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Metabotropic 5-HT receptor-mediated effects in the human submucous plexus

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Abstract

Background: Serotonin (5-HT) is an important mediator in the gastrointestinal tract, acting on different neuronal 5-HT receptors. The ionotropic 5-HT_3 receptor mediates immediate but transient spike discharge in human enteric neurons. We studied the role of the metabotropic 5-HT_{1P}, 5-HT_4, and 5-HT_7 receptors to activate human submucous neurons.

Methods: Neuroimaging using the voltage sensitive dye Di-8-ANEPPS was performed in submucous plexus preparations from human surgical specimens of the small and large intestine. We synthesized a new, stable 5-HT_{1P} agonist, 5-benzyloxyhydrazonoindalpine (5-BOHIP).

Key Results: 5-HT evoked a fast and late-onset spike discharge in enteric neurons. The fast component was blocked by the 5-HT_3 receptor antagonist cilansetron, while the remaining sustained response was significantly reduced by the 5-HT_{1P} receptor antagonist 5-hydroxytryptophanyl-5-hydroxytryptophan amide (5-HTP-DP). The newly synthesized 5-HT_{1P} agonist 5-BOHIP induced a slowly developing, long-lasting activation of submucous neurons, which was blocked by 5-HTP-DP. We could not demonstrate any 5-HT_7 receptor-induced spike discharge based on the lack of response to 5-carboxamidotryptamine. Similarly, the 5-HT_4 agonists 5-methoxytryptamine and prucalopride evoked no immediate or late-onset spike discharge.

Conclusions & Inferences: Our work demonstrated for the first time the presence of functional 5-HT_{1P} receptors on human submucous neurons. Furthermore, we found no evidence for a role of 5-HT_4 or 5-HT_7 receptors in the postsynaptic activation of human submucous neurons by 5-HT.

KEYWORDS
5-HT_{1p}, 5-HT_4, 5-HT_7, human submucous neurons, metabotropic serotonin receptors

1 | INTRODUCTION

Serotonin (5-HT) is among the most prominent mediators involved in regulation of gut functions.[1] Many of its effects are mediated by 5-HT receptors on neurons of the enteric nervous system (ENS).[2] There are numerous 5-HT receptors expressed on enteric neurons, interstitial cells of Cajal or smooth muscle cells.[3–5] Most of what is known about expression and function of different 5-HT receptors is based on studies in rodent ENS. Knowledge about 5-HT receptors in human intestine is very limited but the available data highlight some important species differences. The best studied target is the ionotropic 5-HT_3 receptor. Activation of 5-HT_3 evokes neuronal spike discharge both in rodent[6] and human[7] enteric neurons. However, the function is vastly different. While activation of 5-HT_3 is relevant for normal peristaltic reflex activity in rodents, 5-HT_3 antagonists have no effect on the excitatory or inhibitory components of the peristaltic reflex in isolated human intestine and in healthy volunteers.[8,9] Moreover, 5-HT_3 activation evokes nerve mediated secretion in rodent[10] but not in human[7] intestine. In both species, the peristaltic reflex activity involves 5-HT_4/5-HT_{1P} pathways.[8] 5-HT_{1P} as well as 5-HT_7 receptor activation causes long-lasting spike discharge in rodent enteric neurons.[11,12] The 5-HT_{1P} receptor is special in that it has only been identified *via* its high 5-HT affinity and particular pharmacology, which is different from all other metabotropic 5-HT receptors.[13] Till today, this receptor has not been cloned but is listed in the 2019 official International Union of Pharmacology (IUPHAR) nomenclature as a potential orphan receptor.[14] 5-HT_{1P} receptors are localized to the peripheral nervous system and were found in enteric neurons of the gut,[13,15] in the skin and in the heart (probably localized to nerve fibers)[15] and in the pancreas.[16] They are part of the 7-transmembrane domain G-protein-coupled receptor family.[17,18] Most information on the intracellular signal transduction mechanism was obtained in myenteric neurons, showing that the coupled G-protein is a G_0 protein,[18] and the signal transduction involves protein kinase C and A as well as adenylate-cyclase.[19,20] Based on a very similar Gαo and PKCα immunostaining, an analogous signaling mechanism is suspected in submucous neurons.[19] In the guinea pig, sensory neurons of the submucous plexus are activated *via* 5-HT_{1P} and are probably cholinergic and contain also calbindin and substance P. [21]

5- and 6-hydroxyindalpine (5- and 6-OHIP) have been identified as agonists and 5-hydroxytryptophanyl-5-hydroxytryptophan amide (5-HTP-DP) is known as a specific antagonist of the 5-HT_{1P} receptor.[13] There are no studies on the functional expression of neuronal 5-HT_4, 5-HT_7, or 5-HT_{1P} receptors in human enteric neurons. We aimed at filling this gap by studying the effect of agonists and antagonists selective for the three 5-HT receptor subtypes. Agonists and antagonists are commercially available for the 5-HT_4 and 5-HT_7 receptors. However, this is not the case for the 5-HT_{1P} receptor. The synthesis of 5- and 6-OHIP is complex and very challenging as the compounds themselves as well as intermediate products are highly unstable, which prevents routine preparation of these agonists. Therefore, we sought to facilitate its synthesis and, during this process, we discovered the new and stable 5-HT_{1P} agonist 5-benzyloxyhydrazonoindalpine (5-BOHIP).

Key Points

- Serotonin (5-HT) acts as a mediator on different neuronal 5-HT receptors in the GI tract.
- The 5-HT3 receptor is ionotropic and evokes an immediate, short term activation of human enteric neurons.
- Our aim was to explore the role of metabotropic 5-HT receptors in the activation of human submucous neurons.
- We used neuroimaging in human submucous plexus preparations of the small and large intestine.
- While the fast component of neuronal activation by 5-HT was mediated by 5-HT3, only 5-HT1P, but not 5-HT4 and 5-HT7, were involved in the remaining sustained response.
- We have synthesized a novel, stable 5-HT1P agonist, 5-benzyloxyhydrazonoindalpine (5-BOHIP).

2 | MATERIALS AND METHODS

2.1 | Human tissue: sampling and tissue preparation

The human intestinal samples were collected from patients who underwent surgery in the Rechts der Isar Hospital of the Technical University of Munich and at the Department of Surgery of the Hospital in Freising. The collection and use of human surgical specimens for neuroimaging experiments was approved by the Ethics Commission of the TUM (1746/07). Tissue samples from 34 patients (age range: 35–88 years, mean age: 65.7 ± 2.3 years, sex: 19 females [55.9%], 14 males [41.2%], 1 not revealed [2.9%]) were used for the study, 31 samples from the large and 3 samples from the small intestine. Patients were diagnosed with the following diseases: carcinoma (21), diverticulitis (6), polyps (2), stenosis (1), and angiodysplasia (1). In 3 cases, the diagnosis was not revealed. After removal, macroscopically normal surgical specimens were immediately placed in a transport solution (oxygenated sterile Krebs solution containing 1% antibiotics/antimycotics [Z-18 from CCPro, Oberdorla, Germany composed of 25 mg L^{-1} Amphotericin B, 20.5 mg L^{-1} Na-desoxycholat, 8000 mg L^{-1} NaCl, 10^7 U L^{-1} Penicillin G and 10 000 mg L^{-1} Streptomycinsulphat], kept at 4°C, pH: 7.4; components: 117 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$ 6 H_2O, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$ 2 H_2O, 11 mM glucose). The tissue samples were constantly kept at 4°C during transport. Immediately after arrival, the tissue specimens were transferred into fresh, carbogen-bubbled (95% O_2, 5% CO_2) Krebs solution and kept further at 4°C. For the preparation, tissues were placed in a Petri

dish covered by a Sylgard layer and fixed with insect pins with their mucosal side up, and were continuously perfused with the carbogen-bubbled ice-cold Krebs solution. Under a stereomicroscope, the layers of the bowel wall were carefully separated with fine preparation instruments to obtain submucous plexus preparations. The preparation was mounted on a silicon ring with insect pins and placed in a recording chamber with an ultra-thin glass bottom on the inverted microscope. During the experiment, the chamber was continuously perfused with 37°C carbogen-bubbled Krebs solution.

2.2 | Optical recording with the multi-site optical recording technique

Multi-Site Optical Recording Technique (MSORT) is a technology developed to detect the activity of enteric neurons with high spatial and temporal resolutions.[22,23] The technique used in our study has been described previously in details, suitable to record action potentials in individual cells in a single ganglion simultaneously.[24] Briefly, the set-up consisted of an IX50 inverted epifluorescence microscope (Olympus, Hamburg, Germany) placed on a vibration-isolation table, equipped with Hoffmann modulation optics. The detection of action potentials was allowed by the voltage sensitive fluorescent dye di-8-ANEPPS (Pyridinium, 4-[2-[6-(dioctylamino)-2-naphthalenyl] ethenyl]-1-(3-sulfopropyl)-, inner salt) as it incorporates into the cell membrane and changes its absorption and emission spectra with the membrane potential. A microejection pipette, loaded with 20 μM di-8-ANEPPS dissolved in DMSO and pluronic F-127 containing Krebs solution, was inserted in the intraganglionic fiber tracts and individual ganglia were stained with a pressure ejection application of a 300–800 ms. After injection into the fiber tract, the dye was allowed to distribute within the ganglion for 10 min. Antioxidants and radical scavengers were not used in the experiments in order to avoid their negative influence on neurons. It has been previously demonstrated that the dye has no influence on the electrophysiological properties of the neurons.[22] The dye was excited by a light emitted by a 150 W xenon arc lamp (Osram, Munich, Germany) or a green LED (PT 39 Green, Luminus Devices Inc., Billerica, USA), modified by a Cy3 fluorescence filter. The duration of the light exposure is critical because of bleaching and phototoxicity. This can be prevented by using only short light exposures, which was limited to 0.6 s for the electrical stimulations and 1.8 s for the pharmacological experiments. The light exposure of the preparation was controlled with a software operated shutter (Uniblitz D122, Vincent Associates, NY, USA) or by switching of the LED. Fluorescent signals obtained with a frequency of 1.6 kHz were processed by an array of 464 photodiodes (RedShirt Imaging, Decatour, GA, USA). The relative changes in fluorescence (ΔF/F) are linearly related to changes in the membrane potential.[22] With the 40x objective of the microscope, the system has a spatial resolution of 280 μm^2 per diode, which allows the identification of individual cells. The images of the individual ganglia with the outlines of the neurons were overlaid with the measured signals, which allowed the analysis of the activity of individual neurons.[7] Computer analysis was performed by the Neuroplex 9.1.0 software, which has been already described in several previous publications.[25]

2.3 | Pharmacology

Agonists were applied with a microinjection glass pipette directly onto the ganglion. Based on previous calculations, the applied substances are diluted to approximately 1:10 until they reach the ganglion.[24] The application was performed *via* pressure ejection pulses with 0.8 to 1.0 bar and 55 ± 27 nl s^{-1} with a custom-made PicoSpritzer. The indicated concentrations correspond to those in the pipette. Serotonin (serotonin creatinine sulfate monohydrate, 5-HT; Sigma, Schnelldorf, Germany) was dissolved in Krebs solution to obtain a stock solution of 10 mM (stored at 4°C for maximally one week). The microinjection pipettes were filled with a 1 mM working solution, which was applied for 400 ms onto the ganglia. Two different 5-HT_4 receptor agonists were used: 5-methoxytryptamine (5-MeOT; Sigma, Schnelldorf, Germany) and prucalopride (provided by GlaxoSmithKline, Harlow, UK). Both stock and working solutions were prepared in Krebs solution. Stock solutions of 100 mM were stored at 4°C for a maximum of two weeks. Working solutions used in the pipettes were 100 μM in case of 5-MeOT and 10 μM in case of prucalopride, duration of application 300–1000 ms. The 5-HT_7 receptor agonist 5-carboxamidotryptamine (5-CT; Tocris, Cologne, Germany) was dissolved in distilled water to obtain a stock solution of 100 mM. Different working concentrations (1 μM, 5 μM, 10 μM, 50 μM, 100 μM, 500 μM, and 1 mM) were all prepared in Krebs solution. We synthesized 5-BOHIP (see Figures S1–S10), which turned out to be a novel 5- HT_{1P} receptor agonist (see Section 3). 5-BOHIP was freshly dissolved in water by adding a droplet of HCl and then diluted in Krebs solution for each experiment to obtain a stock solution of 100 mM. A working solution of 50 μM was tested for neuronal stimulation. The HCl addition did not change the pH of the Krebs solution.

Antagonists were added to perfusing Krebs solution. In the first step, the agonist was applied to the ganglion with a microinjection pipette and the response of the neurons were recorded, then the tissue was perfused with the antagonist and after 20 min, the application of the agonist was repeated. The recovery of the response was also tested by a third application after wash out of the antagonist. From the 5-HT_3 receptor antagonist cilansetron (Solvay, Hannover, Germany), a stock solution of 100 μM was prepared and the aliquots were stored at −20°C. The tissue was perfused for 20 min with 0.1 μM cilansetron dissolved in the perfusing Krebs solution. Standard wash-out time was 40 min, prolonged for up to 2 h in some experiments. The stock solution of the 5-HT_{1P} receptor antagonist n-acetyl-5-hydroxytryptophyl-5-hydroxytryptophanamide (5-HTP-DP, synthesized by the Department of Biological Chemistry of the Technical University of Munich) was dissolved at 10 mM in Krebs solution containing 3–5% DMSO and stored at 4°C for a maximum of one week. The tissue was perfused with a working solution of 10 μM 5-HTP-DP for 20 min, followed by a wash-out of 60 min.

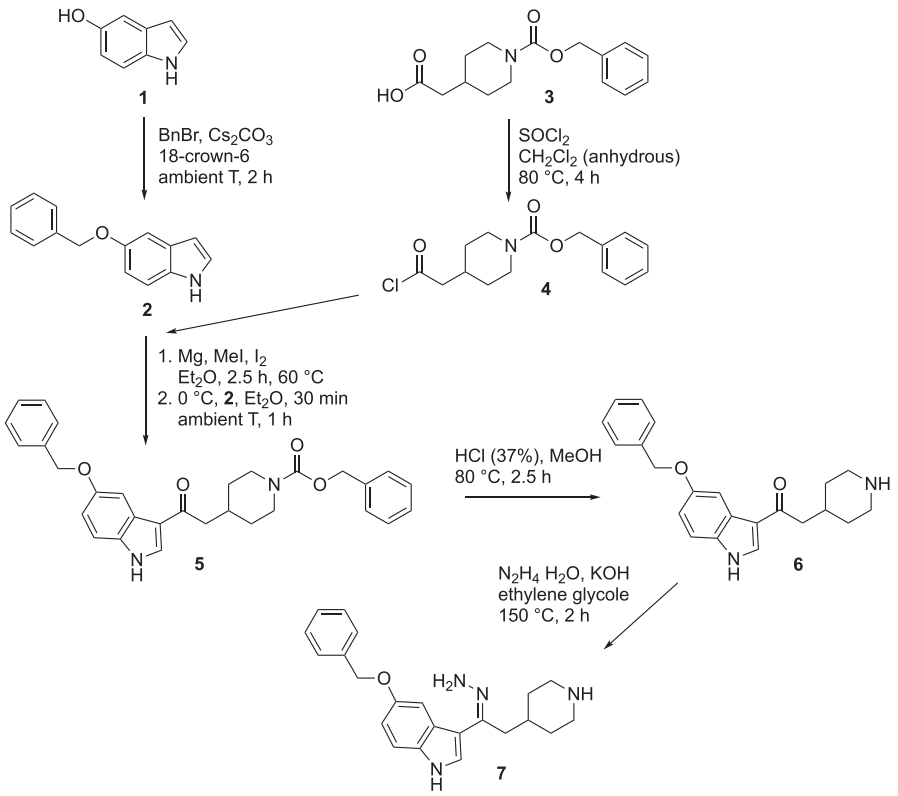

FIGURE 1 Schematic illustration of the synthesis of 5-benzyloxyhydrazonoindalpine (5-BOHIP)

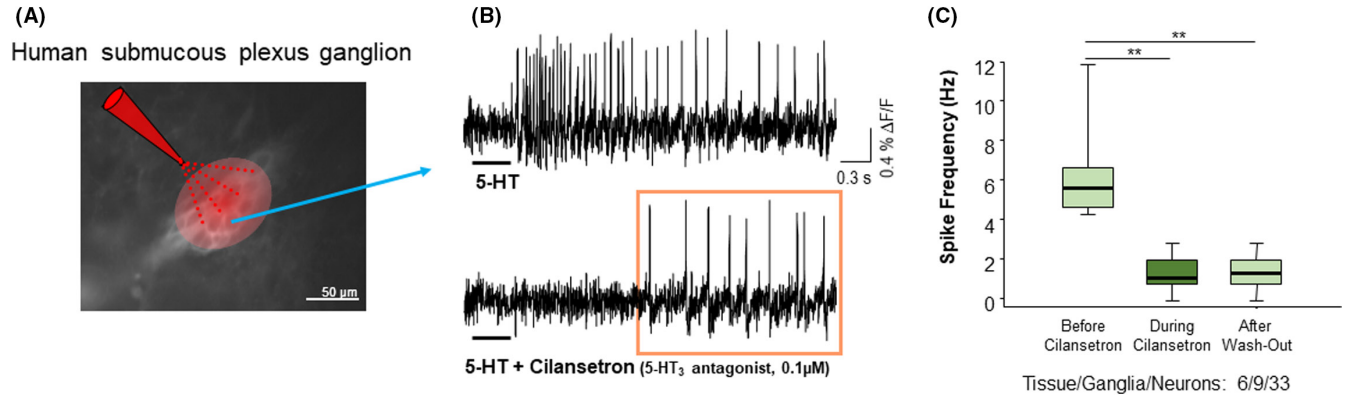

FIGURE 2 Response to micro ejection of 5-HT (1mM) in the human submucous plexus. (A) Spritz application of 5-HT (red) on a human submucous ganglion. (B) Original traces representing the neuronal response. The time and duration of the application (400 ms) are shown by a black line below the trace. The application of 5-HT induced an immediate spike discharge (upper trace). In the presence of the 5-HT_3 antagonist cilansetron (0.1 µM) the fast 5-HT response disappeared, but the late response persisted. (C) Perfusion of the tissue with cilansetron significantly reduce the action potential frequency in neurons activated by 5-HT, but did not completely abolish the response. The response did not recover after wash-out (**$p \leq 0.001$, Kruskal–Wallis One Way ANOVA on Ranks)

2.4 | Synthesis of 5-Benzyloxyhydrazonoindalpine (5-BOHIP)

5-Benzyloxyhydrazonoindalpine (**7**) was synthesized in 5 steps starting from commercially available 5-hydroxy-indole, which was first alkylated with benzylbromide using cesium carbonate and crown ether (Figure 1). The protected indole (**2**) was coupled in a *Grignard* reaction with the acid chloride of commercially available piperidinyl acetic acid (**4**). Removal of the benzyl carbamate protecting group in **5** was performed with conc. HCl to provide **6**. In a last step, the carbonyl group was converted to a hydrazone in an incomplete *Wolff–Kishner* reduction to yield 5-benzyloxyhydrazonoindalpine **7** (For details see Supplementary Information).

2.5 | Data analysis and statistics

The incorporation of the fluorescent dye into the outer membrane revealed the outlines of neurons within one ganglion. Overlay of this image with the neuronal signals allowed analysis of the responses in

individual neurons. The number of neuronal cells within the field of view, the number of responding cells per ganglion and the number and frequency of action potentials were analyzed. Signals were analyzed by Neuroplex 9.1.0 (Redshirt Imaging), Igor Pro 8 (Wavemetrics Inc, Lake Oswego, OR, USA), and Image J 1.43i software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA).[26] Statistical analysis was performed with Sigmaplot 12.5 (Systat Software Inc, Erkrath, Germany). All data are presented as mean ± standard deviation or in case of non-gaussian distribution as median together with 25/75 quartiles. For the comparison between two groups, Student's *t*-test, in case of a paired design, a paired t-test or in case of non-gaussian distribution, a Mann–Whitney rank sum test was performed. For the comparison of more than two groups, one way analysis of variance, or in case of non-normally distributed data, a Kruskal–Wallis analysis of variance on ranks was used. Dunn's method and Tukey test were applied for the post hoc analysis. Difference was considered statistically significant if the *p*-value was ≤0.05.

3 | RESULTS

In the presence of the 5-HT_3 antagonist cilansetron, microejection of 5-HT onto human submucous neurons evoked a late-onset spike discharge (69 neurons in 9 ganglia from 6 samples; Figure 2). In 80.5% of the neurons, the spike discharge was blocked by cilansetron, while the remaining neurons still fired action potentials. The inhibitory response of cilansetron did not fully recover after wash-out, even in case of extended wash-out periods. To further investigate this late response to 5-HT, we used multi-recordings to analyze the spiking after a 400 ms spritz application for up to 13.8 s (Figure 3). The multi-recording protocol consisted of five acquisitions, each lasting 1.8 s, with non-recording intervals of 1.2 s in between. A continuous recording was not feasible, as this would have caused phototoxicity. Almost 43% of the 77 neurons studied (8 ganglia from 5 samples) responded during the first 1.8 s recording period with a spike frequency of 4.6 ± 2.1 Hz. During the second recording period, which was 3–4.8 s after the 5-HT application, still 21.5% of the neurons responded with an average spike frequency of 1.4 ± 0.4 Hz. During the later recording periods, the proportion of responding neurons gradually decreased to 4.2, 4.6, and 0.8%, respectively. In these later periods, the spiking frequency was only between 0.1 and 0.4 Hz. This set of experiments suggested that the peak of the non-5-HT_3-mediated spike discharge occurred 2–5 s after the 5-HT application.

To study the pharmacology of the late response, we, therefore, used a single recording period of 3.1 s starting 2 s after the 5-HT application. This effectively represented an extended period of the second time period of the multi-recording sessions during which the late response was most prominent. Analysis revealed that about 44% of 76 neurons (3 patients, 6 ganglia) showed a late response with a spike frequency of 4.0 Hz. The 5-HT_{1P} antagonist 5-HTP-DP (10 μM) decreased the proportion of late-spiking neurons to 31.5% and those showed a significantly decreased discharge frequency of 1.1 Hz (Figure 4). After 1 h wash out, the response started to resume.

Microejection of the newly synthesized agonist 5-BOHIP evoked no immediate but a late response in about 31% of 61 neurons (8 ganglia from 5 patients; Figure 5). They fired with an average frequency of 1.6 Hz. 5-HTP-DP significantly reduced the proportion of responding neurons to 9.4%, which now fired at a maximal frequency of 0.5 Hz. After wash out, the response to 5-BOHIP recovered.

The 5-HT_7 receptor is involved in the late response to 5-HT in guinea pig myenteric neurons.[12] However, in the human submucous plexus, the 5-HT_7 agonist 5-CT showed no response (71 neurons out of 9 ganglia from 6 patients; Figure 6).

In the human submucous plexus, spritz application of the 5-HT_4 agonist 5-MeOT evoked neither an immediate nor a late-onset spike discharge (tested in 15 neurons from 5 ganglia and 4 samples, Figure 6). Likewise, the selective 5-HT_4 agonist prucalopride evoked no spike discharge (tested in 9 neurons from 2 ganglia and 2 samples).

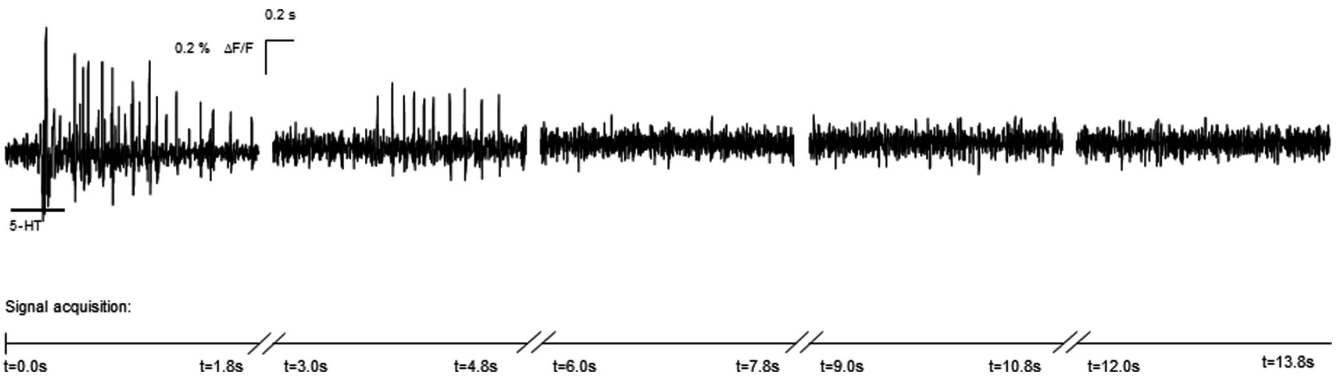

FIGURE 3 Multi-Captioning after application of 5-HT. Original traces of five consecutive acquisitions recorded from one submucous neuron. The first acquisition started at t = 0 s, followed by application of 5-HT (1 mM for 400 ms, illustrated by a black line below the first trace). Each acquisition lasted 1.8 s with 1.2 s pause in between as indicated by the timeline at the bottom. The application of 5-HT evoked action potential discharge starting in the first (t = 0 s–1.8 s) time frame, referred to as immediate response, and from the second time frame on (t = 3.0 s–4.8 s) as late-onset response

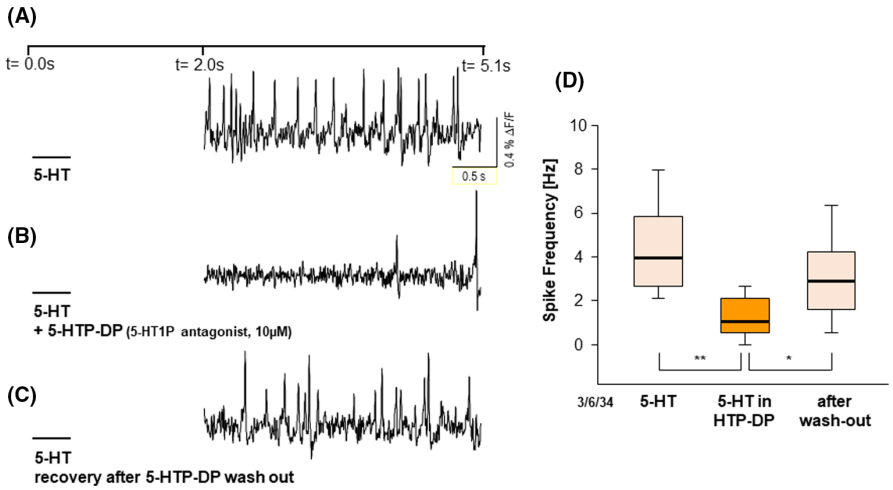

FIGURE 4 Influence of 5-HTP-DP on the late-onset 5-HT response. (A) The spritz application of 5-HT (black line before the trace, followed by 2 s without recording) evoked a late-onset response in the depicted neuron. (B) Perfusion of the 5-HT_{1P} receptor antagonist 5-HTP-DP (20 min, 10 μM) dramatically reduced the frequency of action potentials. (C) After 60 min wash-out the late response to 5-HT recovered. (D) Statistical analysis of the 5-HTP-DP effects. 5-HTP-DP significantly reduced the action potential frequency evoked by 5-HT (**$p < 0.01$, *$p < 0.05$, Kruskal–Wallis One Way ANOVA on Ranks). The effect of 5-HT recovered after wash out

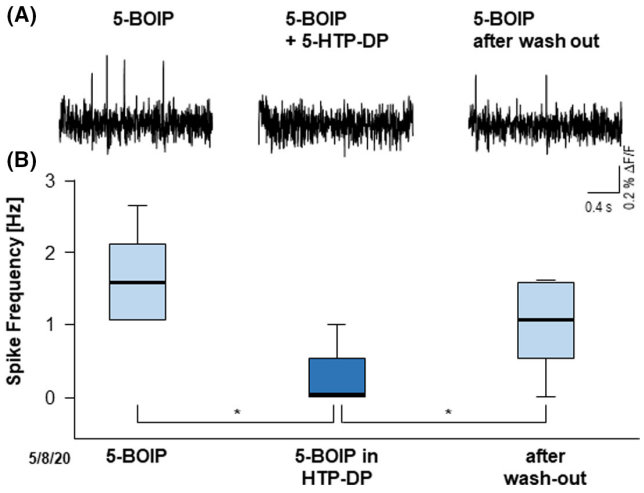

FIGURE 5 Influence of 5-HTP-DP on the neuronal response evoked by 5-BOHIP. (A) Original traces showing late-onset response after spritz application of 5-BOHIP (50 μM, 400 ms) before and after perfusion of the 5-HT_{1P} antagonist 5-HTP-DP (10 μM, 20 min) and after wash out. 5-BOHIP was administered 2 s before starting the recording. (B) The application of 5-BOHIP induced a late-onset spike discharge. 5-HTP-DP significantly blocked the effect of 5-BOHIP, while the spike frequency is restored after wash out (*$p < 0.05$, Kruskal–Wallis One Way ANOVA on Ranks)

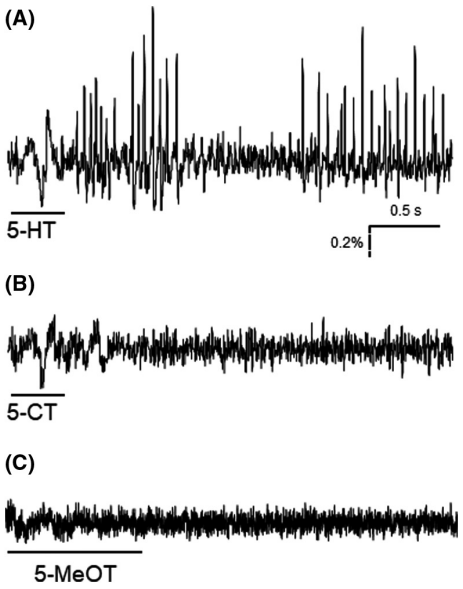

FIGURE 6 Effect of 5-CT and 5-MeOT on human submucous neurons. (A and B) Original traces showing the response of the same neuron to 5-HT (A, 1 mM, 400 ms) and 5-CT (B, 50 μM, 400 ms). While 5-HT initiates action potential firing, 5-CT has no effect on the neuron. The duration of the application is indicated by the black bars below the traces. (C) Original trace demonstrating the lack of effect after 5-MeOT application (1000 ms) on a different neuron. The duration of the application is indicated by the black bars below the trace

4 | DISCUSSION

The present work has explored for the first time the functional expression of metabotropic 5-HT receptors in human enteric neurons and their role in the action potential discharge evoked by 5-HT. In accordance with findings in rodents, the activation of 5-HT_{1P} receptors evoked a late-onset response which consisted of a long-lasting spike discharge in neurons of the human submucous plexus. Similar to data obtained in rodents, 5-HT_4 receptors did not evoke a postsynaptically mediated spike discharge. Contrary to animal studies, the activation of 5-HT_7 did not cause any response in human submucous neurons.

It has been previously shown that 5-HT evoked action potential discharge in human submucous neurons.[7] The immediate effect

was blocked by the HT_3 receptor antagonist cilansetron. Already in this study, we observed late-onset spike discharge which remained in the presence of cilansetron and was, therefore, most likely mediated by metabotropic 5-HT receptors. The present study revealed that nearly 20% of neurons in the human submucous plexus responded to 5-HT with a late action potential discharge in the presence of cilansetron. The specific antagonist of the $5\text{-}HT_{1P}$ receptor, 5-HTP-DP, significantly decreased this late response, demonstrating the involvement of this receptor. This is in accordance with animal studies showing that 5-HT induced a slowly developing, long-lasting activation of enteric neurons, which was mediated by the $5\text{-}HT_{1P}$ receptor.[13,15] The receptor has been suggested to play a role in the initiation of peristalsis and the sensitization of the peristaltic reflex in rodents.[16,27] Neuronal $5\text{-}HT_{1P}$ receptors are activated by 5-HT release from nerves and endocrine cells.[28,29] The finding that synaptically evoked slow EPSPs were blocked by 5-HTP-DP further suggested that neuronally released 5-HT also activated neuronal $5\text{-}HT_{1P}$ receptors.[30]

Our present study has demonstrated the presence of functional $5\text{-}HT_{1P}$ receptors on human enteric neurons and their role in late action potential discharge similar to animal experiments. However, we cannot conclude on their role in peristaltic reflex activity as we recorded from the inner submucous plexus. 5-HTP-DP substantially decreased but did not block the late-onset spike discharge evoked by 5-HT. Moreover, 5-HTP-DP dramatically reduced the spike discharge induced by 5-BOHIP. The affinity of 5-HTP-DP to human $5\text{-}HT_{1P}$ receptors may not be sufficient to block the 5-HT response or other metabotropic receptors may be involved in the late response; our results would rule out $5\text{-}HT_4$ and $5\text{-}HT_7$ receptors. Although we cannot exclude involvement of other metabotropic 5-HT receptors, their role must be rather small based on the finding that 5-HTP-DP decreased 5-HT-induced spike frequency by ~80%. This is a clear species difference as $5\text{-}HT_7$ receptor activation causes the late spike discharge in rodent enteric neurons.[12] Low affinity of 5-CT to the human $5\text{-}HT_7$ receptor may be ruled out as the pK_i value of 9.91–9.94 was even higher than the one of serotonin itself (8.78–8.93).[31] Receptor internalization during tissue handling, although possible, was unlikely as other 5-HT receptors remained functional. So far, mRNA expression of the $5\text{-}HT_7$ receptor has been limited to the circular muscle of the human colon.[32] In colonic biopsies from Crohn's disease patients a significantly increased immunofluorescent staining for $5\text{-}HT_7$ could be detected in the inflamed compared with the non-inflamed area, and most of this staining occurred in intestinal CD11c/CD86 double-positive cells, possibly mature dendritic cells.[33] There is no data so far demonstrating the expression of $5\text{-}HT_7$ receptors in the human ENS. In accordance with our data, the receptor has been shown to mediate relaxation of human colonic circular muscle by a myogenic rather than a neurogenic action.[34]

We discovered 5-BOHIP as a new $5\text{-}HT_{1P}$ receptor agonist. This substance is closely related to the established $5\text{-}HT_{1P}$ receptor agonist 5-OHIP, but it is more stable as it lacks an acidic proton at position 5 and, therefore, the formation of oxidized quinine imine species is less likely (for stability tests see Supplementary Information). 5-OHIP is quite susceptible to oxidation, which makes it difficult to work with during experiments in live tissue which require constant oxygen supply. Due to its higher stability, the usage of 5-BOHIP is better suitable in such experimental settings. Notably, 5-BOHIP caused a late-onset postsynaptic activation of human submucous neurons, and its effect was significantly reduced by addition of the $5\text{-}HT_{1P}$ receptor antagonist 5-HTP-DP.

In humans, the presence of $5\text{-}HT_4$ receptors was shown in the gut wall by *in vitro* receptor autoradiography, but this technique did not allow precise localization of the receptor.[35] A more recent study in human colon has shown high mRNA expression levels of $5\text{-}HT_4$ receptors with qPCR, most prominent in the circular muscle, less in the longitudinal muscle and even less in the myenteric ganglia.[36] In the same study, $5\text{-}HT_4$ receptors were shown by immunohistochemistry in the circular and longitudinal muscle layer, on smooth muscle cells of the muscularis mucosae and the lamina propria. Furthermore, $5\text{-}HT_4$ immunoreactivity was present in the submucous and myenteric plexus neurons both on neuronal somata and neuronal processes, but the receptor must have roles others than postsynaptic activation of submucous neurons.

In conclusion, our study demonstrates the functionality of $5\text{-}HT_{1P}$ receptors on human enteric neurons and describes a new, more stable $5\text{-}HT_{1P}$ receptor agonist, 5-BOHIP, which provides an important addition to species-related 5-HT functions and may contribute to the development of new drugs acting on serotoninergic receptors in the human GI tract.

DISCLOSURE

No conflict of interest exists.

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AUTHOR CONTRIBUTIONS

Anita Annaházi, analyzed and interpreted the data and drafted the manuscript; Thomas Erwin Berger, performed the acquisition and analysis of the data and critically revised the manuscript; Ihsan Ekin Demir and Florian Zeller, contributed to the conception of the work, provided human surgical samples and critically revised the manuscript; Michael Müller, contributed to the conception of the data, synthetized the chemical compounds and critically revised the manuscript; Markus Anneser, contributed to the conception of the data, performed the supplementary experiments and drafted the manuscript; Arne Skerra, contributed to the design of the work, interpreted the data and drafted the manuscript; Klaus Michel, contributed to the design of the work, analyzed and interpreted the data and drafted the manuscript; Michael Schemann, designed the work, analyzed and interpreted the data and drafted the manuscript; All authors read and acknowledged the final manuscript.

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SUPPORTING INFORMATION

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