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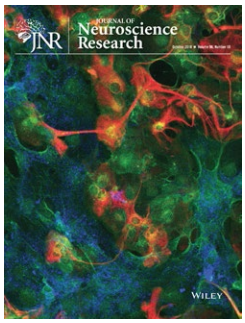

Role of 5-HT1A and 5-HT3 receptors in serotonergic activation of sensory neurons in relation to itch and pain behavior in the rat

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Abstract

Serotonin (5-hydroxytryptamine, 5-HT) released by platelets, mast cells, and immunocytes is a potent inflammatory mediator which modulates pain and itch sensing in the peripheral nervous system. The serotonergic receptors expressed by primary afferent neurons involved in these sensory functions are not fully identified and appear to be to a large extent species dependent. Moreover, the mechanisms through which 5-HT receptor activation is coupled to changes in neuronal excitability have not been completely revealed. Using a combination of *in vitro* (calcium and voltage imaging and patch-clamp) and *in vivo* behavioral methods, we used both male and female Wistar rats to provide evidence for the involvement of two 5-HT receptor subtypes, 5-HT1A and 5-HT3, in mediating the sustained and transient effects, respectively, of 5-HT on rat primary afferent neurons involved in pain and itch processing. In addition, our results are consistent with a model in which sustained serotonergic responses triggered via the 5-HT1A receptor are due to closure of background potassium channels, followed by membrane depolarization and action potentials, during which the activation of voltage-gated calcium channels leads to calcium entry. Our results may provide a better understanding of mammalian serotonergic itch signaling.

KEYWORDS
dorsal root ganglion, itch sensing, pain behavior, serotonin receptor

1 | INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is one of the major neurotransmitters in the central nervous system (CNS) and is involved in the modulation of many brain circuits, having a substantial influence on behavior (Berger, Gray, & Roth, 2009). However, it has also been recognized that a major fraction of 5-HT in the body is generated outside of the CNS, where it regulates all major organs, including the gastrointestinal tract, heart, blood vessels, lungs, and the endocrine system. Peripheral 5-HT also acts as an inflammatory mediator and as such modulates the activity of primary nociceptors and itch receptors in the skin (Loyd, Henry, & Hargreaves, 2013). During tissue inflammation, a complex "inflammatory soup" is generated which includes 5-HT, nerve growth factor, histamine, bradykinin, prostaglandins, and other endogenous lipid metabolites. All these mediators act on nociceptive nerve endings and trigger neuronal hypersensitivity, leading to allodynia (pain sensation evoked by non-noxious stimuli) and hyperalgesia (enhanced response to a noxious stimulus; Coutaux, Adam, Willer,

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& Le Bars, 2005). Inflammatory 5-HT released by mast cells, platelets, and immune cells plays a key role in nociceptor sensitization and itch signaling (Akiyama & Carstens, 2013; Sommer, 2004). 5-HT has long been known to elicit itch sensation in humans (Fjellner & Hägermark, 1979; Hägermark, 1992) and scratching behavior in rodents (Akiyama, Carstens, & Carstens, 2010; Akiyama et al., 2016; Akiyama, Merrill, Zanotto, Carstens, & Carstens, 2009; Berendsen & Broekkamp, 1991; Jinks & Carstens, 2002; Klein, Carstens, & Carstens, 2011; Moser & Giesler, 2014b; Thomsen, Petersen, Benfeldt, Jensen, & Serup, 2001; Yamaguchi, Nagasawa, Satoh, & Kuraishi, 1999). Intradermal injection of 5-HT activates neurons in the superficial spinal dorsal horn (Akiyama et al., 2009; Akiyama, Merrill, Zanotto, et al., 2009; Jinks & Carstens, 2002; Klein et al., 2011), including those with ascending parabrachial and thalamic projections (Jansen & Giesler, 2015; Moser & Giesler, 2014a), as well as in somatosensory thalamus (Lipshetz et al., 2018) in a manner that parallels scratching. Several receptor subtypes have been implicated in 5-HT-evoked scratching behavior, including 5-HT1A (Tian et al., 2016), 5-HT1F (Stantcheva et al., 2016), 5-HT2 (Akiyama et al., 2016; Nojima & Carstens, 2003; Yamaguchi et al., 1999), 5-HT2B (Lee et al., 2018), 5-HT3 (Ostadhadi, Kordjazy, Haj-Mirzaian, Mansouri, & Dehpour, 2015), 5-HT6 (Tian et al., 2016), and 5-HT7 (Morita et al., 2015).

Serotonergic receptors are classified in seven receptor families, one of which (5-HT3) is ionotropic, while the rest are metabotropic (reviewed by Marin, Becamel, Dumuis, & Bockaert, 2012). The expression and function of the various 5-HT receptor subtypes in primary afferent neurons have not been fully clarified, and important species differences may be responsible for complicating the issue. Thus, an RT-PCR investigation in rat dorsal root ganglia (DRG) neurons provided evidence for the mRNA expression of 5-HT1B, 5-HT1D, 5-HT2A, 5-HT2B, 5-HT3B, and 5-HT4, while 5-HT1A, 5-HT2C, and 5-HT7 (among others) were not detected (Nicholson, Small, Dixon, Spanswick, & Lee, 2003). In another RT-PCR study carried out in human DRG tissue there was evidence for the expression of 5-HT1D, E and F, 5-HT2A, and 5-HT7, while 5-HT1A expression appeared to be highly variable, but nonetheless detectable (Pierce, Xie, Meuser, & Peroutka, 1997). Recent reports confirmed 5-HT1A expression at mRNA level in small diameter rat DRG neurons (Paddock, Sheppard, & Gardiner, 2018; Usoskin et al., 2015) while an immunohistochemical approach led to the detection of 5-HT1A and 5-HT1B receptors in Merkel cell-associated nerve endings in sinus hair follicles of the rat whisker pad (Tachibana, Endoh, Fujiwara, & Nawa, 2005). Furthermore, 5-HT1A transcripts were found to be highly expressed in a population of Ret-positive, IB4-negative DRG neurons from the mouse (Stantcheva et al., 2016).

Using patch-clamp electrophysiology, Todorovic and Anderson (1992) have shown that 5-HT treatment hyperpolarizes a subpopulation of capsaicin-sensitive rat DRG neurons via activation of 5-HT1A receptors, and an involvement of the same 5-HT1A receptors in serotonergic modulation of voltage-gated calcium channels in DRG neurons was reported by other investigators (Cardenas, Mar, & Scroggs, 1997). However, more recent functional evidence has emphasized key roles for the metabotropic 5-HT2 and 5-HT7 receptors in pain and itch signaling in the peripheral nervous system. Activation of both receptors in rat DRG neurons leads to potentiation of TRPV1 function, which is likely to contribute to inflammatory hyperalgesia (Ohta et al., 2006). A more recent paper provided evidence for the involvement of the 5-HT2C isoform in mediating not only TRPV1 sensitization in rat DRG, but also opening of Ca^{2+}-activated chloride channels, leading to 5-HT-induced increased neuronal excitability (Salzer, Gantumur, Yousuf, & Boehm, 2016). The role of these receptors in serotonergic itch has also been addressed using the mouse model, but the findings were not in agreement: thus, one group reported that 5-HT activates 5-HT7 receptors in primary afferent neurons, followed by opening of TRPA1 channels and calcium entry in itch-sensing nerve endings (Morita et al., 2015), while another group identified 5-HT2 receptors and TRPV4 channels as responsible for serotonergic itch (Akiyama et al., 2016). A third group recently reported that the 5-HT analogue α-methyl-5-HT elicited scratching via the 5-HT2B receptor linked to TRPC4 (Lee et al., 2018).

Using a combination of *in vitro* (calcium imaging and patch-clamp) and behavioral approaches, we investigated the signaling pathways responsible for both the transient and the sustained activation of primary afferent neurons by 5-HT in the Wistar rat model. While the ionotropic 5-HT3 receptors are responsible for the transient activation, our results indicate that the sustained effect involves the 5-HT1A receptor isoform, followed by neuronal depolarization associated with a decrease in membrane conductance, most likely determined by the closure of potassium channels. In addition, we demonstrate that both 5-HT3 and 5-HT1A mediate the itch and pain behavior induced by 5-HT in Wistar rats. Our data further support the conclusion that the signaling pathways and receptor subtypes involved in serotonergic effects on primary afferent neurons are strongly species dependent.

Significance

Serotonin is an inflammatory molecule involved in pain and itch sensing by peripheral nerve endings. Our work identifies some of the key molecules and mechanisms which mediate the cellular actions of serotonin and participate in the activation and sensitization of nerve fibers, leading to the perception of pain and itch. These results contribute to a better understanding of the biological basis of itch and pain and may be useful in designing novel therapies to counteract these undesired sensory perceptions.

2 | MATERIAL AND METHODS

2.1 | Solutions and reagents

Standard extracellular solution (ES) had the following composition (in mM): NaCl 140, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, NaOH 4.54, and

glucose 5, pH 7.4 at 25°C. The pharmacological compounds were added from stock solutions (concentrations and solvents inside brackets). The reagents were from: Tocris Bioscience: BRL-15572 hydrochloride (1 mM in DMSO), MDL-100907 (100 mM in DMSO), ketanserin (10 mM in H_2O), RS 23597-190 hydrochloride (100 mM in H_2O), SB-204070 (100 mM in DMSO), SB-206553 hydrochloride (100 mM in DMSO), SB-269970 hydrochloride (20 mM in H_2O), HC-030031 (100 mM in DMSO), HC-067047 (100 mM in DMSO), A-967079 (100 mM in DMSO), BIMU8 (50 mM in H_2O), RO 60-0175 fumarate (5 mM in H_2O), TCB-2 (20 mM in H_2O), DOI hydrochloride (50 mM in H_2O), (R)-(+)-8-Hydroxy-DPAT hydrobromide (8-OH-DPAT, 70 mMin H_2O), PNU-142633 (100 mM in DMSO), LY 344864 hydrochloride (50 mM in H_2O), 5-Carboxamidotryptamine maleate (5-CT, 100 mMin H_2O), BW-723C86 hydrochloride (100 mM in DMSO), cisapride (100 mM in DMSO), tegaserod maleate (100 mM in DMSO), RS 67506 hydrochloride (80 mM in H_2O), WAY-181187 oxalate (100 mM in DMSO), granisetron hydrochloride (1 mM in H_2O), m-Chlorophenylbiguanide hydrochloride (mCPBG, 20 mMin H_2O), SR-57227 hydrochloride (100 mM in DMSO), LP-44 (100 mM in DMSO), MONNA (10 mM in DMSO); Sigma-Aldrich: serotonin hydrochloride (5-HT, 50 mMin H_2O), serotonin creatinin sulfate monohydrate (5-HT, 25 mMin 0.9% saline), RS 102221 hydrochloride (100 mM in DMSO), SB-69955 dihydrochloride (10 mM in H_2O), SB-399885 hydrochloride (100 mM in H_2O), Ruthenium Red (10 mM in H_2O), capsaicin (5 mM in ethanol), lanthanum chloride ($LaCl_3$, 250 mMin H_2O), pertussis toxin (100 μg/ml in H_2O); Fluka: allyl isothiocyanate (allyl isothiocyanate [AITC], 100 mMin DMSO); Cayman Chemical: SB-224289 hydrochloride (1 mM in DMSO), zolmitriptan (100 mM in DMSO); Abcam: WAY-100635 maleate (10 mM in H_2O).

2.2 | DRG and trigeminal ganglia (TG) cell culture

DRG were obtained from all spinal levels of adult male Wistar rats, as described elsewhere (Reid, Babes, & Pluteanu, 2002). TG were harvested after removing the brain, from rats of both sexes. Male and female rats (150–200 g; 8–10 weeks) were killed by inhaling increasing concentrations of CO_2, followed by decapitation, in accordance with the European guidelines on laboratory animal care and with the approval of the institutional ethics committee of the Faculty of Biology, University of Bucharest. Upon excision, the ganglia were incubated in a mixture of 2 mg/ml collagenase (type XI, Sigma Aldrich) and 3 mg/ml dispase (Gibco) in IncMix solution for 1 hr at 37°C. Neurons were dissociated mechanically by trituration, plated on 24 mm round borosilicate coverslips (0.17 mm thick) treated with poly-D-lysine (0.1 mg/ml; 30 min) and maintained at 37°C with 5% CO_2 in a 1:1 mixture of DMEM and Ham's F12 medium (D8900, Sigma-Aldrich), supplemented with 10% horse serum, 50 μg/ml gentamicin, and 7.4 mM glucose. The cells were used for experiments 12 to 24 hr after plating. For the pertussis toxin (PTX) experiment, neurons from one male rat were split into control and PTX pre-treated conditions (seven dishes each). PTX (500 ng/ml) was added to the culture medium and the cells were imaged the following day (after 15–20 hr).

2.3 | Intracellular non-ratiometric calcium imaging

DRG neurons cultured on 24-mm coverslips were incubated in standard extracellular solution (see Section 2.1) containing 2 μM Calcium Green-1 AM and 0.02% Pluronic F-127 (both from Invitrogen) for 30 min at 37°C and left to recover for 30 min before use. Coverslips were mounted in a Teflon chamber (MSC TD, Digitimer, Welwyn Garden City, UK) on the stage of an Olympus IX70 inverted microscope. Cells were illuminated at 470 nm with a Dual OptoLED light source (Cairn Research, Faversham, UK) controlled by the Axon Imaging Workbench 2.2 software (Axon Instruments, Union City, CA, USA), which was also used for image acquisition and analysis. Fluorescence changes were acquired with a CCD camera (Cohu 4910, Pieper GmbH, Schwerte, Germany) and digitized to 8 bits with a frame grabber (DT-3155, Data Translation, Marlboro, MA, USA). The change in the calcium indicator fluorescence was analyzed with custom software, and the maximal fluorescence change during stimulation relative to the initial fluorescence ($\Delta F/F_0$) was measured with individual F_0 values before each stimulation. In the graphs we displayed the raw absolute fluorescence data measured in arbitrary units (a.u.). The area under the curve (AUC) was computed from the response start until the amplitude decreased by 80% from the peak ΔF value or, if this value was not reached, until the next chemical stimulus occurred. AUC was expressed in arbitrary units multiplied by seconds (a.u.s). For statistical analysis, $\Delta F/F_0$ and AUC from the second to the last 5-HT challenge were normalized for each neuron to the $\Delta F/F_0$ and AUC values of the first 5-HT exposure. For each calcium imaging experiment described in the Results section, the *n* number provided refers to the total number of neurons recorded. For certain experiments we only used neurons from one primary culture. For most experiments we used neurons from between 2 and 16 animals, which were pooled together. The actual numbers of animals used for each experiment are indicated in the Figure Legends.

2.4 | Voltage imaging

Voltage imaging was performed with the voltage-sensitive dye from the FLIPR Membrane Potential Assay Kit Red (Molecular Devices, Sunnyvale, CA, USA). DRG neurons were plated on 24-mm diameter round coverslips 24 hr before the experiments, using the protocol described for calcium imaging. The content of a dye bottle was dissolved in 15 ml extracellular solution (the same as used for calcium imaging) and cells were incubated with this solution for 15 min at room temperature (about 22°C). The coverslips were imaged on the platform of an inverted microscope with a 20x 0.75 NA objective, by exciting the fluorescence at 470 nm and collecting the emission through a 520 nm long-pass filter. During the recordings, cells were continuously superfused at a 0.5 ml/min flow rate, either with the loading solution (washout) or with the test solutions (5-HT and KCl) made in the loading solution as well.

2.5 | Patch-clamp recordings

For patch-clamp recordings, DRG neurons were plated on 24-mm borosilicate glass coverslips coated with poly-D-lysine and used within 24 hr. Whole-cell patch-clamp currents were recorded using a WPC-100 patch-clamp amplifier (E.S.F. Electronic, Göttingen, Germany), filtered at 3 kHz, and digitized at 5 kHz with an Axon Instruments DigiData 1322A interface driven by pCLAMP 8.2 (Molecular Devices, Sunnyvale, CA, USA). All capacitive transients were compensated using the amplifier compensation module (C-slow, C-fast and Rs). The extracellular solution was the same as the solution for calcium imaging. The intracellular solution had the following composition (in mM): 140 KCl, 1 $MgCl_2$, 2 Na, 2 ATP, 5 EGTA, 10 HEPES; pH 7.25 at 25°C adjusted with KOH. For simultaneous patch-clamp and calcium imaging recordings of the same neurons, the pipette solution was supplemented with 3.3 μM Calcium Green-1 AM that was previously de-esterified for 1 hr with KOH, diluted at 1.6 mM in the final solution. After entering the whole-cell configuration, Calcium Green-1 diffused inside the cell until a plateau of the intracellular fluorescence was reached, point after which the recordings were started. Borosilicate capillaries with filament (BF150-86-10HP, Sutter Instrument, Novato, CA, USA) were pulled using a horizontal micropipette puller (P-1000, Sutter Instrument) and tip polished for resistances of 2–4 MΩ. To obtain symmetrical junction potentials at the two electrodes, the reference electrode was placed in the same intracellular solution as the recording electrode and connected to the bath trough an agar bridge. All recordings were performed at room temperature (21–24°C).

2.6 | Behavior

Behavioral experiments were conducted at UC Davis and were approved by the UC Davis IACUC. Both male (n = 8) and female (n = 8) Wistar rats were used. Each set of eight animals was tested with saline and each of the seven drugs (see below). The animals were housed in pairs wherever possible, with food and water available ad libitum. The animal housing area was maintained on a 12:12 light–dark cycle. Vehicle (0.9% saline), or the 5-HT1A antagonist WAY-100635 (10 mM/10 μl) and/or the 5-HT3 antagonist granisetron (10 μM/10 μl) were injected intradermally (i.d.) in the cheek, followed 15 min later by the injection of serotonin creatinine sulfate monohydrate (5-HT, 1% ≈ 25 mM/10 μl) at the same site. The investigator was blinded as to the injected chemicals. The animals were videorecorded for 60 min after injecting 5-HT. For analysis, the hind limb scratch bouts (indicative of itch) and forelimb wipes (indicative of pain; Shimada & LaMotte, 2008) directed to the injection site were scored offline by at least two observers who were blinded as to treatment. The 5-HT1A agonists 5-CT (50 mM/10 μl) and 8-OH-DPAT (10 mM/10 μl), and the 5-HT3 agonist SR-57227 (1 mM/10 μl) were injected i.d. in the cheek as above.

2.7 | Statistical analysis

Data are presented as mean ± standard error of the mean (*SEM*). Statistical significance was calculated using Student's *t*-test or one-way repeated measures ANOVA with post hoc Tukey's HSD. Statistical analysis was performed with OriginPro 8 software (OriginLab Corporation, Northampton, MA, USA). A value of $p < 0.05$ was considered statistically significant. No data were excluded or are missing from the analysis.

3 | RESULTS

3.1 | Serotonin elicits distinct types of responses in cultured rat DRG neurons

We investigated the effect of 5-HT on cultured rat DRG neurons in primary culture using non-ratiometric calcium microfluorimetry-based on the fluorescent indicator Calcium Green-1 AM The cells were challenged with 5-HT (50 μM, 2 min) three times at 4-min intervals, followed by KCl (50 mM) to identify viable neurons responding to KCl-induced depolarization (Figure 1a,b).

Application of 5-HT induced distinct types of $[Ca^{2+}]_i$ responses which were classified in two categories according to their kinetics: *transient*, displaying a fast return to their baseline fluorescence while still in the presence of 5-HT (Figure 1a) and *sustained*, showing a slow fluorescence decrease which reached baseline only after 5-HT was washed out and often presenting multiple spikes (Figure 1b). In order to categorize the 5-HT-induced increases in $[Ca^{2+}]_i$ in a clear quantitative manner, we used a histogram of the AUC of the responses to the first application of 5-HT from 420 5-HT-sensitive neurons, which was fitted with a two-peak Gaussian function. As shown in Figure 1c, the histogram reveals two distinct populations of 5-HT responses: the first group, with an AUC of 126 ± 5 a.u.s (n = 120), corresponded to the 5-HT-sensitive neurons with transient responses, while the second group, with an AUC of 2,400 ± 27 a.u.s (n = 300) consisted of 5-HT-sensitive neurons with a sustained response. To assign neurons to the population displaying sustained responses to 5-HT, the threshold value was chosen at the distribution peak (mean) for the transiently responding cells plus two standard deviations of the same distribution. Thus, we established an AUC threshold of 250 a.u.s for the sustained responses (Figure 1b) and all neurons with an AUC less than this value were assigned to the purely transient group (Figure 1a). In a large fraction of the sustained responses there was also a transient component present immediately after applying 5-HT (Figure 1b, black arrow). To simplify the analysis, all neurons displaying both transient and sustained responses (mixed responders) were joined with the purely sustained responding neurons. Of the neurons tested in DRG cultures from male rats (N = 16 animals), 23.4% responded transiently to 5-HT and 8.5% had a sustained response (in a total of n = 3,658 KCl-sensitive neurons).

To record fluorescence signals associated with the depolarizations evoked by 50 μM 5-HT, the neurons were loaded with a

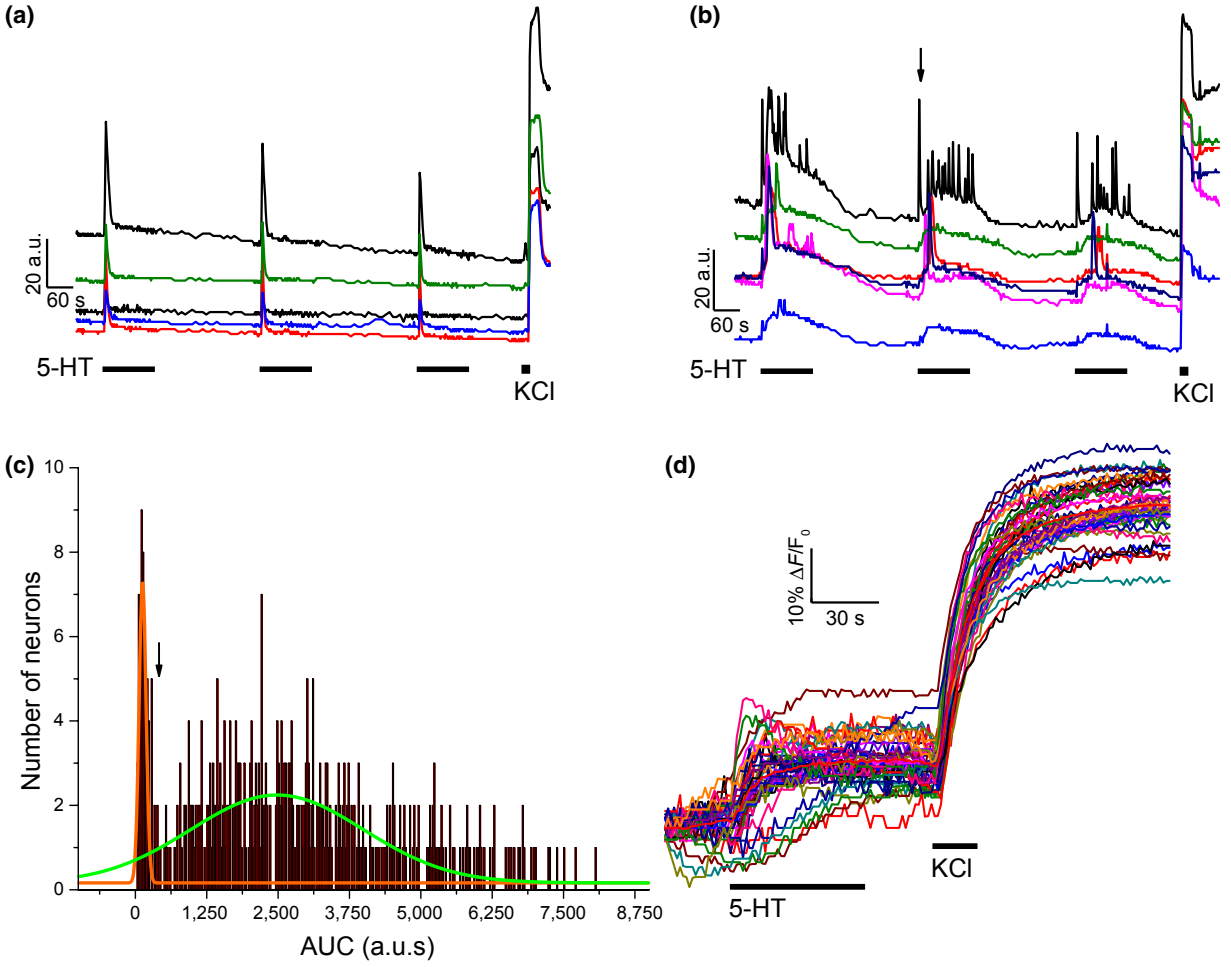

FIGURE 1 5-HT elicits distinct types of $[Ca^{2+}]_i$ responses and depolarizations in rat DRG neurons. (a) Illustrative examples of calcium imaging traces of rat DRG neurons with a transient response to 5-HT (50 μM, 2 min). (b) Examples of sustained $[Ca^{2+}]_i$ responses elicited in the same conditions as Figure 1a. Note the transient component at the beginning of the 5-HT application (black arrow). (c) The histogram of representative responses to 5-HT from 420 rat DRG neurons (from 16 animals) was fitted with two Gaussian functions. A demarcation threshold was set as the mean of the first peak plus two times its standard deviation (mean of first peak = 125 + 2 × *SD* = 250 a.u.s). The transiently responding group included all responses under this threshold (< 250 a.u.s), while the sustained group included responses over this value (>250 a.u.s). The mixed responses, sustained with initial transient components, were included in the sustained group based on their AUC. (d) Examples of 5-HT-evoked (50 μM, 1 min) depolarizations recorded using a membrane potential assay. The threshold for identifying responses was set at 15% $\Delta F/F_0$. From the total number of neurons responding to KCl, 33.75% were depolarized by 5-HT. No hyperpolarizing responses were recorded

voltage-sensitive dye (FLIPR Membrane Potential Assay Kit Red). When superfusing solutions contained both the test compounds and the loading solution, the method delivered robust depolarizing responses (Figure 1d), confirming its usefulness for imaging DRG neurons (Fairless et al., 2013). Although the indicator can decrease in fluorescence with membrane hyperpolarization, 5-HT evoked no such hyperpolarizing responses. In order to select all responses to 5-HT, the $\Delta F/F_0$ threshold was set to 15% from baseline. From the total of neurons showing an increase of fluorescence to KCl (50 mM), 33.7% were activated by 5-HT, with an average $\Delta F/F_0$ = 0.21 ± 0.005 (n = 139 neurons from one animal). The slow kinetics of the voltage indicator did not allow to further split this population into transiently and sustained depolarizing neurons. However, these results are in good agreement with the calcium imaging data, which yielded a very similar fraction of 5-HT-sensitive neurons (31.9%). Moreover, they demonstrate that both types of 5-HT responses evoke depolarizations in DRG neurons.

We investigated the source of the 5-HT-evoked Ca^{2+} increase by switching to a Ca^{2+}-free extracellular solution during the second 5-HT challenge. In Ca^{2+}-free conditions, the transient responses were completely absent while the sustained responses were reduced by 58.7%, from an average AUC of 2,559.7 ± 124.6 a.u.s (n = 74) in control conditions to 1,055.1 ± 193.6 a.u.s (n =14; two-sample Student's t-test, p < 0.0001; Figure 2a,b). Furthermore, the spiky components of the sustained responses were abolished under Ca^{2+}-free conditions. This indicates that the increases in $[Ca^{2+}]_i$ induced by 5-HT, either transient or sustained, are mainly due to calcium entry, while Ca^{2+} release from intracellular stores also contributes to the sustained responses.

3.2 | Characterization of 5-HT-sensitive neurons from DRG and TG

The diameter of DRG neurons with a transient response to 5-HT (20.6 ± 0.2 μm; n = 106) was significantly smaller (two-sample Student's t-test, $p < 0.001$) than the diameter of neurons with a sustained response (26.0 ± 0.2 μm; n = 103). The 5-HT-insensitive neurons had a diameter of 31.1 ± 0.6 μm (n = 109) (Figure 3a). The 5-HT-sensitive neurons were further characterized by applying capsaicin (0.3 μM, a selective TRPV1 agonist) and AITC (100 μM, a selective TRPA1 agonist) on cultured DRG neurons from male rats and trigeminal neurons (TG) from rats of both sexes. In male rat DRG/TG neurons, 5.9%/25% of the transiently responding neurons and 44.1%/95.6% of the sustained responding neurons were sensitive to AITC (Figure 3b). In these neurons, 87.5%/86.1% of transiently responding neurons and 100%/100% of sustained responding neurons were sensitive to capsaicin. In female TG neurons that showed a sustained response to 5-HT, 87% were sensitive to AITC and 87.5% to capsaicin (Figure 3b). Both the small diameter and the sensitivity to TRPV1 and TRPA1 agonists support the classification of 5-HT-sensitive neurons as nociceptors.

3.3 | Transient responses to 5-HT are mediated by the 5-HT3 ionotropic receptor

In order to identify the 5-HT receptor subtypes responsible for the 5-HT-induced increases in $[Ca^{2+}]_i$ in DRG neurons, we used the same protocol with three 5-HT challenges (as in Figure 1a,b). Antagonists selective for subtypes of 5-HT receptors were pre-applied for 1 min and co-applied during the second exposure to 5-HT. The increase in $[Ca^{2+}]_i$ measured by the change in fluorescence during stimulation relative to baseline ($\Delta F/F_0$) was compared to control conditions (i.e., without antagonist). Granisetron (10 nM), a selective 5-HT3 antagonist, produced a complete and long-lasting inhibition of the transient responses to 5-HT, while having no effect on the sustained responses (Figure 4a,b). In order to further confirm the expression of the 5-HT3 ionotropic receptor we applied the selective 5-HT3 agonist SR-57227 (1, 10 μM), which elicited a short-lasting $[Ca^{2+}]_i$ increase in 96% (26/27) of neurons that also responded transiently to 5-HT (Figure 4c). Another 5-HT3 agonist, mCPBG (20 MM), evoked transient responses while it also activated a population of 5-HT-insensitive neurons (data not shown), therefore it was not further used.

3.4 | Sustained responses are inhibited by a specific 5-HT1A antagonist

To investigate the putative contribution of other 5-HT receptor subtypes to the sustained responses evoked by 5-HT in DRG neurons we further tested a set of selective antagonists and agonists. The antagonists were pre- and co-applied with the second application of 5-HT (50 μM) and the AUC of these responses was compared with the control condition. Of all the antagonists tested, WAY-100635, a 5-HT1A antagonist, was the only one that significantly inhibited the sustained responses to 5-HT, and acted in a concentration-dependent manner. All other antagonists had no significant inhibitory effect or even enhanced the sustained responses (Figure 5a, Figure S1a). The purely sustained responses were almost

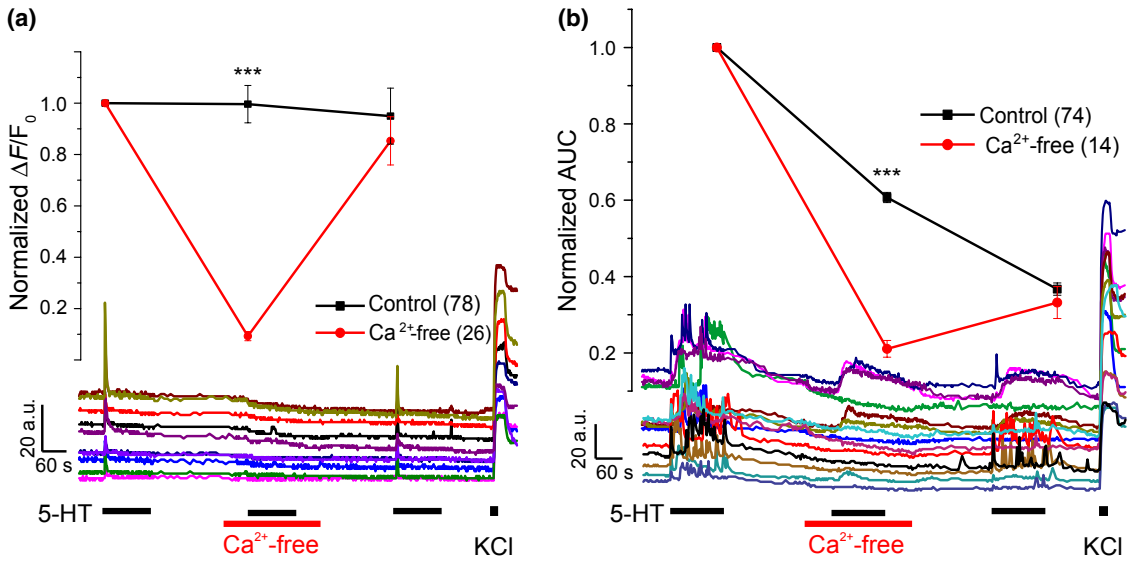

FIGURE 2 Calcium-free conditions abolished the transient responses and significantly inhibited the sustained ones. (a) Representative transient responses, abolished under Ca^{2+}-free conditions. Top: Statistical analysis of the $\Delta F/F_0$ ± *SEM* measured for the transient responses (***, $p < 0.001$, two-sample Student's t-test). The number of neurons (all from one animal) for each condition is indicated in brackets. (b) Illustrative recordings of sustained responses, inhibited under Ca^{2+}-free conditions. Top: Statistical analyses of the AUC measured in Ca^{2+}-free conditions compared with control (***, $p < 0.001$, two-sample Student's t-test). Neurons from three different animals were pooled together

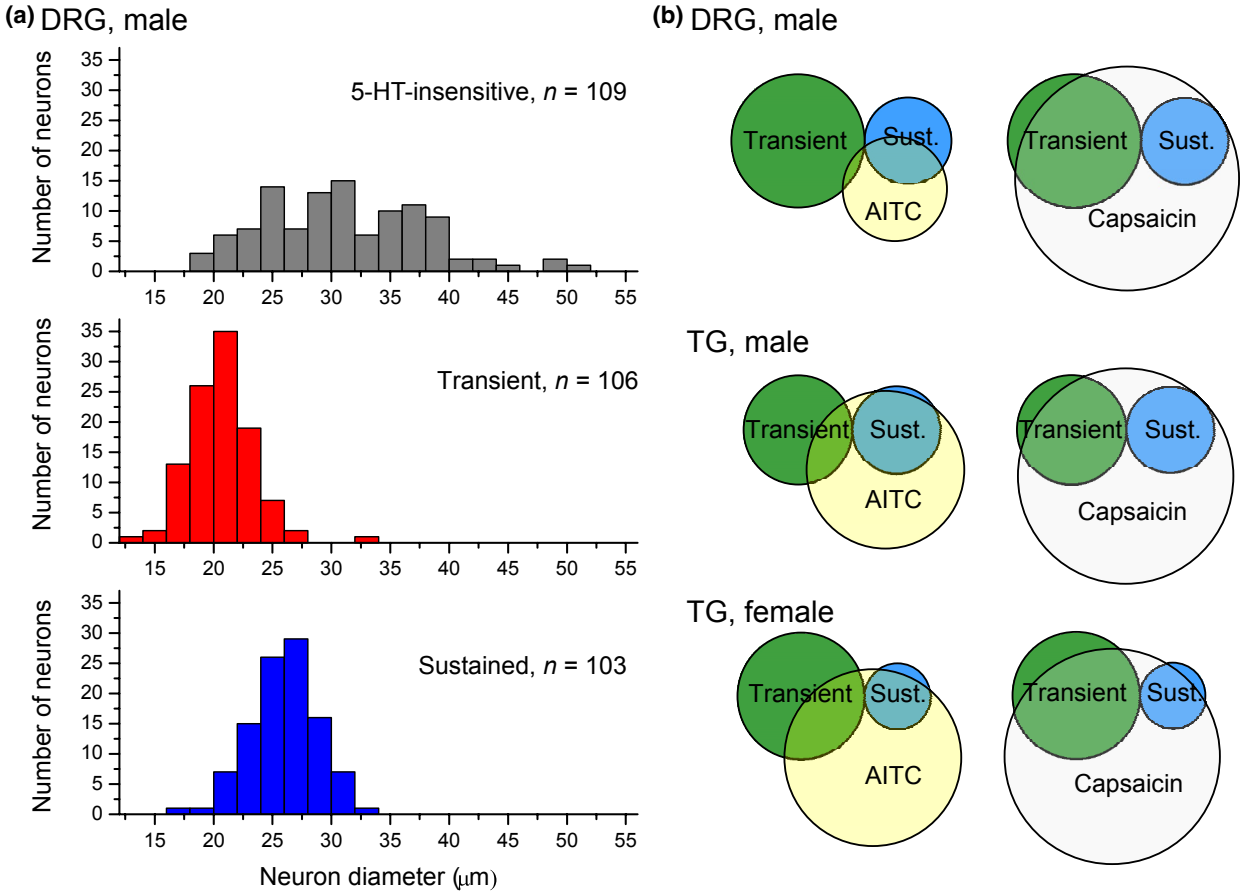

FIGURE 3 Characterization of 5-HT-sensitive rat dorsal root ganglia (DRG) neurons. Distribution of neurons and their sensitivity to 5-HT, AITC, and capsaicin. (a) Histograms showing the diameter of 5-HT-insensitive (upper), transiently responding (middle) and sustained responding neurons (lower). The 5-HT-insensitive neurons had a diameter of 31.1 ± 0.6 μm. The DRG neurons with a transient response to 5-HT had a diameter of 20.6 ± 0.2 μm, which differed significantly ($p < 0.001$, two-sample Student's *t*-test) compared with the diameter of sustained responding neurons (26.0 ± 0.2 μm). Neurons from six different animals were pooled together. (b) Venn diagrams showing the relationship between neurons displaying transient (green) and sustained responses (blue) to 5-HT and their sensitivities to AITC (100 μM) (yellow) and capsaicin (300 nM) (white) in DRG neurons from two male rats (top) and trigeminal ganglia (TG) neurons from two male and two female rats (middle and bottom). Note the large overlap of 5-HT and capsaicin sensitivities in all conditions and the larger overlap with AITC-sensitivity in neurons responding sustained to 5-HT compared with neurons responding transiently

completely abolished by WAY-100635, while the mixed responses still maintained the transient component, which is likely mediated by 5-HT3 (Figure 5b). WAY-100635 inhibited the AUC of sustained responses from 2,559.71 ± 124.68 a.u.s ($n = 74$), in control conditions, to 1,049.49 ± 113.39 a.u.s ($n = 31$; two-sample Student's *t*-test, $p < 0.0001$) at 0.1 μM and to 669.38 ± 70.12 a.u.s ($n = 40$; two-sample Student's *t*-test, $p < 0.0001$) at 10 μM (Figure 5b).

3.5 | Sustained responses are elicited by a specific 5-HT 1A agonist

To determine the specificity of the agonists, we quantified the co-expression between the response to 5-HT and the response to the agonist in DRG neurons for each compound tested. Due to the sometimes large off-target effects that we recorded when using 5-HT agonists, we introduced a so-called *specificity coefficient* to quantify the selectivity of these compounds in our experiments. For this purpose, the number of neurons that were activated solely by the agonist and not by 5-HT was subtracted from the number of neurons that were activated by both the agonist and 5-HT. The result was divided by the total number of 5-HT-sensitive neurons. The closer the *specificity coefficient* is to 1, the more selective the agonist (Figure 6a, Table S1). Our results show that, excepting 5-CT, none of the tested agonists closely mimicked the effects of 5-HT. 8-OH-DPAT, the 5-HT1A receptor agonist, and WAY-181187, a 5-HT6 receptor agonist, were the sole promising ligands to activate neurons that displayed sustained responses to 5-HT, based on their positive specificity coefficients. The specificity of 8-OH-DPAT responses correlated well with the inhibition elicited by WAY-100635 recorded previously, while the 5-HT6 antagonist SB-399885 had no significant effects on 5-HT responses (Figure 5a).

5-Carboxamidotryptamine (5-CT), a relatively non-selective agonist, which also activates 5-HT1B, 5-HT1D, 5-HT5, and 5-HT7

receptors, and the selective 5-HT1A agonist (R)-(+)-8-OH-DPAT (DPAT) induced a long-lasting $[Ca^{2+}]_i$ entry in 88% and, respectively, 76% of the neurons that also showed a sustained response to 5-HT (Figure 6b).

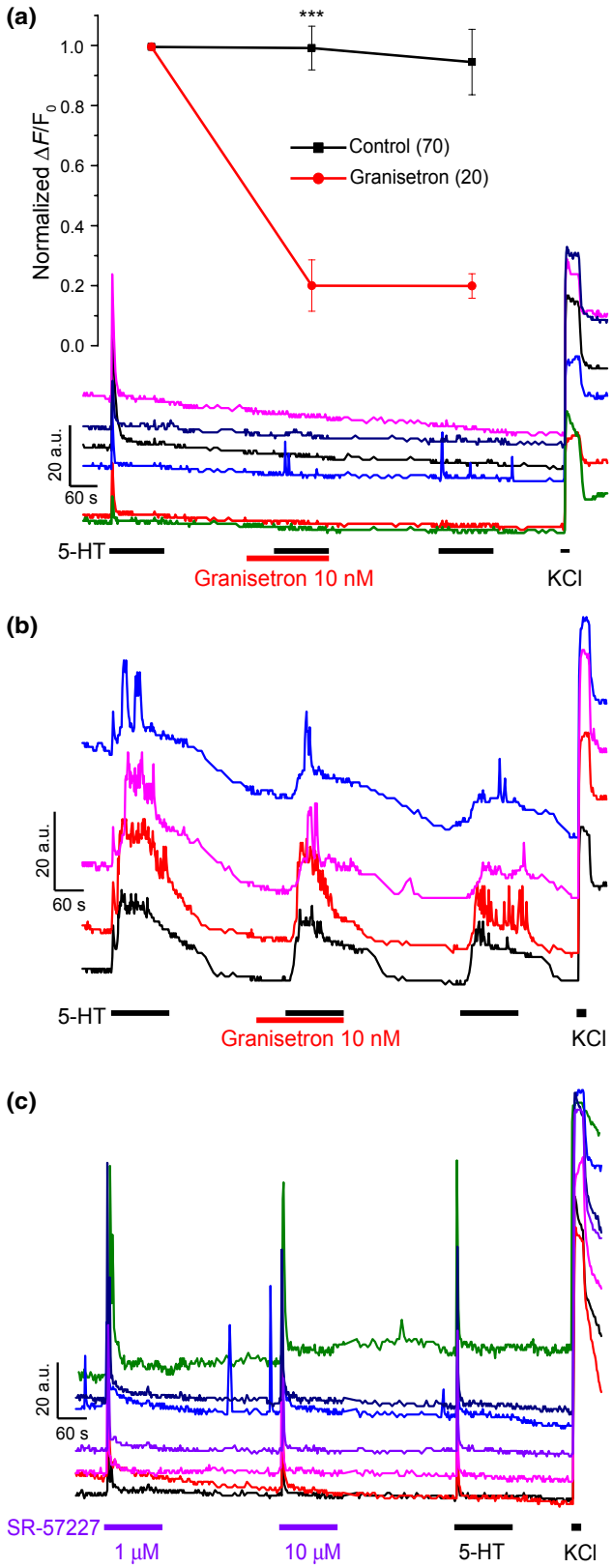

FIGURE 4 Transient responses were inhibited by granisetron, a selective 5-HT3 antagonist and elicited by SR-57227, a selective 5-HT3 agonist. (a) Representative transient responses of 5-HT-sensitive DRG neurons that were irreversibly inhibited by granisetron (10 nM). Top: normalized mean $\Delta F/F_0 \pm SEM$ of the three 5-HT (50 μM) challenges. The difference between control (black) and granisetron (10 nM; red) conditions was statistically significant (***, $p < 0.001$, two-sample Student's *t*-test). The number of neurons for each condition is indicated in brackets. The neurons were pooled from three different animals. (b) Granisetron (10 nM) had no effect on the sustained responses to 5-HT. DRG neurons were recorded from one animal. (c) The 5-HT3 agonist SR-57227 (1 and 10 μM, 2 min) elicited transient responses solely in neurons that showed a transient $[Ca^{2+}]_i$ increase to 5-HT (50 μM). DRG neurons were recorded from one animal

3.6 | Sustained responses to 5-HT are strongly inhibited in the presence of the $G\alpha_{i/o}$ inhibitor PTX

Pretreatment (for 15–18 hr) of cultured rat DRG neurons with the $G\alpha_{i/o}$ subunit inhibitor PTX (500 ng/ml) reduced the amplitude of sustained $[Ca^{2+}]_i$ responses evoked by 5-HT from an average AUC of 2,719 ± 238 a.u.s ($n = 64$) to 1,002 ± 236 a.u.s ($n = 19$; a 64% reduction, Student's unpaired *t* test, $p < 0.01$, Figure 7a,b), while also decreasing the fraction of such responses (from 8.1% in control conditions (64/793) to 2.3% following PTX treatment (19/819), chi-squared test, $p < 0.001$). PTX had no effect on the amplitude and fraction of transient responses evoked by 5-HT (Figure 7b), nor on the responses elicited by AITC (100 μM) or capsaicin (300 nM; data not shown).

3.7 | Transient receptor potential (TRP) ion channels are not involved in mediating responses to 5-HT

Given that 5-HT1A is a member of the G protein-coupled receptor (GPCR) superfamily, and thus not directly responsible for calcium entry, we wanted to investigate the effector channels involved in the generation of calcium signals in DRG neurons. The transient receptor potential (TRP) channel family includes several plasma membrane ion channels acting as cellular sensors for a variety of physical and chemical agents. These ion channels also play a major role in signal transduction and amplification, are widely expressed in sensory neurons, and show a high permeability for Ca^{2+} ions. Selective antagonists for TRPA1 (A-967079 and HC030031), TRPV4 (HC067047), and a broad-spectrum TRP channel blocker, Ruthenium Red were tested. None of the TRP channel antagonists inhibited the increase in $[Ca^{2+}]_i$ induced by 5-HT, while Ruthenium Red enhanced some of the sustained responses (Figure S1b).

MONNA (1 μM), an inhibitor of the Ca^{2+}-activated chloride channel anoctamin-1, was not able to inhibit the sustained responses to 5-HT (Figure S1b), while at 10 μM it elicited responses in a large proportion of the neurons.

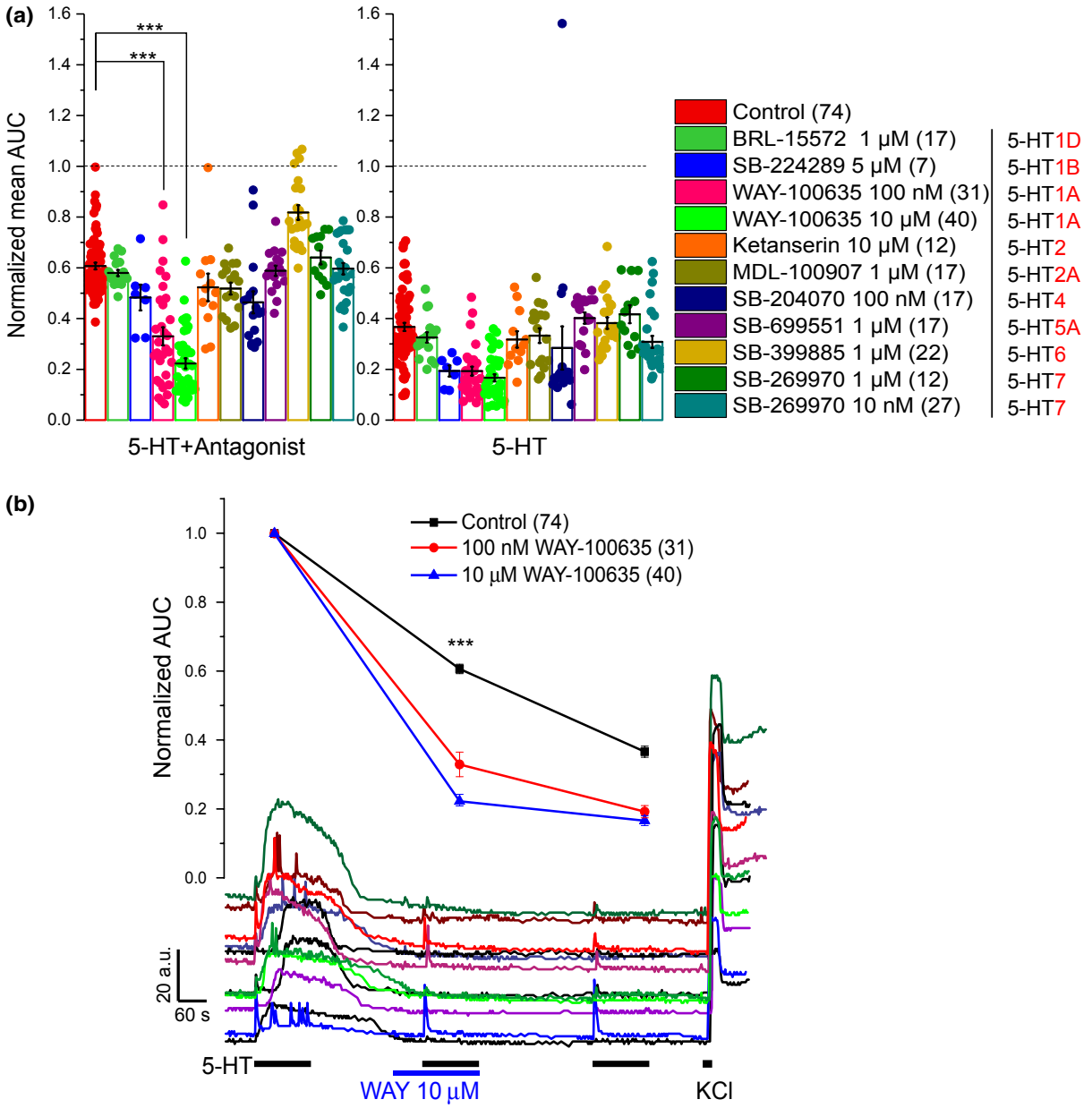

FIGURE 5 WAY-100635, a selective 5-HT1A antagonist, inhibited the sustained responses to 5-HT. (a) The list of tested 5-HT receptor antagonists, concentrations and their targets. The effect of antagonists specific to 5-HT receptors tested for their inhibitory effects on the second application of 5-HT (50 µM). The AUC of the three consecutive responses was normalized to the first. The statistical analysis of the difference between each antagonist and control for the second 5-HT response was calculated using two-samples Student's *t*-test (***, $p < 0.001$). The number of neurons for each condition is indicated in brackets. DRG neurons from five different animals were pooled. For all the antagonists tested see supplementary Figure 3. (b) Pre- and co-application of WAY-100635 (WAY, 0.1, 10 µM) inhibited in a concentration-dependent manner the sustained responses to 5-HT (50 µM). Top: Normalized mean AUC ± *SEM* of the sustained responses to 5-HT. The inhibitory effect of WAY-100635 (0.1, 10 µM) was compared with control conditions (***, $p < 0.001$, two-sample Student's *t*-test). DRG neurons from four different animals were pooled. Bottom: Examples of seven neurons that showed a sustained response to the first application of 5-HT and the inhibitory effect of WAY-100635 (10 µM) on the second 5-HT challenge. Note the transient component which is still visible in the mixed-responding neurons

3.8 | Voltage-gated calcium ion channels contribute to the transient and sustained responses evoked by 5-HT

To test the contribution of voltage-gated ion channels on the Ca^{2+} increase following 5-HT receptor activation, we co-applied 5-HT with the non-selective calcium channel blocker, lanthanum chloride ($LaCl_3$ 100 µM; Lansman, 1990). The transient responses were inhibited by $LaCl_3$ (100 µM) from $\Delta F/F_0$ 0.99 ± 0.07, in control conditions ($n = 78$) to 0.28 ± 0.04 ($n = 35$, $p < 0.001$, two-sample Student's *t*-test; Figure 8a). For the sustained responses, the 5-HT-induced increase in $[Ca^{2+}]_i$ was inhibited by 77.8% with $LaCl_3$ (100 µM), from

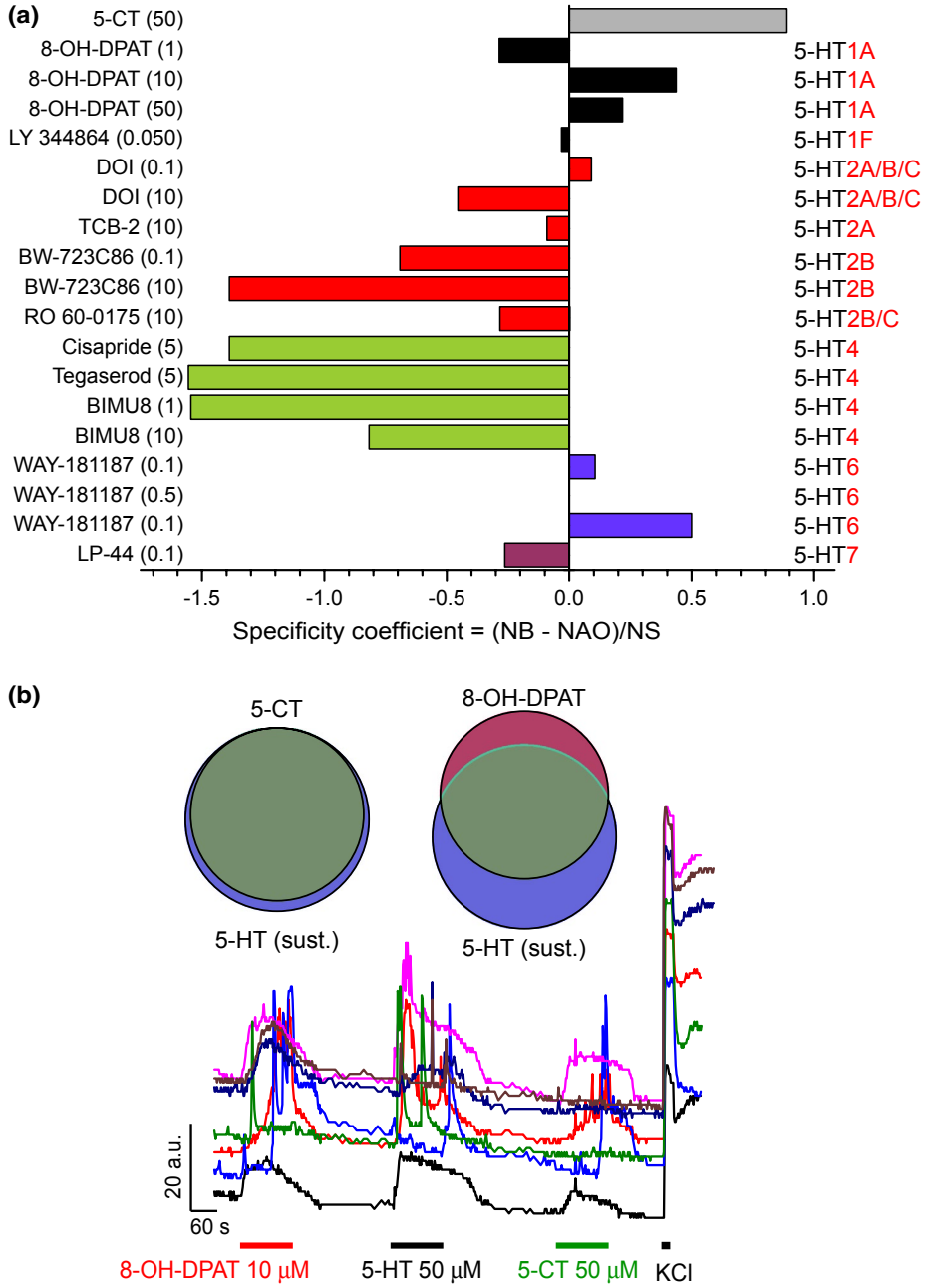

FIGURE 6 The selective 5-HT1A agonist 8-OH-DPAT activated 5-HT-sensitive neurons with sustained responses. (a) The list of tested 5-HT receptor agonists, concentrations (µM, in brackets) and their targets. To evaluate the specificity of the tested agonists, a specificity coefficient was computed as follows: the number of neurons activated by the agonist only (NAO) was subtracted from the number of neurons activated by both the same agonist and 5-HT (NB), and the result was divided to the total number of neurons responding to 5-HT (NS). Only 5-CT and the selective agonists for 5-HT1A (8-OH-DPAT) and 5-HT6 (WAY-181187) showed large positive specificity coefficients. These experiments were carried out on DRG neurons from 11 different animals. (b) Representative calcium imaging traces showing sustained responses to 5-HT (50 µM), 5-CT (50 µM), and 8-OH-DPAT (10 µM). 5-CT activated 88% of 5-HT sustained neurons, while 8-OH-DPAT activated 76%. DRG neurons from two different animals were pooled

an AUC of 1,069.11 ± 178.52 a.u.s (n = 35) in control conditions to 237.12 ± 48.76 a.u.s (n = 27; two-sample Student's t-test, $p < 0.001$; Figure 8b). The spiky component of the sustained responses was abolished, similar to Ca^{2+}-free conditions. Lanthanum had little effect on the amplitude of the transient currents elicited by 5-HT (Figure 8c). This supports the hypothesis that $LaCl_3$ inhibits the Ca^{2+} influx by blocking voltage-gated calcium ion channels activated downstream of the initial depolarization.

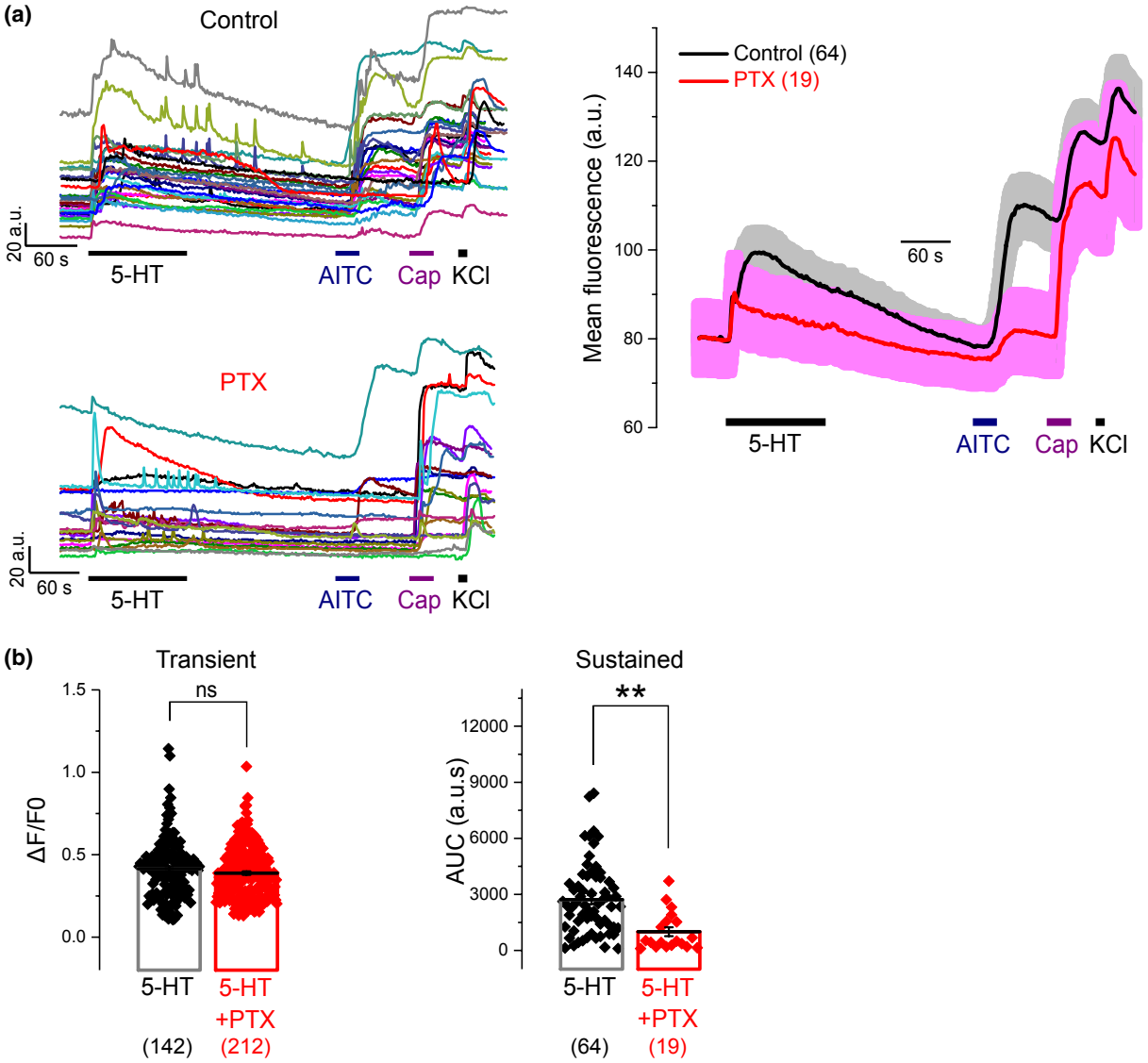

FIGURE 7 Pertussis toxin (PTX) inhibited the sustained responses to 5-HT. (a) Left: calcium imaging traces of sustained responses to 5-HT (50 μM) in control conditions (left, top) and after pre-treatment with the $G\alpha_{i/o}$ subunit inhibitor, PTX (500 ng/ml, overnight; left, bottom). Right: mean fluorescence ± *SEM* of all the neurons that showed a sustained $[Ca^{2+}]_i$ to 5-HT (50 μM) in control conditions (black) and pre-incubated with PTX (red). (b) Left: the mean $\Delta F/F_0$ of transient responses was not significantly different between PTX-treated and control conditions. Right: pre-treatment with PTX significantly reduced the AUC of sustained responses. Statistical analysis for $\Delta F/F_0$ and AUC ± *SEM* (**, $p < 0.01$, two-sample unpaired Student's *t*-test). The number of neurons for each condition is indicated in brackets. All neurons were recorded from one animal

3.9 | Serotonin elicits inward currents and action potentials in rat DRG neurons

We used calcium microfluorimetry to identify neurons activated by 5-HT (50 μM), which were further used for patch-clamp experiments in the whole-cell configuration. In some of the experiments, the recorded neuron was also filled with Calcium Green-1 AM from the pipette to simultaneously record electrical activity and changes in $[Ca^{2+}]_i$. All neurons that showed an initial 5-HT-induced transient increase in $[Ca^{2+}]_i$ ($n = 15$) also showed an inward current in voltage clamp mode at a holding potential of −80 mV, accompanied by no discernable fast transient increase in $[Ca^{2+}]_i$, suggesting the important role of voltage-gated channels for the responses recorded using calcium imaging (Figure 9a). In current clamp mode, 5-HT induced depolarizations in the transiently responding neurons ($n = 9$ mean $\Delta V = 13.2 \pm 1.9$ mV) and elicited 1 and respectively 3 action potentials in 2 of these neurons, while $[Ca^{2+}]_i$ displayed a fast increase. In experiments with three successive 5-HT challenges (1–2 min at 2-min interval), the transient inward current elicited by 5-HT was blocked by co- and pre-applying granisetron (10 nM) in both transient ($n = 10$, Figure 9b) and mixed-responding neurons ($n = 3$, not shown). The inhibition was statistically significant against control experiments ($n = 6$), for both the second and third application of 5-HT ($p < 0.01$ and $p < 0.05$, respectively, Student's two sample *t*-test, Figure 9b).

In cells that showed both sustained and transient increases in $[Ca^{2+}]_i$, 5-HT (50 μM) evoked only transient currents at a holding potential of −80 mV and no $[Ca^{2+}]_i$ increase, while in current clamp mode 5-HT evoked an average 30 ± 11 action potentials (n = 10) accompanied by an increase in $[Ca^{2+}]_i$ (Figure 9c). In other experiments, performed in the presence of granisetron (10 nM), 5-HT evoked an average of 13 ± 6 action potentials (n = 6, not shown). All neurons were tested for viability in the current-clamp mode, by applying KCl (50 mM) for 20 s, which evoked robust depolarizations and bursts of action potentials.

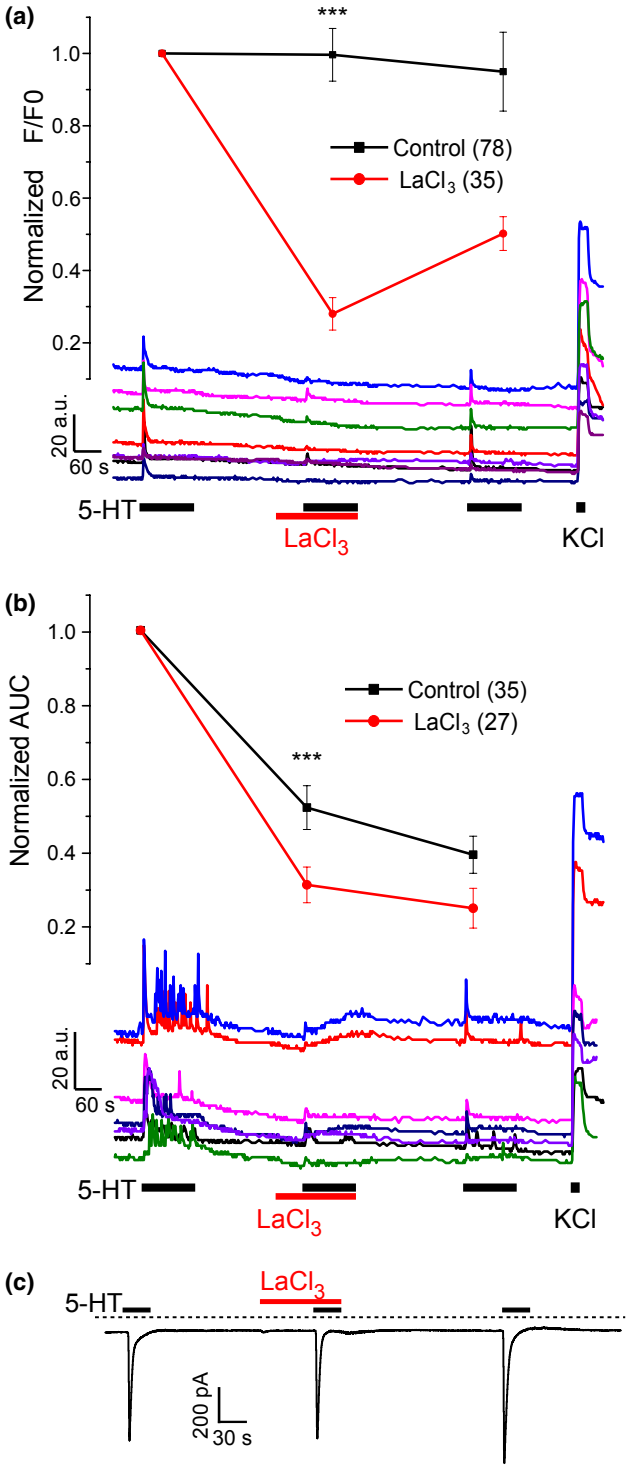

FIGURE 8 Lanthanum inhibited both transient and sustained responses to 5-HT. A. The voltage-gated calcium ion channel blocker, lanthanum ($LaCl_3$ 100 μM) inhibited the transient responses. Statistical analysis for $\Delta F/F_0 \pm SEM$ (***, $p < 0.001$, two-sample Student's t-test). (b) Lanthanum ($LaCl_3$ 100 μM) also inhibited the sustained responses. Statistical analysis for AUC ± *SEM* (***, $p < 0.001$, two-sample Student's t-test). (c) Whole-cell currents recorded at −80 mV in transiently responding neurons were little affected by lanthanum, suggesting that $LaCl_3$ is not acting by inhibiting 5-HT3 but rather by voltage-gated ion channels. Experiments in parts A, B, and C were carried out in neurons from three different animals

3.10 | Serotonin decreases membrane conductance in neurons with sustained responses

In order to assess the mechanism of 5-HT-induced depolarizations independent of 5-HT3, we used a series of 5 s hyperpolarizing voltage steps from −60 to −70 mV and calculated the membrane conductance at each step. Granisetron (10 nM) was applied for 30 s followed by 1 min of 5-HT (50 μM) in the presence of granisetron, in order to exclude the contribution of 5-HT3 receptors (Figure 10a). Serotonin induced a decrease in membrane conductance in all 5-HT-sensitive cells tested, from a mean baseline of 9.3 ± 2.0 nS and 9.0 ± 2.0 nS during granisetron (10 nM) to 8.5 ± 2.0 nS (n = 12) when 5-HT was co-applied with granisetron. When normalized to the baseline conductance, this corresponds to a conductance decrease of approx. 10% induced by 5-HT (from 0.97 ± 0.1 in granisetron to 0.88 ± 0.02 in granisetron + 5-HT; one-way ANOVA with post hoc Tukey's HSD **, $p < 0.01$, Figure 10a,b). This suggests that 5-HT depolarizes these neurons by inhibiting the background potassium leak conductance.

3.11 | The 5-HT1A and 5-HT3 receptors mediate the scratching behavior evoked by 5-HT in Wistar rats

Based on the *in vitro* results, we selected 5-HT1A and 5-HT3 as the most likely receptors involved in the response of sensory neurons to 5-HT. Further, we used a behavioral model to investigate whether these receptors play a role in itch or pain signaling. Intradermal injections (i.d.) of algogens into the cheek induce brief forepaw wipes across the injected cheek thus indicating pain, while pruritogens produce hind limb scratch bouts directed to the injection site (Shimada & LaMotte, 2008). This behavioral model was used to test if 5-HT and selective 5-HT1A and 5-HT3 agonists elicit scratching and/or wiping. In a separate set of experiments, selective antagonists for 5-HT1A and 5-HT3 were injected i.d. in the cheek 15 min prior to 5-HT.

Intradermal injection of 5-HT (1%) into the cheek did not produce a robust wiping behavior, indicating a limited amount of pain evoked by 5-HT. The specific 5-HT1A and 5-HT3 antagonists diminished significantly the behavior indicative of pain in animals of both

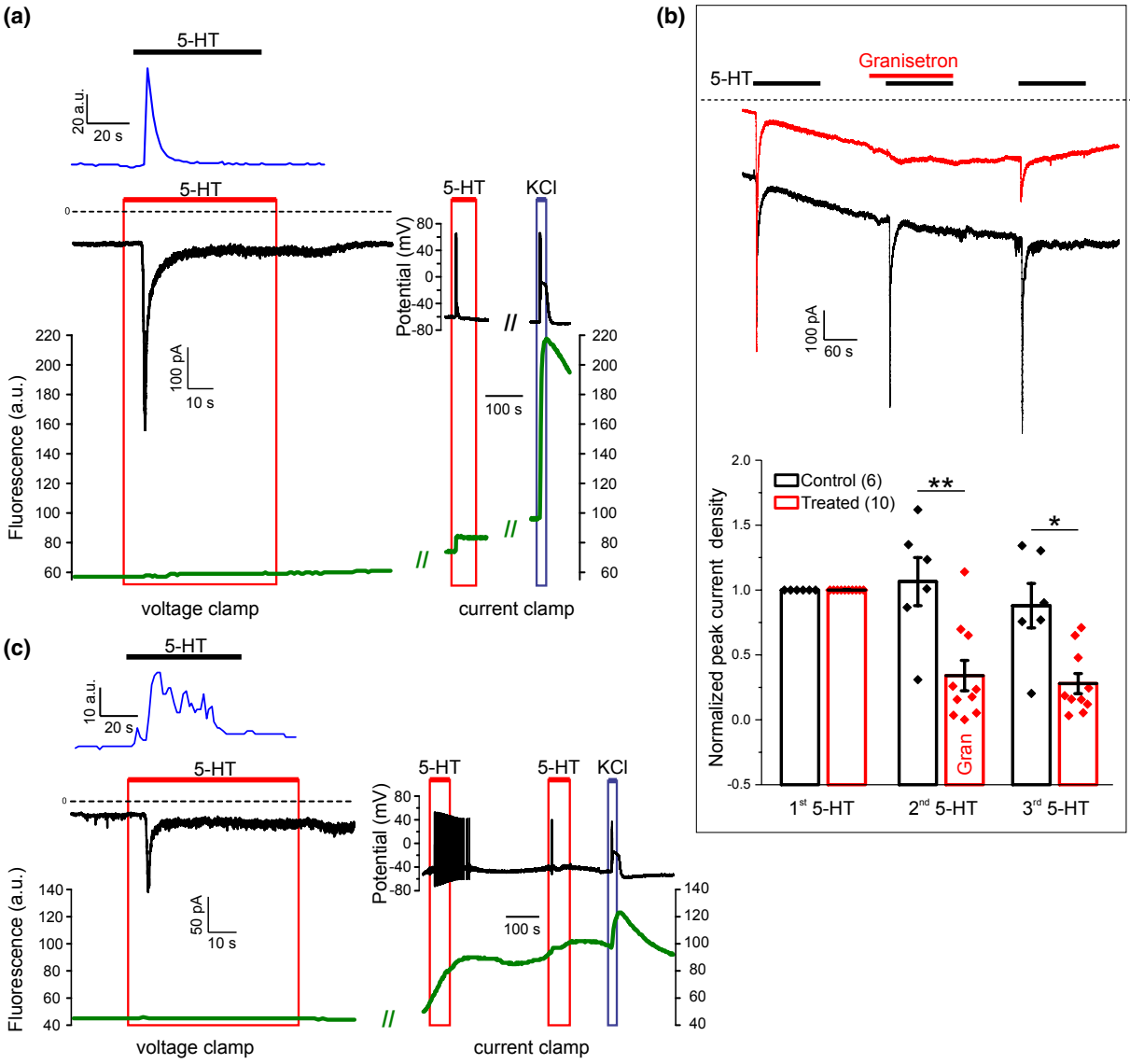

FIGURE 9 Serotonin evokes inward currents and depolarization in both transient and mixed-responding neurons. (a) Example of a transiently responding neuron, selected using calcium imaging (blue trace) and subsequently recorded using whole-cell patch-clamp simultaneous with calcium imaging. In voltage clamp, at a holding potential of −80 mV, 5-HT (50 μM) activated only single transient currents and no calcium increase. In current clamp, a very short burst of action potentials was recorded simultaneously with a small increase in calcium. KCl (50 mM) was applied at the end of the experiment and elicited a burst of action potentials accompanied by $[Ca^{2+}]_i$ increase, confirming neuronal viability. (b) Neurons that showed transient responses in calcium imaging were challenged three times with 5-HT (50 μM), for 1 min at 2-min intervals. Exposure to granisetron (10 nM) before and during the second 5-HT application inhibited the 5-HT-evoked currents recorded at −80 mV. Below: peak current densities normalized to the first response showing that granisetron significantly inhibited the second and third peak currents elicited by 5-HT (*, $p < 0.01$; **, $p < 0.05$, two-sample Student's *t*-test, control $n = 10$, granisetron-treated $n = 6$). Recordings were made on neurons from three different animals. (c) Example of a mixed responding neuron, selected using calcium imaging (blue trace) and then patch-clamped. In voltage clamp (−80 mV), 5-HT evoked only single transient currents and no $[Ca^{2+}]_i$ increase. In current clamp, the application of 5-HT elicited a large number of action potentials accompanied by a substantial $[Ca^{2+}]_i$ increase. KCl (50 mM) applied at the end of the recording confirmed neuronal viability

sexes (Figure 11a,b). To a smaller extent, the wiping behavior was also elicited by 5-CT (50 mM) in males and by 5-CT and SR-57227 in females (Figure 11c,d).

5-HT (1%; 10 μl) evoked robust hind limb scratching of the injected cheek. Pre-treatment with the 5-HT1A antagonist WAY-100635 (WAY; 10 mM) led to a significant reduction of scratching behavior in males (Figure 12b). The co-injection of granisetron (10 μM) and WAY-100635 (10 mM) significantly reduced scratching behavior evoked by 5-HT, in both male and female rats (Figure 12a,b). In males, WAY-100635 alone was sufficient to inhibit the number of scratch bouts to similar levels as the co-injection with granisetron (Figure 12a,b). The less selective 5-HT1A agonist 5-CT

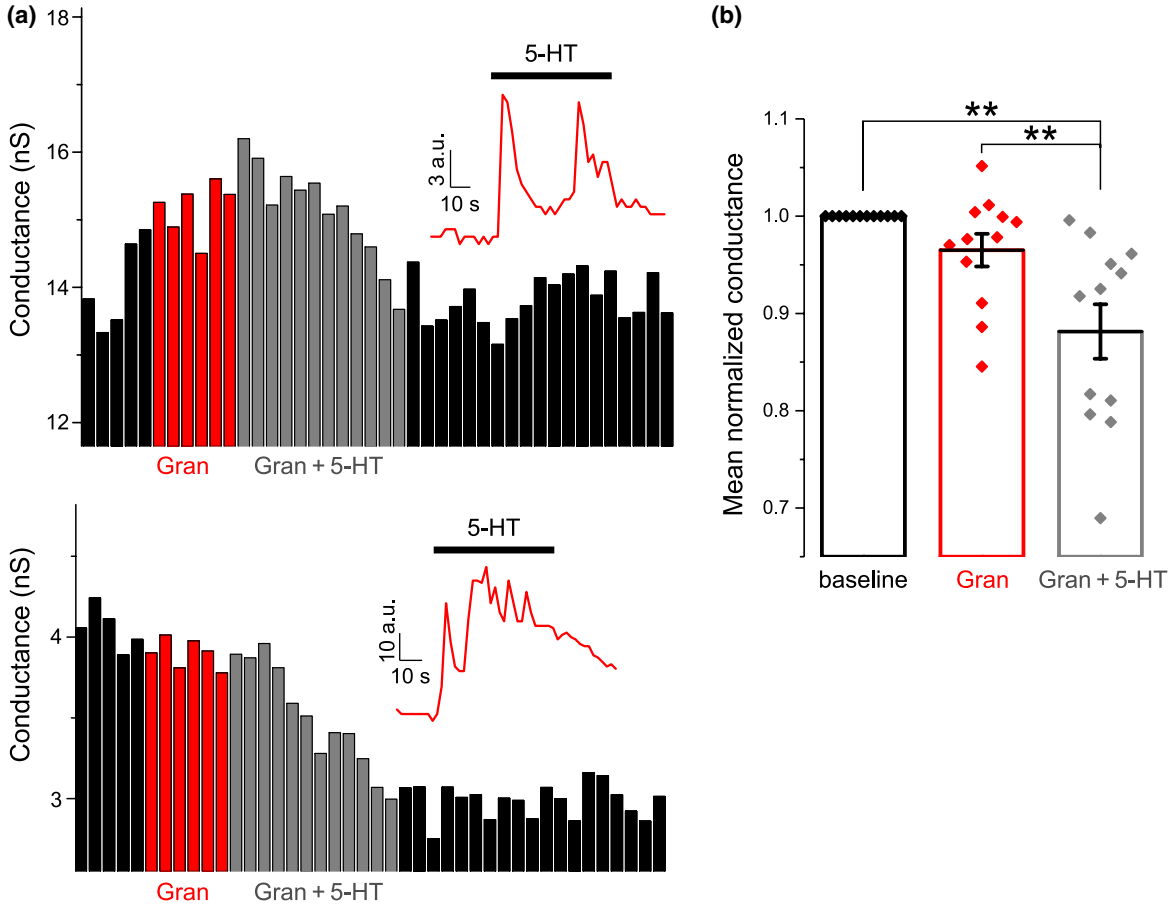

FIGURE 10 5-HT decreases neuronal membrane conductance in voltage clamp recordings. (a) Example of two neurons that showed both the transient and sustained components in calcium imaging and were recorded during repeated voltage steps from −60 to −70 mV (5 s each). (b) Normalized mean of the conductance measured using the above protocol. Note the decrease in conductance induced by 5-HT (50 μM). Data represent mean conductance for each condition normalized to baseline. Statistical significance of the difference in mean conductance between Ringer (control, 4 steps, 20 s), granisetron (Gran, 4 steps, 20 s), and 5-HT + granisetron (last 4 steps, 20 s) was calculated with one-way ANOVA with post hoc Tukey's HSD **, $p < 0.01$ ($n = 12$, from two different animals)

(50 mM) produced significantly more scratching than vehicle in rats of both sexes (Figure 12c,d), while the more selective agonist 8-OH-DPAT (10 mM) and the 5-HT3 agonist, SR-57227 (1 mM) elicited more scratching compared to vehicle only in female rats (Figure 12c).

4 | DISCUSSION

The aim of this work was to investigate the excitatory action of 5-HT on primary afferent neurons in a rat model in order to provide a better understanding of the acute sensory effects of 5-HT, especially in relation to itch. Our *in vitro* calcium imaging and patch-clamp results show that in rat DRG, 5-HT activates two populations of small diameter neurons displaying either purely transient responses, consistent with inward transient currents, or sustained/mixed responses in neurons lacking sustained inward currents. This supports previous findings showing that 5-HT-evoked depolarizations can occur via either an increase or a decrease in membrane conductance and that these mechanisms can coexist in the same neurons (Todorovic & Anderson, 1990a, 1990b). Both of the 5-HT-sensitive neuronal populations had a nociceptor profile, based on their mean diameter and pharmacological characterization. Our *in vitro* results demonstrate a key role of the metabotropic 5-HT1A receptor in mediating the sustained responses to 5-HT, while the ionotropic 5-HT3 receptor appears to be fully responsible for the transient responses elicited by 5-HT. To confirm receptor identity, we have tested a multitude of 5-HT receptor agonists and antagonists. The sustained responses to 5-HT were only inhibited by the selective 5-HT1A antagonist WAY-100635, while antagonists of 5-HT1B, 5-HT1D, 5-HT2A, 5-HT2, 5-HT4, 5-HT5A, 5-HT6, and 5-HT7 had no inhibitory effects. In addition, transient responses induced by 5-HT were completely abolished by the 5-HT3 receptor antagonist granisetron. These findings were in good agreement with the effects of selective 5-HT receptor agonists, as the prolonged excitatory action of 5-HT was mimicked by the selective 5-HT1A receptor agonist 8-OH-DPAT. Similarly, the sustained action of 5-HT was reproduced by 5-CT, a high-affinity agonist of 5-HT1A, 5-HT1B, 5-HT1D, 5-HT5, and 5-HT7, while selective agonists of

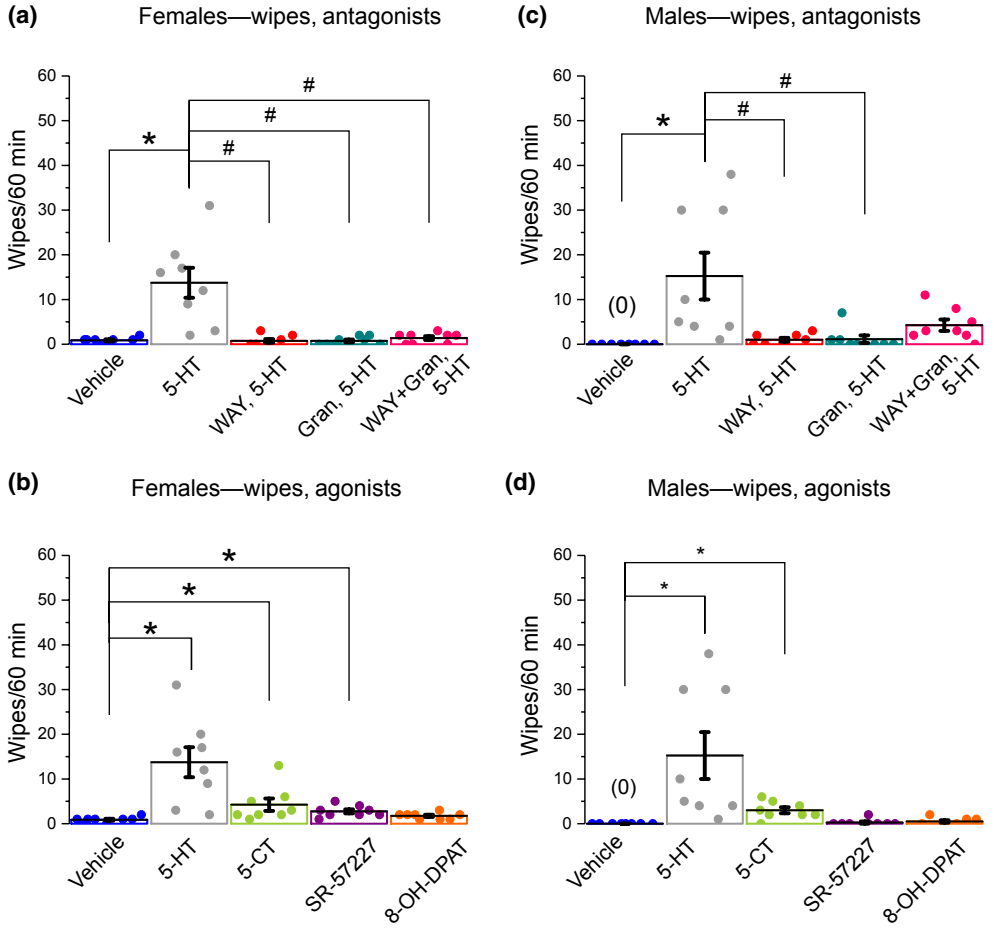

FIGURE 11 Effects of i.d. 5-HT1A and 5-HT3 antagonists and agonists on the wiping behavior in rats of both sexes. (a) The 5-HT1A antagonist WAY-100635 (WAY, 10 mM, 10 µl), the 5-HT3 antagonist granisetron (Gran, 10 µM, 10 µl), and their combination significantly inhibited the number of wipes by front paw (manifestation of pain) evoked in 60 min by 5-HT (1%, 10 µl) in female rats. (b) In male rats, the 5-HT receptor antagonists had similar effects as in female rats, except for a smaller inhibition of wiping behavior by the co-injection of WAY-100635 and granisetron. (c) Wiping behavior significantly different from the effects of vehicle was evoked in female rats by 5-CT (50 mM, 10 µl) and SR-57227. (d) Activating 5-HT3 receptors with SR-57227 did not produce wiping behavior in male rats. Only 5-CT was able to increase this behavior (*, $p < 0.05$ compared to vehicle; #, $p < 0.05$ compared to 5-HT, paired-sample Student's *t*-test, $n = 8$ females, m = 8 males)

5-HT1F, 5-HT2A, 5-HT2C, 5-HT4, and 5-HT7 receptors were unable to specifically activate 5-HT-sensitive neurons. The only exception was the 5-HT6 agonist WAY-181187, which was able to activate 5-HT-sensitive neurons with a sustained response; however, the 5-HT6 antagonist SB-399885 was not able to inhibit the responses to 5-HT. Considering the overall effect of 5-HT receptor agonists and antagonists, 5-HT1A emerges as the only target responsible for the sustained serotonergic activation of primary afferent neurons from Wistar rats. While the functional expression of the ionotropic 5-HT3 receptor in the PNS has been clearly shown, the issue of 5-HT1A expression in primary sensory neurons is a rather controversial one (see Introduction). However, several studies reported the presence of this receptor in the central endings of first-order sensory neurons in the superficial dorsal horn in rat spinal cord (Daval, Vergé, Basbaum, Bourgoin, & Hamon, 1987; Ito et al., 2000; Jeong, Mitchell, & Vaughan, 2012; Laporte, Fattaccini, Lombard, Chauveau, & Hamon, 1995). Although the expression of 5-HT1A receptor in the central presynaptic terminals of primary afferent neurons appears to be an accepted fact, there is limited agreement concerning the effect of receptor activation. While a substantial number of studies provide evidence for an inhibitory role of 5-HT1A in pain signaling, following the release of 5-HT by descending axons originating in the rostroventral medulla (Ito et al., 2000; Jeong et al., 2012; reviewed by Viguier, Michot, Hamon, & Bourgoin, 2013), there are also reports of 5-HT1A-mediated facilitation of pain and itch transmission in the spinal cord (Alhaider, Hamon, & Wilcox, 1993; Ali, Wu, Kozlov, & Barasi, 1994; Zhao et al., 2014; reviewed by Cortes-Altamirano et al., 2018). It is not yet fully understood to what extent this facilitation of nociceptive signaling is mediated pre- or post-synaptically, or even by 5-HT1A receptors on spinal interneurons. Interestingly, in a recent mouse study, the 5-HT1A and 5-HT1F receptors were found highly expressed in a subpopulation of Ret-positive DRG neurons. 5-HT1F, similar to 5-HT1A, would be expected to inhibit neuronal activity via a reduction of intracellular cyclic AMP levels.

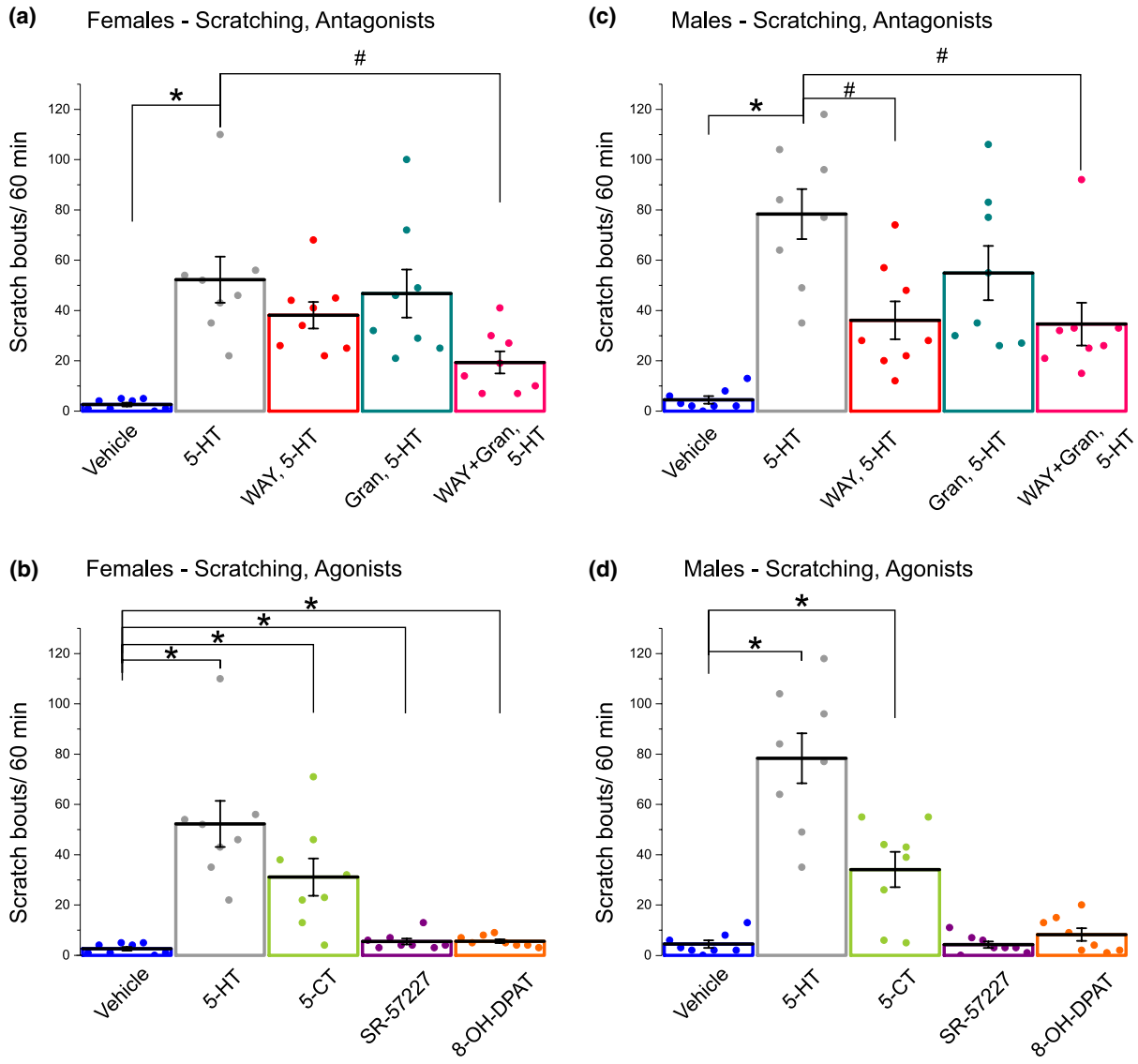

FIGURE 12 Effects of i.d. 5-HT1A and 5-HT3 antagonists and agonists on the scratching behavior in rats of both sexes. (a) The inhibitory effect of WAY-100635 (WAY, 10 mM, 10 µl), granisetron (Gran, 10 µM, 10 µl), and their combination on the scratching behavior evoked in 60 min by 5-HT (1%, 10 µl) in female rats. Only the combination of both antagonists significantly inhibited the scratching behavior. (b) In male rats, WAY-100635 (WAY) inhibited by itself to significant levels the scratching behavior evoked by 5-HT and the addition of granisetron did not inhibit further the number of scratch bouts. (c) The number of scratch bouts elicited by 5-HT (1%, 10 µl), 5-CT (50 mM, 10 µl), SR-57227 (1 mM, 10 µl), and 8-OH-DPAT (10 mM, 10 µl) were all significantly higher than the effects of vehicle in female rats. (d) In male rats, only 5-CT evoked a significantly higher number of scratch bouts compared to vehicle (*, $p < 0.05$ compared to vehicle; #, $p < 0.05$ compared to 5-HT, paired-sample Student's *t*-test, $n = 8$ females, m = 8 males)

Unexpectedly, 5-HT1F activation evoked responses in calcium imaging and scratching behavior following intradermal injections of the agonist (Stantcheva et al., 2016).

We further investigated the signaling pathway activated downstream of 5-HT1A receptors and involved in mediating the sustained 5-HT responses in rat DRG neurons. Heterologously expressed and also neuronal 5-HT1A receptors have been reported to activate $G_{i/o}$, leading to an inhibition of adenylyl cyclase (reviewed by Albert & Vahid-Ansari, 2019). The same signaling pathway seems to be triggered by 5-HT1A receptors in rat DRG neurons, as the 5-HT-induced sustained responses were strongly reduced in both amplitude and frequency following pre-treatment with the $G_{i/o}$ inhibitor PTX (Figure 7).

Our results are not in agreement with those obtained by Ohta et al. (2006), using Fura-2 ratiometric calcium imaging of cultured rat DRG neurons. While in our study approx. 30% of rat DRG neurons responded to 5-HT with a transient, sustained, or mixed response, Ohta et al. (2006) observed that 5-HT elicited transient responses in only 6.5% of DRG cells and did not elicit sustained responses in any cells. The reason for this discrepancy is not clear, but it may reside in the fact that Ohta et al. (2006) used DRG neurons from neonatal Wistar rats, while we used cells from adults.

In addition, the concentration of 5-HT used by Ohta et al. was smaller than that used in our study (10 μM compared to 50 μM). Another group used patch-clamp electrophysiology to record slow and sustained inward currents evoked by the application of 10 μM 5-HT in approx. 50% of rat DRG neurons, but attributed these currents to opening of Ca^{2+}-activated chloride channels following the activation of 5-HT2C receptors (Salzer et al., 2016). These latter investigators did not report the strain, age, or sex of the rats, thus limiting comparisons with the present data. Moreover, in our present calcium imaging experiments the sustained 5-HT-induced responses were not affected by the selective anoctamin-1 antagonist MONNA (1 and 10 μM).

Intradermal cheek injection of 5-HT in Wistar rats produced robust itch-related scratching behavior and only limited pain-related wiping behavior, consistent with previous studies using Sprague-Dawley rats (Klein et al., 2011; Moser & Giesler, 2014b; Spradley, Davoodi, Carstens, & Carstens, 2012). We observed that the 5-HT-evoked scratching and wiping behaviors were attenuated by antagonists of 5-HT1A and 5-HT3 receptors, while the 5-HT1A agonist 5-CT elicited scratching behavior. The behavioral data are consistent with the calcium imaging data showing that sustained and transient 5-HT-evoked responses were reduced by 5-HT1A and 5-HT3 antagonists, respectively, and that agonists of 5-HT1A and 5-HT3 elicited sustained and transient responses, respectively. We speculate that the sustained responses evoked by 5-HT are likely to be more relevant for itch signaling. In a related study, intracellular recording from C-type DRG neurons in Sprague-Dawley rats revealed two types of spiking responses elicited by 5-HT—either transient or prolonged firing. The scratching behavior induced by topical application of 5-HT had a time-course similar to that of the prolonged 5-HT-evoked firing (Hachisuka, Furue, Furue, & Yoshimura, 2010). Both the 5-HT1A agonist 8-OH-DPAT and the 5-HT3 agonist SR-57227 reproduced the effect of 5-HT and increased the scratching behavior in female rats, but not in males. We do not have an explanation for this gender difference. The data suggest, however, that simultaneous activation of both 5-HT3 and 5-HT1A receptors may be required to induce scratching behavior, as indicated by the fact that the combination of 5-HT3 and 5-HT1A antagonists was able to diminish this behavior in both males and females, while the 5-HT1A antagonist alone produced a significant reduction in males only and the 5-HT3 antagonist was ineffective in animals of both sexes.

Our results indicate that serotonergic itch in Wistar rats is triggered via a different signaling pathway compared to the mouse, in which three different mechanisms were reported, one involving the 5-HT7 receptor followed by the activation of TRPA1 (Morita et al., 2015), a second requiring opening of TRPV4 channels downstream of 5-HT2 receptors (Akiyama et al., 2016), and a third involving activation of the 5-HT2B (by α-methyl-5-HT) linked to TRPC4 (Lee et al., 2018). In our experiments with rats, the sustained responses evoked by 5-HT were neither inhibited by the TRPA1 antagonist/A967079, nor by the TRPV4 antagonist HC-067047, which might be explained by a species difference. In conclusion, our pharmacological investigations rule out the involvement of either TRPA1 or TRPV4 in mediating 5-HT-induced sustained responses in rat primary afferent neurons.

Our voltage-sensitive dye and electrophysiological experiments show that application of 5-HT leads to membrane depolarization of rat DRG neurons (Figure 1d), followed by a high-frequency train of action potentials (Figure 9c), associated with a sustained decrease in membrane conductance (Figure 10). Taking together all these results, we propose a signaling pathway in which these sustained responses evoked by 5-HT in rat primary afferent neurons are triggered by the closure of leak potassium channels downstream of the $G_{i/o}$-coupled 5-HT1A receptor activation, followed by depolarization and calcium entry via voltage-gated calcium channels. Our results indicate that the 5-HT1A receptor may be considered a putative target to alleviate serotonergic itch.

DECLARATION OF TRANSPARENCY

The authors, reviewers and editors affirm that in accordance to the policies set by the *Journal of Neuroscience Research*, this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Conceptualization, T.S., E.C., and A.B.; *Methodology*, T.S., L.C.C., M.I.C., E.C., and A.B.; *Formal Analyses*, D.D., T.S., E.C., and A.B.; *Investigation*, D.D., T.S., and M.I.C.; *Resources*, E.C. and A.B.; *Writing – Original Draft*, T.S., E.C., and A.B.; *Visualization*, D.D. and T.S.; *Project Administration*, A.B.; *Funding Acquisition*, E.C. and A.B.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

FIGURE S1 Summary of the experiments investigating a number of 5-HT receptor antagonists and ion channel blockers. (a) The effect of selective 5-HT receptor antagonists tested for their inhibitory effect on the second application of 5-HT (50 μM). The AUC of the three consecutive responses was normalized to the first one. The number of neurons for each condition is provided in brackets. (***, $p < 0.001$, two-sample Student's *t*-test). The experiments were carried out on neurons from eight different animals. (b) Normalized AUC ± *SEM* of the three consecutive applications of 5-HT (50 μM). The second 5-HT application was performed in the presence of either TRPA1 antagonists (A-967079, 5 μM and HC030031, 10 μM), TRPV4 antagonist (HC067047, 500 nM), anoctamin-1 antagonist (MONNA, 1 μM), or the broad spectrum TRP channel inhibitor, Ruthenium Red (RuR, 5 μM). None of the antagonists tested significantly inhibited the sustained responses to 5-HT. The number of neurons for each experimental condition is provided in brackets. The experiments were carried out on neurons from six different animals

TABLE S1 Specific agonists tested and the co-expression of their effect with 5-HT responses. Co-expression coefficient = [No. of neurons activated by both 5-HT and agonist] − [No. of neurons activated by the agonist only]/[Total No. of 5-HT responding neurons]

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