Merkel disc is a serotonergic synapse in the epidermis for transmitting tactile signals in mammals

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The evolution of sensory systems has let mammals develop complicated tactile end organs to enable sophisticated sensory tasks, including social interaction, environmental exploration, and tactile discrimination. The Merkel disc, a main type of tactile end organ consisting of Merkel cells (MCs) and Aβ-afferent endings, are highly abundant in fingertips, touch domes, and whisker hair follicles of mammals. The Merkel disc has high tactile acuity for an object's physical features, such as texture, shape, and edges. Mechanisms underlying the tactile function of Merkel discs are obscured as to how MCs transmit tactile signals to Aβ-afferent endings leading to tactile sensations. Using mouse whisker hair follicles, we show herein that tactile stimuli are transduced by MCs into excitatory signals that trigger vesicular serotonin release from MCs. We identify that both ionotropic and metabotropic 5-hydroxytryptamine (5-HT) receptors are expressed on whisker Aβ-afferent endings and that their activation by serotonin released from MCs initiates Aβ-afferent impulses. Moreover, we demonstrate that these ionotropic and metabotropic 5-HT receptors have a synergistic effect that is critical to both electrophysiological and behavioral tactile responses. These findings elucidate that the Merkel disc is a unique serotonergic synapse located in the epidermis and plays a key role in tactile transmission. The epidermal serotonergic synapse may have important clinical implications in sensory dysfunctions, such as the loss of tactile sensitivity and tactile allodynia seen in patients who have diabetes, inflammatory diseases, and undergo chemotherapy. It may also have implications in the exaggerated tactile sensations induced by recreational drugs that act on serotoninergic synapses.

Merkel cells | touch | serotonin | 5-HT receptors | whisker hair follicles

Sensory systems for detecting tactile stimuli have evolved from mechanosensitive free nerve endings in invertebrates to complicated tactile end organs in mammals, although mechanosensitive free nerve endings are conserved in mammals mainly for noxious mechanical stimuli. Tactile end organs, including Merkel discs, Pacinian corpuscles, Meissner's corpuscles, and Ruffini endings, are highly specialized structures in the periphery of mammals (1, 2). These organs are crucial to the performance of sophisticated sensory tasks, such as environmental explorations, social interactions, and tactile discrimination (2). The Merkel disc, also known as Merkel cell-neurite complex, is a main type of tactile end organ highly abundant in human fingertips, whisker hair follicles, touch domes, and other tactile-sensitive spots throughout mammalian bodies (3, 4). Structurally, Merkel discs are composed of Merkel cells (MCs) and their associated Aβ-afferent nerve endings to form a structure of disc-shaped expansion (3, 5). Merkel discs have high tactile acuity and are very sensitive to skin indentation, pressure, hair movement, and other tactile stimuli. Tactile stimuli to Merkel discs in the touch domes of the skin and whisker hair follicles result in slowly adapting type 1 (SA1) responses, the characteristic Aβ-afferent impulses for tactile encoding (2, 5). Functionally, SA1 responses in fingertips and whisker hair follicles are essential for tactile discrimination of an object's texture, shape, and other physical properties.

Despite the recent progress in demonstrating the essential role of MCs via Piezo2 channels in mechanotransduction and tactile behaviors in mammals (6–8), it is currently not known how the mechanotransduction in MCs can subsequently result in Aβ-afferent SA1 impulses leading to tactile sensations. It has been hypothesized that Merkel discs may be sensory synapses and tactile signals are transmitted synaptically from MCs to Aβ-afferent nerve endings (3, 9, 10). However, it has long been believed that the first sensory synapses for somatosensory signals are located centrally in the dorsal horn of the spinal cord and brainstem, where sensory signals are transmitted synaptically using glutamate (Glu) as a principal neurotransmitter (11, 12). Thus, the hypothesis of Merkel discs being sensory synapses is challenged by the classic view about somatosensory transmission.

The idea that Merkel discs are sensory synapses has been further challenged by the finding that the generation of Aβ-afferent impulses by tactile stimulation is faster than the latency of chemical synapses (13). Interestingly, transcripts of synaptic release machinery, such as synapsin, synaptotagmin, and vesicular glutamate transporter 2, have been observed in MCs (14). Dense-core vesicles, which are thought to contain Glu, ATP, serotonin, substance P (SP), enkephalin (Enk), and other chemical messengers, have also been observed in MCs (3, 5). Although SA1 responses were found to be substantially suppressed by some antagonists for Glu and 5-hydroxytryptamine (5-HT) receptors at very high concentrations (15–17), the inhibitory effects with the 5-HT receptor antagonists used in the previous studies were found to be a result of nonspecific effects. Moreover, previous studies have failed to show the presence of any excitatory receptors for the hypothetic transmitters at Aβ-afferent endings of Merkel discs (18). Alternatively, the chemical messengers, including Glu, ATP, and serotonin in MCs have been thought to be autocrine or

Significance

The Merkel disc is a main type of tactile end organ for sensing gentle touch and is essential for sophisticated sensory tasks, including social interaction, environmental exploration, and tactile discrimination. Despite recent studies showing that Merkel cells in Merkel discs are main sites of mechanotransduction in response to tactile stimuli, it remains unclear how Merkel cells transmit tactile signals to Aβ-afferent endings, leading to tactile sensations. Here we show that Merkel discs are serotonergic synapses in the epidermis, that tactile stimuli trigger serotonin release from Merkel cells to excite their associated whisker Aβ-afferent endings, and that this epidermal serotonergic transmission is critical to both electrophysiological and behavioral responses to tactile stimulation.

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paracrine to modify Merkel disc's functions (3, 10, 19) rather than to be transmitters to directly elicit tactile impulses at Aβ-afferent endings in Merkel discs. Thus, molecular mechanisms underlying tactile signaling at Merkel discs remain largely obscured.

In the present study we set out to study whether Merkel discs are sensory synapses uniquely located in the epidermis to transmit tactile signals from MCs to Aβ-afferent endings, and if so, what is the transmitter released from MCs in response to tactile stimulation and how this transmitter initiates tactile impulses at Aβ-afferent endings to result in tactile sensations.

Results

Robust SA1 whisker afferent impulses (SA1 responses) could be evoked following a small deflection of whisker hair in our mouse whisker hair follicle preparation (Fig. 1 *A* and *B* and Fig. S1 *A*

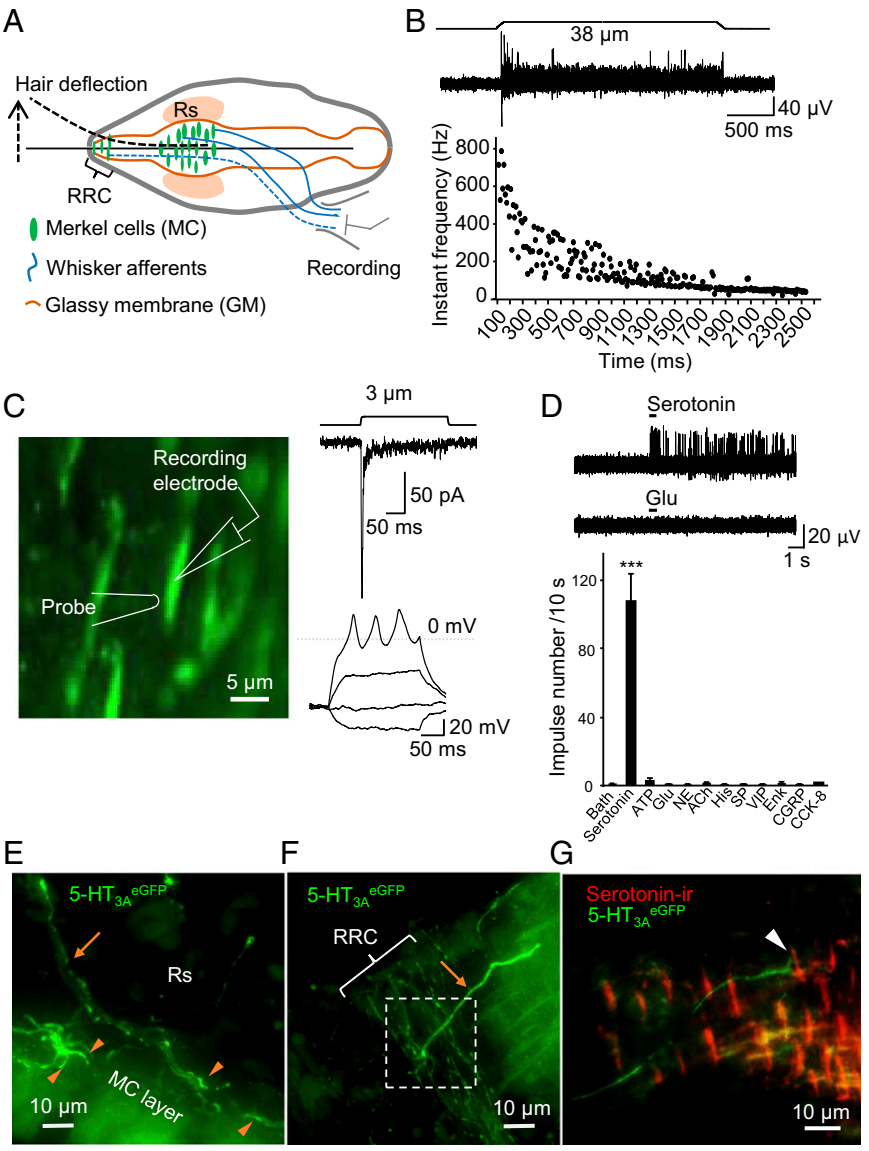

Fig. 1. Tactile SA1 responses can be mimicked by focal application of serotonin to Merkel discs where 5-HT$_{3A}$ receptors are identified at whisker afferent endings. (*A*) Experimental set-up and basic structures of a whisker hair follicle related to the present study. Whisker afferent impulses were recorded using a suction electrode. Tactile stimuli were delivered by deflecting the whisker hair. MC, Merkel cells; GM, glassy membrane; Rs, ring sinus; RRC, rete ridge collar. MCs are located at two places, underneath the Rs and around the RRC. (*B*) Sample trace (*Upper*) shows typical SA1 impulses elicited by a 38-μm displacement to deflect the whisker hair. Instantaneous frequency of SA1 impulse over time is shown in the *Lower* panel. (*C*, *Left*) Image shows MCs labeled by quinacrine vital staining in a fresh whisker hair follicle preparation. A patch-clamp recording electrode and a mechanical stimulation probe are indicated in the image. (*Right*) Sample traces show MA currents (*Upper*) and membrane responses (*Lower*) of a MC recorded in situ in a whisker hair follicle preparation. (*D*) Two sample traces show focally puffing serotonin (*Upper*) but not Glu (*Lower*) to Merkel disc region elicits whisker afferent impulses. Bar graph below the sample traces is summary data of whisker afferent impulses following the focal application of bath solution (control, $n = 30$), serotonin (1 mM, $n = 12$), ATP (1 mM, $n = 12$), Glu (1 mM, $n = 6$), NE (1 mM, $n = 6$), Ach (1 mM, $n = 6$), His (1 mM, $n = 6$), SP (358 μM, $n = 6$), VIP (30 μM, $n = 6$), Enk (0.9 mM, $n = 6$), CGRP (26 μM, $n = 6$), and CCK-8 (87 μM, $n = 6$). (*E*) Image shows that a 5-HT$_{3A}^{eGFP}$-positive whisker afferent fiber (arrow indicated) extends its processes (arrowheads indicated) from the Rs to the underneath MC region. (*F*) A 5-HT$_{3A}^{eGFP}$-positive whisker afferent fiber (arrow indicated) innervates the RRC Merkel cell region. The boxed region shows 5-HT$_{3A}^{eGFP}$ terminal processes. (*G*) Image shows the presence of 5-HT-ir cells and 5-HT$_{3A}^{eGFP}$-whisker afferent endings in Merkel disc region. The surface section of a coronal cut follicle was used and the outer root sheath in this image is seen as a sheet of cells. Arrowhead indicates a 5-HT$_{3A}^{eGFP}$ whisker afferent ending that is in close contact with a serotonin-ir cell in the Merkel disc region of a whisker hair follicle. Data represent the mean ± SEM; ***$P < 0.001$; compared with bath, one-way ANOVA with Bonferroni post hoc tests.

and *B*). We performed patch-clamp recordings from MCs in the Merkel disc region to determine whether mouse MCs were mechanically sensitive and electrically excitable (Fig. 1*C*). We found that these cells were indeed mechanically sensitive and electrically excitable, as was evidenced by mechanically activated currents (MA) and membrane depolarization-evoked action potentials (AP), respectively (Fig. 1*C* and Fig. S1 *C* and *E*). To determine whether a chemical messenger might be used for transmitting MC activity into whisker afferent endings to drive SA1 responses, we tested possible candidates to see if focal application of each candidate could directly evoke whisker afferent impulses that mimicked SA1 responses (Fig. 1*D*). Eleven possible candidates, including six classic transmitters/chemical messengers and five neuropeptides, were tested by briefly (400 ms) puffing individual candidates via a sharp glass pipette to the Merkel disc region in the whisker hair follicle. Of the 11 candidates tested, only serotonin evoked robust whisker afferent impulses (Fig. 1*D*). In sharp contrast, the other 10 candidates, including ATP, Glu, norepinephrine (NE), acetylcholine (Ach), histamine (His), SP, vasoactive intestinal peptide (VIP), Enk, calcitonin gene-related peptide (CGRP), and cholecystokinin octapeptide-8 (CCK-8) did not induce any significant increase in whisker afferent impulses following their focal applications to the Merkel disc region of whisker hair follicles (Fig. 1*D*). As a control, the ability of these candidates to excite trigeminal ganglion (TG) neurons were examined, and in our hands we found that serotonin, ATP, Glu, Ach, and His, but not the remaining compounds, could directly excite some mouse TG neurons (Fig. S1 *F* and *G*).

Although serotonin evoked whisker afferent impulses, it had no effect on MC mechanical sensitivity and membrane excitability (Fig. S1 *C*–*E*), indicating that MCs are not the primary sites of serotonin's action. Whisker afferent impulses evoked by focal serotonin application onto the Merkel disc region may be because of its direct action on whisker afferent endings. To begin to address this question, we used 5-HT_{3A}^{eGFP} transgenic mice, which showed 5-HT_{3A}^{eGFP} fluorescence in TG neurons (Fig. S2 *A* and *B*), to examine whether 5-HT_{3A} was present at whisker afferent endings in the region of Merkel discs. The 5-HT_{3A}^{eGFP}-positive whisker afferent endings were indeed identified within whisker hair follicles using the 5-HT_{3A}^{eGFP} transgenic mice (Fig. 1 *E*–*G*). The 5-HT_{3A}^{eGFP}-positive afferent fibers terminated in the outer root sheath underneath the ring sinus (Rs) (Fig. 1*E*) and around the rete ridge collar (RRC) (Fig. 1*F*), the two Merkel disc regions in a whisker hair follicle (see schematic diagram Fig. 1*A*) (20). Moreover, 5-HT_{3A}^{eGFP}-positive afferent endings were found to have close contacts with cells that were serotonin-immunoreactive (ir) in the Merkel disc region (Fig. 1*G*).

To understand the mechanisms underlying the long excitatory action after a brief focal application of serotonin, we performed patch-clamp recordings from whisker afferent neurons in a whole-mount trigeminal ganglion preparation. In this set of experiments, whisker afferent neurons were retrogradely labeled by microinjection of the fluorescent tracer DiI into individual hair follicles (Fig. 2*A*). All DiI-labeled whisker afferent neurons obtained from WT mice were found to respond to serotonin (100 μM) with inward currents (Fig. 2 *B* and *C*). Interestingly, most (75%) of these whisker afferent neurons responded to serotonin with an initial fast current (I_{fast}) followed by a slow current (I_{slow}) (Fig. 2 *B* and *C*); the I_{slow} current was long-lasting after the termination of serotonin application (Fig. 2*B*). The remaining 25% of whisker afferent neurons responded to serotonin with I_{slow} currents only (Fig. 2*C*). We next determined whether serotonin-evoked inward currents were mediated by 5-HT_3 receptors because they are the only ion channels in the 5-HT receptor family and all other 5-HT receptors are metabotropic receptors (21). In this set of experiments, 5-$HT_{3A}^{-/-}$ mice were used and serotonin effects were tested on retrogradely labeled whisker afferent neurons. Because 5-HT_{3A} subunits are essential for forming functional 5-HT_3 receptors, genetic deletion of 5-HT_{3A} subunits completely knocks out all functional 5-HT_3 receptors (21, 22). Interestingly, we found that all retrogradely labeled whisker afferent neurons in 5-$HT_{3A}^{-/-}$ mice still responded to serotonin with inward currents (Fig. 2 *B* and *C*). However, only I_{slow} currents were observed (Fig. 2 *B* and *C*) and I_{fast} currents were completely lost (Fig. 2 *B*–*D*). This result indicates that I_{fast} currents seen in whisker afferent neurons of WT mice are mediated by 5-HT_3 receptors, and I_{slow} currents seen in whisker afferent neurons in both WT and 5$HT_{3A}^{-/-}$ mice must be mediated by metabotropic 5-HT receptors.

Because activation of 5-HT_{2A} receptors have been shown to increase neuronal excitability (23), we tested whether serotonin-evoked I_{slow} currents in whisker afferent neurons were a result of the activation of 5-HT_{2A} receptors. We found that application of the 5-HT_{2A} selective agonist TCB-2 (10 μM) evoked I_{slow} currents in whisker afferent neurons of both WT and 5-$HT_{3A}^{-/-}$ mice (Fig. 2*E*). Interestingly, the I_{slow} currents in 5-$HT_{3A}^{-/-}$ mice were larger than those of WT mice (Fig. 2*E*), which is likely a result of compensatory up-regulation. Because 5-HT_{2B} receptors are functionally similar to 5-HT_{2A} receptors (24), we tested the 5-HT_{2B} selective agonist BW-723C86 (BW, 10 μM) and found that it also evoked I_{slow} currents in whisker afferent neurons of both WT and 5-$HT_{3A}^{-/-}$ mice (Fig. 2*E*). We further tested effects of the 5-HT_{2A} antagonist ketanserin (KT, 100 μM) and the 5-HT_{2B} antagonist LY-266097 (LY, 100 μM) on serotonin-evoked I_{slow} currents in whisker afferent neurons of 5-$HT_{3A}^{-/-}$ mice; serotonin-evoked I_{slow} currents were significantly inhibited by both KT and LY (Fig. 2*F*).

The involvement of 5-HT_3, 5-HT_{2A}, and 5-HT_{2B} receptors in serotonin-evoked responses was further supported by single-cell RT-PCR experiments, which showed the expression of mRNAs of these three 5-HT receptors in retrogradely labeled whisker afferent neurons (Fig. 2 *G* and *H*). All individual whisker afferent neurons expressed mRNAs of both 5-HT_{2A} and 5-HT_{2B} receptors, and the majority of these neurons (60%) coexpressed 5-HT_{3A} mRNAs (Fig. 2 *G* and *H*). This result is consistent with serotonin-evoked I_{fast} and I_{slow} currents in whisker afferent neurons of WT mice (Fig. 2*C*). The expression of 5-HT_{3A}, 5-HT_{2A}, and 5-HT_{2B} receptors in whisker afferent neurons was also consistent with immunostaining results, which showed presence of immunoreactivity for each of these three receptors in retrogradely labeled whisker afferent neurons (Fig. 2*I* and Fig. S2 *C*–*F*).

We determined whether ionotropic and metabotropic 5-HT receptors may play differential roles in evoking whisker afferent impulses because whole-cell inward currents mediated by 5-HT_3 and 5-HT_{2A}/5-HT_{2B} receptors were distinct in whisker afferent neurons. In whisker hair follicles of 5-$HT_{3A}^{-/-}$ mice, focal application of serotonin (1 mM, 400 ms) to Merkel disc regions evoked a delayed response; whisker afferent impulses were not evoked during serotonin application and were generated with delay after the termination of serotonin application, and the responses extended for a prolonged period (Fig. 3*A*). In contrast, focal application of the 5-HT_3 agonist SR57227 (1 mM) did not evoke any impulses (Fig. 3*A*). We further tested 5-HT_{2A} agonist TCB-2 (1 mM) and the 5-HT_{2B} agonist BW (1 mM) to see if they could directly elicit whisker afferent impulses. We found that both TCB-2 and BW produced delayed increases of whisker afferent impulses but the effect of BW was very small (Fig. 3*A*). These results in 5-$HT_{3A}^{-/-}$ mice indicate that functional metabotropic 5-HT receptors (5-HT_{2A}/5-HT_{2B}) are present at the whisker afferent endings in the Merkel disc region and their activation elicits delayed impulses.

In contrast to the results obtained from 5-$HT_{3A}^{-/-}$ mice, focal application of serotonin to whisker hair follicles of WT mice induced immediate increases of whisker afferent impulses during serotonin application (Fig. 3*B*). There were also delayed and

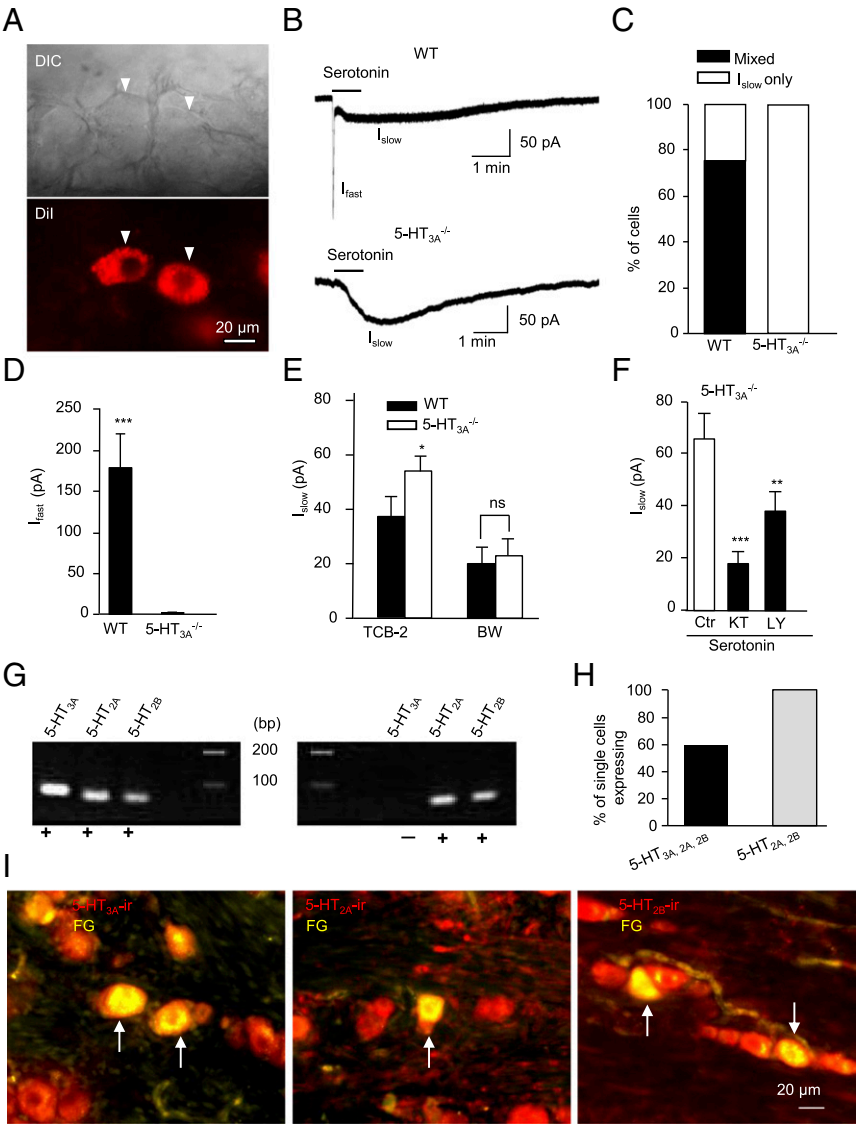

Fig. 2. Serotonin evokes inward currents in whisker afferent neurons through activation of both ionotropic and metabotropic 5-HT receptors. (*A*) Images show two DiI retrogradely labeled whisker afferent neurons (arrowheads indicated) in a whole-mount TG preparation. Both panels are the same field taken under DIC (*Upper*) and fluorescent (*Lower*) microscopy. (*B*) Two sample traces show inward currents evoked by 100 μM serotonin from a whisker afferent neuron of a WT (*Upper*) and a 5-HT$_{3A}^{-/-}$ (*Lower*) mouse. The *Upper* trace shows a mixture of a fast current (I_{fast}) and a slow current (I_{slow}) but the *Lower* trace displays only a I_{slow} current. The horizontal bar above each trace indicates the duration (1 min) of serotonin application. (*C*) Percent of whisker afferent neurons showing mixed I_{fast} and I_{slow} currents (solid bar) and I_{slow} current only (open bar). WT, n = 16; 5-HT$_{3A}^{-/-}$, n = 10. (*D*) Amplitude of I_{fast} currents in whisker afferent neuron of WT mice (n = 12). No significant I_{fast} current was observed in any whisker afferent neuron of 5-HT$_{3A}^{-/-}$ mice (n = 10). (*E*) First set of two bars, I_{slow} currents evoked by the 5-HT$_{2A}$ agonist TCB-2 (10 μM) in whisker afferent neurons of WT mice (solid bar, n = 5) and 5-HT$_{3A}^{-/-}$ mice (open bars, n = 7). Second set of two bars, I_{slow} currents evoked by the 5-HT$_{2B}$ agonist BW (10 μM) in whisker afferent neurons of WT mice (solid bar, n = 5) and 5-HT$_{3A}^{-/-}$ mice (open bars, n = 6). (*F*) Serotonin-evoked I_{slow} currents in whisker afferent neurons of 5-HT$_{3A}^{-/-}$ mice in the absence (open bar, n = 6), presence of 100 μM KT (n = 8) and 100 μM LY (n = 7). I_{slow} currents were evoked by 100 μM serotonin in each test. (*G*) Single-cell RT-PCR results from two whisker afferent neurons; one shows 5-HT$_{3A}$, 5-HT$_{2A}$ and 5-HT$_{2B}$ mRNAs (*Left*) and the other shows only 5-HT$_{2A}$ and 5-HT$_{2B}$ mRNAs (*Right*). (*H*) Summary data show the percent of cells that expressed all three 5-HT receptors (solid bar, n = 10) or only expressed 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors (n = 9). (*I*) Immunoreactivity of 5-HT$_{3A}$, 5-HT$_{2A}$, and 5-HT$_{2B}$ receptors detected on retrogradely labeled whisker afferent neurons (arrows indicated). Data represent the mean ± SEM. NS, no significant difference; *P < 0.05; **P < 0.01; ***P < 0.001; unpaired Student t test or one-way ANOVA with Bonferroni post hoc tests.

prolonged impulses after the termination of serotonin application (Fig. 3*B*). The 5-HT$_3$ agonist SR57227 (1 mM) elicited immediate but brief increases of afferent impulses (Fig. 3*B*). In contrast to the 5-HT$_3$ agonist, application of the 5-HT$_{2A}$ agonist TCB-2 (1 mM) or the 5-HT$_{2B}$ agonist BW (1 mM) induced a delayed increase of whisker afferent impulses; the effect of BW was very small but was significantly higher than control with bath application (Fig. 3*B*). Selective agonists for other 5-HT receptor subtypes (5-HT$_1$, 5-HT$_{2C}$, 5-HT$_4$, 5-HT$_5$, 5-HT$_6$, and 5HT$_7$) were tested and none of them elicited any whisker afferent impulses (Fig. S3).

The effects of serotonin were much greater than the simple summation of the effects induced by individually applying the 5-HT$_3$ agonist SR57227, 5-HT$_{2A}$ agonist TCB-2, and 5-HT$_{2B}$ agonist BW (Fig. 3*B*), raising a possibility that ionotropic 5-HT receptors (5-HT$_3$) may have synergistic effects with metabotropic 5-HT receptors (5-HT$_{2A}$/5-HT$_{2B}$) on generating whisker afferent impulses. Consistent with this idea, when a mixture made with

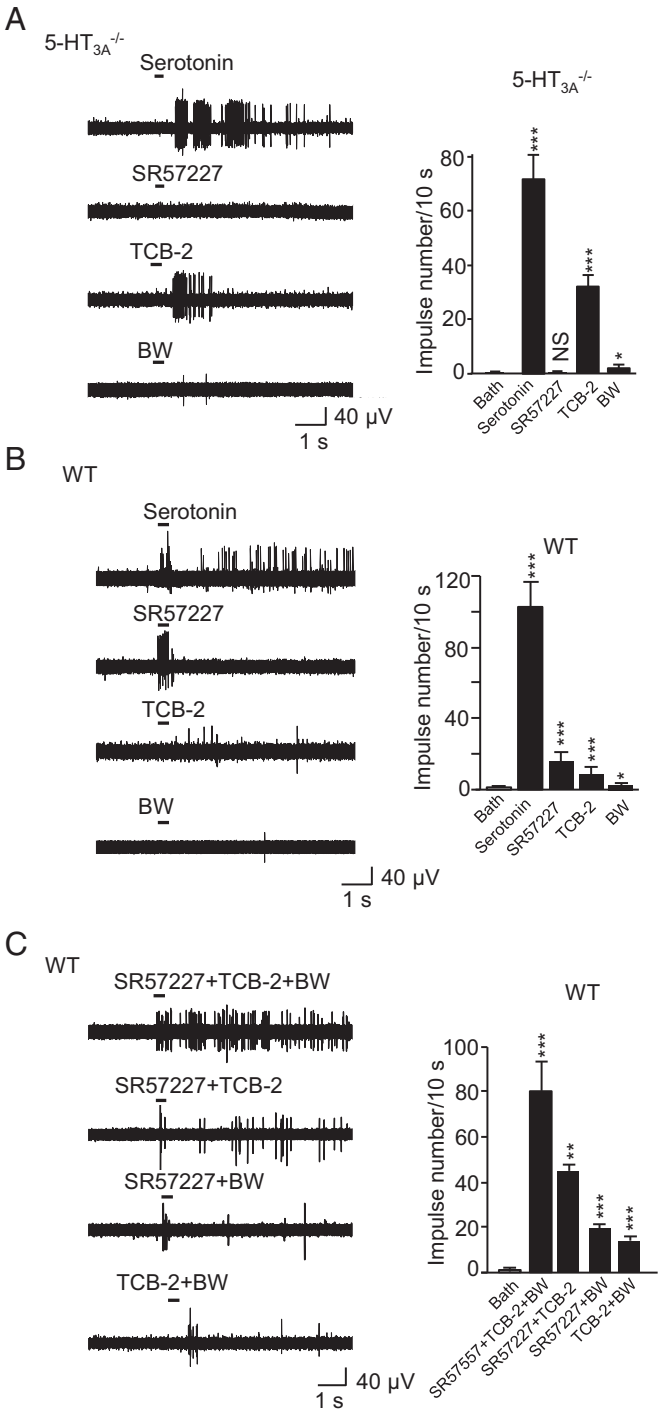

Fig. 3. Ionotropic and metabotropic 5-HT receptors play differential roles with synergism in driving whisker afferent impulses. (*A*, *Left*) Sample traces show whisker afferent impulses elicited by serotonin or 5-HT receptor subtype selective agonists focally applied to whisker hair follicle Merkel disc region of 5-$HT_{3A}^{-/-}$ mice. From top to bottom, the tests are serotonin, the 5-HT_{3A} agonist SR57227, the 5-HT_{2A} agonist TCB-2, and the 5-HT_{2B} agonist BW. (*Right*) Summary data of the impulse numbers induced by the following tests: serotonin ($n = 7$), SR57227 ($n = 7$), TCB-2 ($n = 7$), and BW ($n = 12$). (*B*) Similar to *A* except WT mice were used for the tests of serotonin ($n = 7$), SR57227 ($n = 7$), TCB-2 ($n = 7$), and BW ($n = 12$). (*C*) Similar to *B* except tests were performed with different agonist combinations as follows, SR57227+TCB-2+BW ($n = 7$), SR57227+BW ($n = 7$), SR57227+TCB-2 ($n = 7$), and TCB-2+BW ($n = 7$). For all panels, all compounds were puff-applied at the concentration of 1 mM for 400 ms. Data represent the mean ± SEM; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; one-way ANOVA with Bonferroni post hoc tests.

SR57227, TCB-2, and BW was focally applied, the afferent impulses became greatly increased to the level comparable to those induced by serotonin (Fig. 3*C*). Removing any one of the agonist from the mixture significantly weakened its effects in eliciting whisker afferent impulses (Fig. 3*C*). These results suggest that although ionotropic and metabotropic 5-HT receptors have differential temporal response characteristics in generating whisker afferent impulses, they also produce synergistic effects in driving whisker afferent impulses when both ionotropic and metabotropic receptors are activated simultaneously.

To demonstrate that endogenous serotonin activates 5-HT_3 receptors to drive SA1 impulses, we recorded SA1 impulses in 5-$HT_{3A}^{-/-}$ mice in response to whisker hair movement and found that SA1 frequency was significantly less than those of WT mice from the beginning to middle (100–1,200 ms) of the SA1 response, but not during the later phase (1,300–2,600 ms) of the SA1 response (Fig. 4 *A*–*C*). Electrophysiological and mechanical transduction properties of MCs were not different between 5-$HT_{3A}^{-/-}$ and WT mice (Fig. S4); membrane properties of whisker afferent neurons were also not different between 5-$HT_{3A}^{-/-}$ and WT mice (Table S1). Pharmacological block of whisker afferent neuron 5-HT_3 receptors in WT mice with the two 5-HT_3 selective antagonists Y25130 (2 and 20 μM) (Fig. 4*D* and Fig. S5*A*) and MDL 72222 (10 μM) (Fig. S5*A*) also significantly inhibited SA1 impulses in a manner similar to genetic deletion of 5-HT_3 receptors (Fig. 4*C*). To cross-validate the results obtained from 5-$HT_{3A}^{-/-}$ mice and WT mice, we tested the effects of the two 5-HT_3 antagonists at the above concentrations on SA1 responses in 5-$HT_{3A}^{-/-}$ mice, and found that the inhibitory effects were completely lost (Fig. 4*E* and Fig. S5*B*). This result indicates that the inhibitory effects by these antagonists in WT mice are specifically mediated by 5-HT_3 receptors. We determined whether block of 5-HT_{2A} and 5-HT_{2B} receptors could inhibit SA1 responses, and found that the 5-HT_{2A} antagonist KT (1 and 10 μM) plus the 5-HT_{2B} antagonist LY (10 and 100 μM) suppressed SA1 responses in the later phase SA1 responses (500–2,600 ms) but not in the very early phase (100–400 ms) SA1 responses in WT mice (Fig. 4*F*). The effects of these two antagonists at the concentrations used in the present study were unlikely because of a potential nonspecific effect of them on MCs, because MC electrophysiological and mechanical transduction properties were not significantly affected (Fig. S6). It is worth noting that previous studies used MDL-72222 and KT at much higher concentrations (up to 500 μM) and showed much stronger inhibitory effects on SA1 responses (15, 17), but we found that at these high concentrations the inhibitory effects on SA1 responses by MDL-72222 and KT were a result of their nonspecific actions (Fig. S7).

We investigated whether ionotropic and metabotropic 5-HT receptors had synergism to drive the majority of SA1 impulses. In whisker hair follicles of WT mice, the combination of low concentrations of the 5-HT_3 antagonist Y25130, 5-HT_{2A} antagonist KT, and 5-HT_{2B} antagonist LY produced pronounced inhibition of SA1 responses (Fig. 4*G*). At combined higher concentration, these ionotropic and metabotropic serotonin receptor antagonists almost completely inhibited SA1 responses (Fig. 4*G*). MC electrophysiological and mechanical transduction properties were not significantly affected by these antagonists (Fig. S6). In 5-$HT_{3A}^{-/-}$ mice, combined application of 5-HT_{2A} and 5-HT_{2B} receptor blockers (KT+LY) at a low concentrations produced pronounced inhibitory effects, and at high concentrations almost completely blocked SA1 impulses (Fig. 4*H*). These results indicate that endogenous serotonin drive the majority of electrophysiological tactile responses through the synergism of metabotropic and ionotropic 5-HT receptors.

Endogenous serotonin may be released in whisker hair follicles responding to whisker hair deflections. To test this idea, we determined whether SA1 responses elicited by whisker hair deflections could be diminished by botulinum toxin type A (BoNTA), a

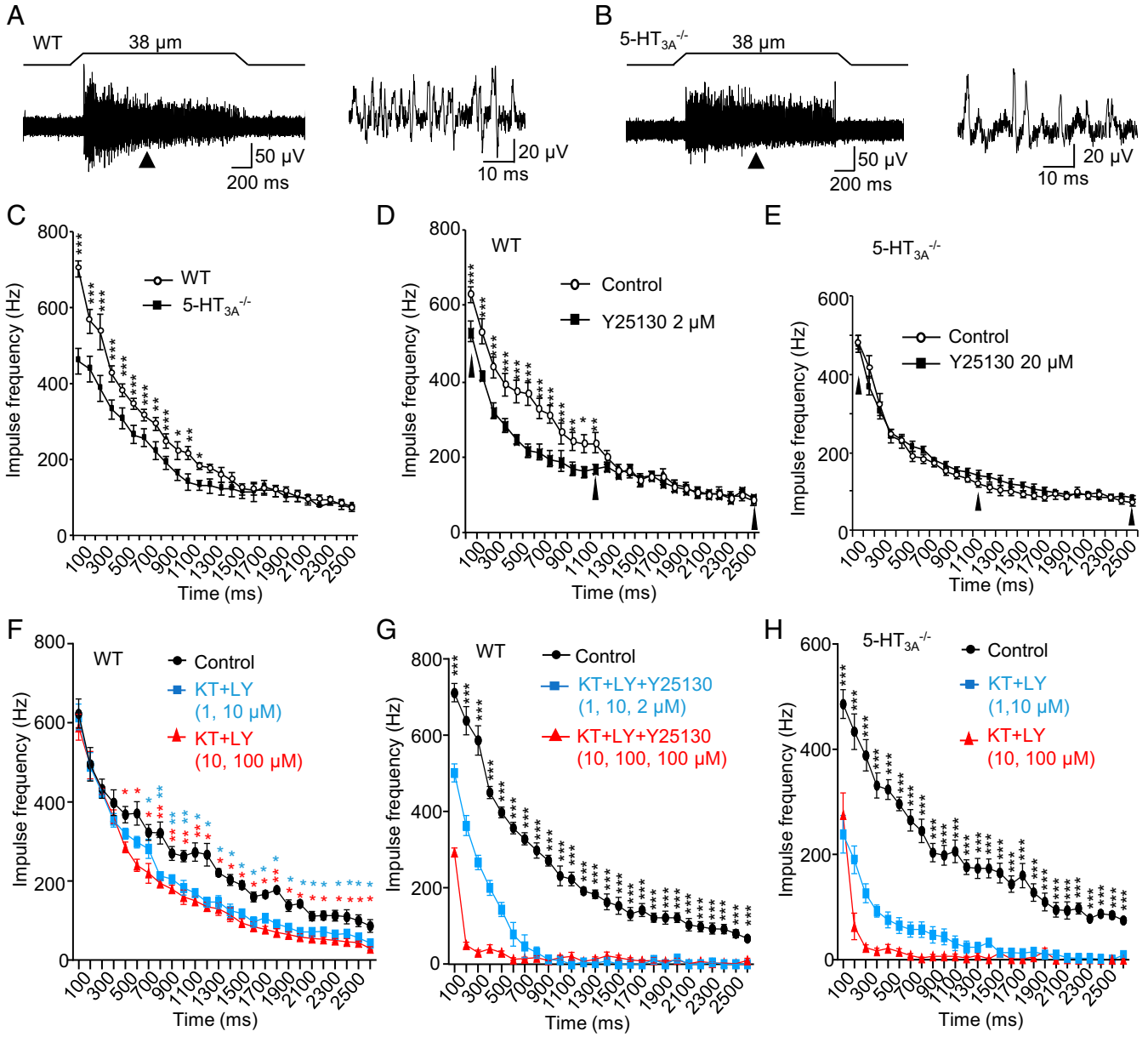

Fig. 4. Synergism of ionotropic and metabotropic 5-HT receptors is essential for electrophysiological whisker tactile responses. (*A*) Sample trace shows SA1 impulses recorded from whisker hair follicles of a WT mouse. Arrowhead-indicated region is expanded on right. (*B*) Similar to *A* except SA1 impulses were recorded from a 5-$HT_{3A}^{-/-}$ mouse. (*C*) Summary of SA1 impulses of WT ($n = 30$) and 5-$HT_{3A}^{-/-}$ ($n = 30$) mice. (*D*) SA1 impulses of WT mice in the absence (control, $n = 10$) and presence ($n = 10$) of 2 μM Y25130. (*E*) Similar to *D* except using 5-$HT_{3A}^{-/-}$ mice and 20 μM Y25130 ($n = 6$). (*F*) SA1 impulses of WT mice in control and the presence of KT+LY ($n = 6$). (*G*) Similar to *F* except the blocker mixture also included Y25130 ($n = 7$). (*H*) Similar to *F* except whisker afferent impulses were recorded from 5-$HT_{3A}^{-/-}$ mice ($n = 6$). Data represent the mean ± SEM; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; two-way ANOVA with Bonferroni post hoc tests.

neurotoxin that specifically cleaves synaptosomal-associated protein (25 kDa) (SNAP-25) to prevent vesicle fusion and neurotransmitter release (25). As shown in Fig. 5*A*, SA1 impulses were significantly suppressed by BoNTA. Moreover, using ELISA, we detected serotonin release from whisker hair follicles in a tactile stimulation-dependent manner (Fig. 5*B*). Serotonin may be released from MCs because mRNAs of tryptophan hydroxylase 1 (an enzyme for serotonin synthesis) were expressed in MCs (Fig. 5*C*) and serotonin-ir cells identified in the MC region (Fig. 5*C*), where these cells were closely contacted by 5-HT_{3A}^{eGFP} whisker afferent endings (Fig. 1*G*).

We then determined whether mechanical transduction in MCs directly coupled to serotonin release. Mechanical stimulation evoked serotonin release from MCs, as was evidenced by the oxidation currents recorded by carbon fiber electrodes (Fig. 5 *D* and *E*; also see Fig. S8 *A* and *B*). In contrast, no significant oxidation current could be detected when the mechanical stimulation was applied to non-MCs (Fig. S8 *C* and *D*). In most MCs, the first release events exhibited the highest amplitudes, and were followed by many smaller release events (Fig. 5 *E–H*). Some MCs only showed single release events with large amplitudes (Fig. 5 *F* and *G*). For cells showing multiple release events, most events had small amplitudes within a narrow range (Fig. 5*H*). Serotonin release was dependent on Ca^{2+} entry and no release event was detected in a low extracellular Ca^{2+} bath condition (Fig. 5*I*). These results indicate that serotonin release is coupled with mechanical transduction in MCs.

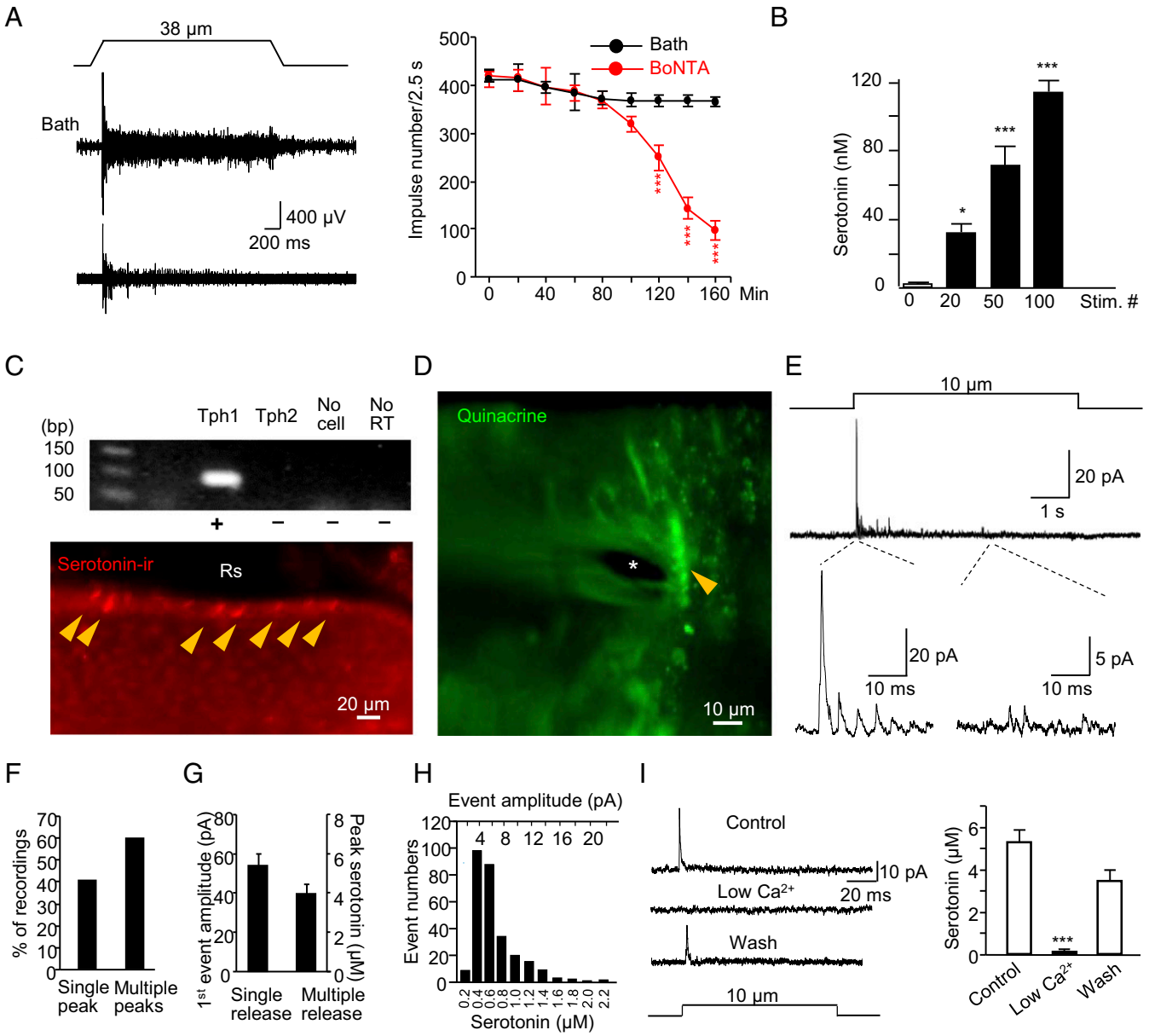

Fig. 5. Tactile stimulation releases serotonin from MCs in whisker hair follicles. (*A*) Two sets of sample traces on *Left* show whisker afferent SA1 responses recorded from whisker hair follicles of WT mice in normal bath (control, *Upper*) and in the presence of 0.5 nM BoNTA for 2 h (*Lower*). (*Right*) Summary data of the changes of whisker afferent SA1 impulses recorded over time in normal bath solution (control, $n = 6$, black circles) and in the presence of 0.5 nM BoNTA ($n = 8$, red circles). (*B*) Serotonin detected in bath solution by ELISA following mechanical stimulation that deflects whisker hairs. Bars from left to right are serotonin concentrations detected following increased tactile stimulation numbers. In each experiment, 10 follicles were used and a tactile stimulus is a displacement at 38 μm. Eight sets of tests ($n = 8$) were performed for each condition. (*C*, *Upper*) RT-PCR shows the expression of tryptophan hydroxylase 1 (Tph1) mRNAs in MCs. Similar results were observed in two other sets of RT-PCR experiments. (*Lower*) Image shows the present of 5-HT-ir cells (arrowheads indicated) in Merkel disc region of the out root sheath. The middle section of a coronal cut follicle was used and the out root sheath (MC region) is a thin layer at the border below the Rs. (*D*) Image shows a Merkel disc region of a fresh whisker hair follicle used for real-time detection of serotonin release from MCs. MCs were vital-stained by quinacrine and a MC is indicated by an arrowhead. The MC is in close contact with the tip of a carbon fiber electrode (star indicated) and the same carbon fiber electrode was used for both mechanical stimulation and amperometric detection of serotonin release. (*E*) Sample trace of oxidation current events detected by a carbon fiber electrode following mechanical stimulation of a Merkel cell by a 10-μm displacement. Two traces on the bottom are at an expanded scale for the two parts indicated by dashed lines. (*F*) Percent of MCs showing multiple release events ($n = 75$) or only a single release event ($n = 47$). (*G*) Peak amplitude (serotonin concentrations) of the first event of multiple release event cells ($n = 40$) and single release event cells ($n = 32$). (*H*) Event-amplitude distribution of multiple release events. The first event is excluded from the plots. Greater than 65% of the release events were in the size range of 0.4–0.6 μM serotonin. (*I*, *Left*) Sample traces show the detection of mechanical stimulation-evoked serotonin release in normal bath (control, *Top*), in a low Ca^{2+} bath solution (*Middle*), and recovery after returning to normal bath (*Bottom*). (*Right*) Summary data ($n = 10$) of serotonin release in control, low Ca^{2+} bath solution and recovery. The value of the biggest event in each test was used for the summary data. Data represent the mean ± SEM; $^*P < 0.05$; $^{***}P < 0.001$; one-way ANOVA with Bonferroni post hoc tests or paired Student's *t* test.

Finally, to determine whether the epidermal serotonergic transmission plays a role in behavioral responses to tactile stimuli, we measured behavioral responses elicited by gently touching a whisker hair (Fig. 6*A*). WT mice responded to the tactile stimuli with a high percent of avoidance reactions in control animals (Fig. 6*B*). Microinjection of the metabotropic

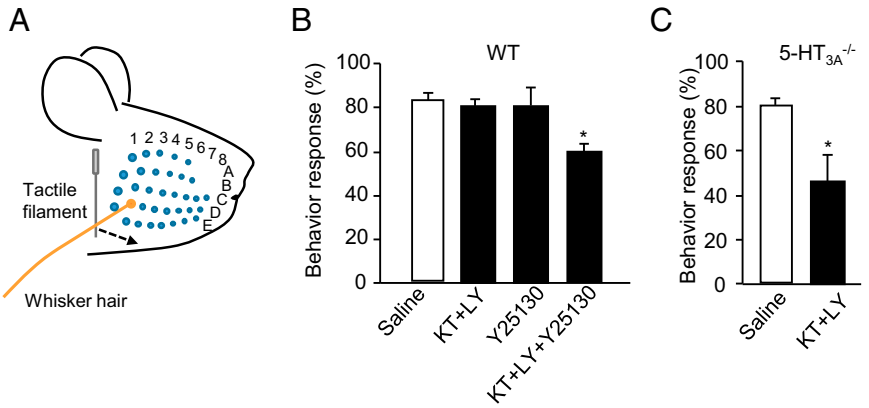

Fig. 6. Ionotropic and metabotropic 5-HT receptors in whisker hair follicles are involved in behavioral whisker tactile responses. (*A*) Diagram illustrates tactile behavioral tests. Dashed arrow indicates the direction of tactile stimulation to move the whisker hair using a tactile stimulation filament. (*B*) Behavioral responses of WT mice to whisker hair tactile stimulation following hair follicle microinjection of saline (control), KT+LY, Y25130, or KT+LY+Y25130. (*C*) Behavioral responses of 5-$HT_{3A}^{-/-}$ mice to whisker hair tactile stimulation following microinjection of saline or KT+LY, $n = 6$ for each experiment in *B* and *C*. The testing drugs were administered at 100 μM with a total volume of 1 μL. Data represent the mean ± SEM; $*P < 0.05$, Student's *t* test.

5-HT receptor antagonists KT + LY or the ionotropic 5-HT receptor antagonist Y25130 separately did not produce a measurable change in tactile behavioral responses (Fig. 6*B*). In contrast, microinjection of all three antagonists together resulted in the significant suppression of tactile behavioral responses (Fig. 6*B*). In 5-$HT_{3A}^{-/-}$ mice, the metabotropic 5-HT receptor antagonists KT + LY could effectively impair tactile responses (Fig. 6*C*). These behavioral results are consistent with the electrophysiological recordings showing that substantial suppression of SA1 responses could be observed only when both the ionotropic and metabotropic 5-HT receptors were blocked (Fig. 4 *G* and *H*).

Discussion

In the present study we show that serotonin meets the criteria of being a transmitter conveying tactile signals from MCs to whisker afferent endings and establish Merkel discs as true sensory synapses uniquely located in the epidermis. This tactile synapse is distinct from the first sensory synapses that transmit other somatosensory signals, such as temperatures and pain in that the latter are located within the CNS and Glu is the principal transmitter (11, 12). The epidermal serotonergic synapses provide novel molecular insights into the tactile functions of the Merkel disc, an important tactile end organ that is crucial to complicated tactile tasks performed by whisker hair follicles of nonprimate mammals and fingertips of humans and other primates (2, 26).

We show that MCs in mouse whisker hair follicles are mechanical transducers responding to mechanical stimulation with MA currents. The MA currents in the present study have the electrophysiological properties identical to the Piezo2-mediated currents in MCs of rat whisker hair follicles (8) and of mouse touch domes in the skin (6, 7). We further show that mouse MCs situated in our whole-mount whisker hair follicle preparations are electrically excitable and fire slow APs in response to membrane depolarization. This interesting property is not shared by other types of cells in the skin and also is not observed in mouse MCs grown in culture (6, 7). Importantly, we have found that the mechanotransduction in MCs triggers serotonin release. This finding provides key evidence indicating that Merkel discs are sensory synapses using a chemical messenger to transmit tactile signals. This idea of serotonergic synapses for tactile transmission is further supported by our findings of the presence of tryptophan hydroxylase 1 for serotonin synthesis and serotonin immunoreactivity in MCs. The kinetics and other properties of mechanical stimulation-triggered serotonin release events shown in our study strongly suggest that MCs use a synaptic mechanism of vesicular exocytosis to release serotonin. This idea is consistent with the previous observation of the presence of dense core vesicles in MCs (3). The idea of synaptic serotonin release from MCs is further strengthened by our findings that the release is Ca^{2+}-dependent and that disruption of synaptic release machinery with BoNTA significantly attenuates SA1 responses.

In our experiments detecting serotonin release from MCs, a large amplitude event of serotonin release usually occurs immediately after mechanical stimulation, and it is often followed by multiple small release events that continually occur for some period. The first large release event may represent synchronized exocytosis of multiple vesicles caused by large Ca^{2+} entry via both Piezo2 channels and voltage-gated Ca^{2+} channels. Multiple vesicular release has been often observed in the synapses formed between hair cells and their afferent endings (27). For those small release events, the event sizes are in a very narrow range of 0.4 μM to 0.6 μM, which may represent quantal releases from individual vesicles. The detected serotonin molecules by our amperometry approach are most likely those that leaked out from synaptic clefts into the bulk bath solution surrounding MCs. Therefore, the measured serotonin concentrations may be substantially lower than actual serotonin concentrations in synaptic clefts of Merkel discs. The synchronized release events at initial stimulation and the multiple small release events over time would generate strong initial actions and prolonged weaker actions over time on whisker afferent endings, respectively. This may be one of the mechanisms underlying the characteristics of SA1 impulses of Merkel discs in response to tactile stimulation.

Serotonin released from MCs acts as a transmitter rather than as an autocrine or a paracrine because it directly elicits impulses in whisker Aβ-afferent endings. We demonstrate that the excitatory action of serotonin is mediated by both ionotropic 5-HT receptors (5-HT_3) and metabotropic 5-HT receptors (5-HT_{2A}/5-HT_{2B}) expressed at whisker afferent endings innervating Merkel discs in whisker hair follicles. Consistently, we show that focal application of serotonin and the agonists to 5-HT_3 and 5-HT_{2A}/5-HT_{2B} receptors to whisker afferent endings directly elicits whisker afferent impulses that mimic tactile-evoked SA1 responses in a manner of fast onset and prolonged excitation. The involvement of 5-HT_3 and 5-HT_{2A}/5-HT_{2B} receptors in the tactile synaptic transmission at Merkel discs is further evidenced by our finding that genetic deletion and pharmacological block of these receptors significantly impair SA1 responses. In our pharmacological tests, we have validated the specificity of 5-HT receptor antagonists at low

concentrations used in the present study. However, we have found that high concentrations of KT (500 μM) and MDL-72222 (100 and 500 μM), which were used in previous studies (15, 17), produced strong nonspecific effects on SA1 responses. This finding indicates that the previous studies with these two antagonists (15, 17) are not valid evidence of serotonergic transmission at Merkel discs. We have demonstrated that the ionotropic and metabotropic 5-HT receptors at whisker afferent endings exhibit the synergism in initiating electrophysiological tactile responses (SA1 impulses). The synergistic effects drive the majority of whisker afferent SA1 impulses, as is evidenced by the large impairment of SA1 impulses after blocking these 5-HT receptors pharmacologically and genetically. In fact, SA1 impulses in static phase appear to be entirely mediated by the serotonergic transmission because these impulses are completely abolished after blocking these 5-HT receptors pharmacologically and genetically. Future studies using mice whose 5-HT_3 and 5-HT_{2A}/5-HT_{2B} receptors are genetically deleted together would help to confirm this point or to reveal other possibilities, such as the involvement of other 5-HT receptor subtypes or other receptor families in mediating SA1 responses.

We have noticed that several initial impulses in the dynamic phase (period during displacement step ramp) of SA1 responses are not significantly suppressed after blocking these 5-HT receptors. These initial impulses may be a result of the direct mechanotransduction at whisker afferent endings because Piezo2 channels may be expressed on these Aβ-afferent endings at Merkel discs (6, 7). However, serotonergic transmission should also significantly contribute to the generation of SA1 impulses in dynamic phase because SA1 impulses in dynamic phase are significantly suppressed when 5-HT_3 and 5-HT_{2A}/5-HT_{2B} receptors are inhibited. Thus, the serotonergic transmission with the synergism of ionotropic and metabotropic 5-HT receptors appears to contribute to most, if not all, SA1 impulses in mouse whisker hair follicles under our experimental conditions. However, chemical messengers including ATP, Glu, SP, and enkephalin may be also present in the vesicles of MCs (3, 5), raising a possibility that these chemical messengers may play some roles in tactile transmission at Merkel discs. The serotonergic transmission at Merkel discs shown by our in vitro experiments is consistent with tactile behavioral responses in the present study; our tactile behavioral tests demonstrate that blocking 5-HT_3 and 5-HT_{2A}/5-HT_{2B} receptors together compromises tactile behavioral responses. Because tactile stimuli to whisker afferents may be partially transduced by Aβ-afferent endings in Merkel discs and also by other afferent endings in whisker hair follicles (e.g., lanceolate endings), it is not unexpected that some degree of tactile behavioral responses will remain even when the serotonergic transmission at Merkel discs is completely blocked.

Serotoninergic synapses for tactile transmission at Merkel discs provide a novel mechanism of somatosensory processing. The serotonergic transmission in the epidermis, probably like that in the CNS, can be regulated by factors affecting serotonin uptake and release. This raises an interesting issue as to whether serotonin uptake inhibitors, such as cocaine, methamphetamine, and other recreational drugs in this category, may act at the epidermal serotonergic synapses to alter tactile sensations. It would also be also interesting to know whether the epidermal serotonergic transmission may be altered under pathological conditions in patients with diabetes, tissue inflammation, and undergoing chemotherapy, because tactile dysfunctions including mechanical allodynia and reduced tactile sensitivity are commonly observed in these patients.

Materials and Methods

Additional experimental details are provided in *SI Materials and Methods*.

Animals. Wild-type C57BL/6 mice, 5-HT_{3A}^{eGFP}, 5-$HT_{3A}^{-/-}$ mice, and GCaMP3 mice were used in the present study. Animal care and use conformed to NIH guidelines for care and use of experimental animals. Experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. Details are provided in *SI Materials and Methods*.

Whisker Afferent Fiber Recordings. Whisker hair follicle preparations and whisker afferent fiber recordings were performed using our previously described method (8). To record whisker afferent impulses elicited by whisker deflections (SA1 responses) or induced by focal applications of candidate chemical messengers, compound action potentials conducted on whisker afferent fibers were recorded using a suction electrode. Details are provided in *SI Materials and Methods*.

Patch-Clamp Recordings from Retrogradely Labeled Whisker Afferent Neurons in Whole-Mount TG. Whisker afferent neurons were retrogradely labeled by microinjection of DiI or Fluoro-Gold (FG) solution into hair follicles. Seven days after the microinjection, TG were obtained for whole-cell patch-clamp recordings from the labeled neurons. To determine whole-cell currents evoked by serotonin or other 5-HT receptor agonists, neurons were held at −73 mV, testing compounds were bath-applied for 1 min, and currents were recorded for 10 min. To determine membrane excitability, step current pulses from −100 to +900 pA (20 pA per step, 250-ms duration) were injected into cells through patch-clamp electrodes. Details are provided in *SI Materials and Methods*.

Patch-Clamp Recordings from MCs in Situ in Whisker Hair Follicles. Whisker hair follicles were dissected out from whisker pad and capsule of each hair follicle removed. Whisker hair follicles (without capsules) were then affixed in a recording chamber. After removing Rs and the glassy membranes, MCs were stained with quinacrine and patch-clamp recordings were made from quinacrine-stained cells. Membrane and AP properties of MCs were determined under the whole-cell current clamp mode. Voltage-evoked and MA currents were recorded with MCs voltage-clamped at −75 mV. Details are provided in *SI Materials and Methods*.

Mechanical Stimulation. Hair deflection was used as a tactile stimulus to elicit whisker afferent SA1 impulses. Unless otherwise indicated, hair deflection was induced by a 38-μm forward step to push the hair follicle for the duration of 2.62 s. MC mechanical sensitivity was tested using a method described in our previous study (8). Details are provided in *SI Materials and Methods*.

Pharmacology. The testing compounds include serotonin, ATP, Glu, NE, Ach, His, SP, VIP, Enk, CGRP, CCK-8, eltoprazine, TCB-2, BW, m-CPP, SR57227, cisapride, 5-carboxamidotryptamine maleate (5-CT), EMD 386088 hydrochloride, AS-19, KT, LY266097, Y25130, MDL-7222, and BoNTA. Details are provided in *SI Materials and Methods*.

Single-Cell RT-PCR for Detecting 5-HT Receptor mRNAs in Whisker Afferent Neurons and Tryptophan Hydroxylase mRNAs in MCs. DiI-labeled whisker afferent neurons or quinacrine-stained MCs were individually aspirated into micropipettes, and the tips of the aspiration micropipettes were broken into microcentrifuge tubes to release individual cells into lysis buffer. After denaturation (1.5 min at 65 °C), RT-PCR was performed using a multiplex strategy with external primers and internal primers. Details are provided in *SI Materials and Methods*.

Immunohistochemistry. For immunostaining of whisker afferent neurons, the following antibodies were used: rat anti-5HT_{2A} (28), rat anti-5HT_{2B} (29), and rat anti-5HT_{3A} (30). For staining on afferent endings in whisker hair follicles, whisker hair follicles of 5-HT_{3A}^{eGFP} mice were used. The following antibodies were used: chicken anti-GFP and rat antiserotonin. Details are provided in *SI Materials and Methods*.

Detection of Serotonin Release by ELISA and Amperometry. Serotonin release from whisker hair follicles in responses to whisker hair displacements was detected using ELISA kit based on the company's instruction. Serotonin release from individual MCs were detected by amperometry (31) using a carbon fiber electrode. To detect serotonin release from a MC in response to mechanical stimulation, a +550-mV oxidation potential was applied to the carbon fiber electrode and mechanical stimulation was applied to the MC by directly touching it using the same carbon fiber electrode. Details are provided in *SI Materials and Methods*.

Behavioral Whisker Tactile Test. The whisker tactile test was performed in a blinded manner in that one examiner conducted drug treatments and animal

grouping (e.g., WT group vs. 5-$HT_{3A}^{-/-}$ group), and another examiner who did not know the grouping performed behavioral whisker tests on these animals. To test whisker tactile responses, a single whisker hair (D1 whisker) was displaced up to 2 mm in a caudal-rostral direction by the tactile stimulation filament, and the whisker tactile test was performed 20 times with an interval of 1 min between trials. A positive behavioral whisker tactile response was considered when the testing animal exhibited an avoidance reaction to the tactile stimulation. Details are provided in *SI Materials and Methods*.

Data Analysis. Data are presented as mean ± SEM. Statistical significance was evaluated using Student's *t* test, one-way or two-way ANOVA with Bonferroni post hoc tests for multiple groups, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Details are provided in *SI Materials and Methods*.

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