2A/2C}$R density can be produced by chronic administration of 5-HT and other 5-HT receptor agonists (Buckholtz et al., 1988; Eison et al., 1989; Anji et al., 2000), as well as antidepressants (Eison and Mullins, 1996). Thus, a long-term reduction in 5-HT_2R density by chronic treatment with SSRIs might play a role in the therapeutic effects of SSRIs in tinnitus. The current study showing the action of 5-HT at a cellular level in the DCN may provide a basis for future studies exploring how SSRIs affect tinnitus.

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