

Fast synaptic excitatory neurotransmission in the human submucosal plexus

Klaus Michel¹  | Dagmar Krüger¹ | Stefanie Schäuuffele¹ | Florian Zeller² | Ihsan Ekin Demir³  | Jörg Theisen⁴ | Michael Schemann¹ 

¹Department of Human Biology, TU München, Freising, Germany

²Department of Surgery, Clinical Center Freising, Freising, Germany

³Department of Surgery, TU München, München, Germany

⁴Department of Surgery, Klinikum Landkreis Erding, Erding, Germany

Correspondence

Michael Schemann, Human Biology, TU Munich, Liesl-Beckmann-Strasse 4, 85354 Freising, Bayern, Germany.
Email: schemann@wzw.tum.de

Funding information

This study was partly funded by NIH/SPARC (1OT2OD0248999).

Abstract

Background: Acetylcholine is the main excitatory neurotransmitter in the enteric nervous system (ENS) in all animal models examined so far. However, data for the human ENS is scarce.

Methods: We used neuroimaging using voltage and calcium dyes, Ussing chamber, and immunohistochemistry to study fast synaptic neurotransmission in submucosal plexus neurons of the human gut.

Key Results: Electrical stimulation of intraganglionic fiber tracts led to fast excitatory postsynaptic potentials (fEPSPs) in 29 submucosal neurons which were all blocked by the nicotinic antagonist hexamethonium. The nicotinic agonist DMPP mimicked the effects of electrical stimulation and had excitatory effects on 56 of 73 neurons. The unselective NMDA antagonist MK-801 blocked fEPSPs in 14 out of 22 neurons as well as nicotine evoked spike discharge. In contrast, the application of NMDA showed only weak effects on excitability or calcium transients. This agreed with the finding that the specific NMDA antagonist D-APV reduced fEPSPs in only 1 out of 40 neurons. Application of AMPA or kainite had no effect in 41 neurons or evoked spike discharge in only one out of 41 neurons, respectively. Immunohistochemistry showed that $98.7 \pm 2.4\%$ of all submucosal neurons ($n = 6$ preparations, 1003 neurons) stained positive for the nicotinic receptor (α_1 , α_2 or α_3 -subunit). Hexamethonium (200 μ M) reduced nerve-evoked chloride secretion by $34.3 \pm 18.6\%$ ($n = 14$ patients), whereas D-APV had no effect.

Conclusion & Inference: Acetylcholine is the most important mediator of fast excitatory postsynaptic transmission in human submucous plexus neurons whereas glutamatergic fEPSPs were rarely encountered.

KEYWORDS

cholinergic nicotinic receptors, fast excitatory postsynaptic potentials, glutamatergic NMDA/AMPA/Kainate receptors, human enteric nervous system

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

2021 The Authors. *Neurogastroenterology & Motility* published by John Wiley & Sons Ltd.

Key Points

- We studied fast excitatory transmission in human enteric neurons.
- All fast excitatory postsynaptic potentials (fEPSPs) in the human submucous plexus was mediated by cholinergic nicotinic mechanisms with a very small contribution of glutamatergic transmission.
- This agreed with the finding that nerve evokes epithelial secretion was inhibited by nicotinic receptor blockers but not by blockers of ionotropic glutamate receptors.
- Acetylcholine is the most important mediator of fEPSPs in the human submucous plexus.

1 | INTRODUCTION

The transmission of information in the enteric nervous system (ENS) is mediated by fast and slow synaptic signaling. Slow excitatory or inhibitory postsynaptic potentials depend on G-protein coupled receptors and have time courses that can last several minutes. They involve the activation of intracellular second messengers and are able to alter the functional state of neurons. Fast synaptic events on the other hand are mediated through ligand-gated ion channels. As no second messengers are involved in this process the onset and duration of fast excitatory synaptic potentials (fEPSPs) are short (~1 and 50 ms, respectively).¹ So far, the vast majority of studies concerning the electrophysiological properties of enteric neurons have been done in the guinea pig. Among the neurotransmitters which mediated fast excitatory synaptic transmission in guinea-pig myenteric and submucous plexus are acetylcholine, serotonin (5-HT), ATP, Glutamate, γ -aminobutyric acid and glycine.¹⁻⁵ The translational relevance of these findings is unclear, which is supported by studies that demonstrate species-dependent neuroanatomical, electrophysiological, and neuropharmacological differences.⁶⁻¹⁵ In particular, there are very few studies on electrophysiological properties of human enteric neurons.^{14,16,17} It is therefore important to investigate the electrophysiological properties of neurons of the human ENS and thus gain insights into its neurophysiology. This knowledge is instrumental to understand the role of the ENS in the physiology and pathophysiology of the human gut. During the last 25 years, we recorded from several thousand neurons in which we evoked thousands of fEPSPs and fEPSP driven action potentials. In the present study, we focused on the pharmacology of fEPSPs in the human submucous plexus. We used a combination of imaging techniques (ultrafast neuroimaging of membrane potential and calcium imaging) and functional experiments (Ussing chamber) to investigate fast synaptic neurotransmission in the human submucosal plexus.

2 | MATERIALS AND METHODS

2.1 | Tissue samples

All experiments were performed with surgical specimens from patients undergoing abdominal surgery in the Departments of Surgery of the Technische Universität München. Samples were taken from macroscopically normal, unaffected areas as determined by visual

inspection of the pathologist. All procedures and the use of the resections were approved by the ethics committee of the Klinikum Rechts der Isar (744/02, 1748/07, and 2595/09) and with the informed patient's consent. Experiments were performed in accordance with the Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects).

2.2 | Tissue preparation

After removal from the patient, the tissue was placed in cold oxygenated sterile Krebs solution containing (in mM): 117 NaCl, 4.7 KCl, 1.2 MgCl₂ 6H₂O, 1.2 NaH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂ 2H₂O, and 11 glucose (all chemicals from Sigma-Aldrich). It was then rapidly transported to the laboratory for experiments. The tissue was cut along the mesenteric border and pinned flat, mucosa down, in a dissection dish containing ice-cold sterile oxygenated Krebs solution that was exchanged every 10 min. The tissue was dissected by carefully removing the mucosa and the muscle layers under a dissection microscope to obtain a preparation of the inner submucosal plexus (next to the mucosa). The final size of the tissue was approximately 5 × 10 mm² and was pinned onto a Sylgard ring (Dow Corning, Midland, USA) that was placed in a recording chamber with a 42 mm diameter glass bottom (130–170 μ m thickness; Sauer, Reutlingen, Germany) and continuously perfused with 37°C Krebs solution gassed with Carbogen (5% CO₂, 95% O₂) equilibrated at pH 7.4. Some preparations were kept overnight in organ culture. For this purpose, the preparations were pinned onto the Sylgard rings, transferred to a sterile Petri dish, and then washed in sterile Krebs solution (3 × 10 min) under a laminar flow workbench. After this, the preparation was covered with culture medium (Dulbecco's Modified Eagle's Medium/F12, supplemented with 10% heat-inactivated fetal calf serum, 100 IU ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 2.75 μ g ml⁻¹ amphotericin B, 20 μ g ml⁻¹ gentamicin, 2.1 mg ml⁻¹ NaHCO₃, all chemicals from Sigma-Aldrich) and placed on a rocking tray in an atmosphere of 95% O₂ and 5% CO₂ in a humidified incubator. Alternatively, preparations were stored overnight in fresh Krebs solution at 4°C.

The tissue chamber was mounted in a self-made recording chamber and placed onto an inverted epifluorescence Olympus IX 50 microscope (Olympus). Fluorescence illumination was achieved with an illumination system from Cairn Research (Optosource 75) with

a 75 W xenon arc lamp (Ushio UXL 75 XE, msscientific). Controlled illumination of the preparation was achieved by a software-operated shutter (Uniblitz D122, Vincent Associates).

2.3 | Voltage-sensitive dye imaging

The multisite optical recording technique (MSORT) was previously described in detail.^{14,18} In the majority of experiments, illumination periods of 1.3–4 s were used. Individual ganglia were stained with the fluorescent voltage-sensitive dye Di-8-ANEPPS (1-(3-sulfonatopropyl)-4-[beta[2-(di-n-octylamino)-6-naphthyl]vinyl]pyridinium betaine, Molecular Probes Mobitec, Göttingen, Germany) by local pressure application through a microejection pipette loaded with 20 μM Di-8-ANEPPS. Di-8-ANEPPS was solubilized in Krebs solution containing pluronic F-127 (final concentration in the application pipette 0.014%; Molecular Probes) dissolved in DMSO (final concentration 0.125% in the application pipette, Sigma-Aldrich). With the aid of a micromanipulator, the glass pipette was brought in close contact with the ganglia and pressure ejection (2 psi) pulses of 400 ms–1.2 s were used to apply the dye. During the staining period, it was possible to follow and to assess the progress of the labeling by briefly illuminating the ganglion. In a previous study combining intracellular and optical recordings, we found that dye staining the nerve cells did not change their electrical or synaptic properties.¹⁸ Di-8-ANEPPS stained neurons were visualized with $\times 40$ or $\times 100$ oil immersion objectives ($\times 40$: UAPO/340, $\times 100$: SPlanApo100, both from Olympus, Hamburg, Germany) by using a filter cube equipped with a 545 ± 15 nm excitation interference filter, a 565 nm dichroic mirror and a 580 nm barrier filter (Olympus).

The fluorescent images of the ganglia were acquired and processed by a Neuro-CCD system or a Neuro-PDA System (both from RedShirtImaging, Fairfield, CT, USA). The Neuro-CCD system consists of a CCD-camera with a resolution of 70×70 pixels and the software for acquisition, processing, and storage of the data. Frames were acquired at frequencies of 1 or 1.6 kHz. To reduce noise and minimize the effects of bleaching the data of individual pixels were usually filtered offline with a bandpass 3–326 Hz. With the $\times 40$ objective, a spatial resolution of $22 \mu\text{m}^2$ was achieved. The Neuro-PDA system uses the same software and is based on an array consisting of 464 photodiodes instead of a CCD camera. Although the spatial resolution is lower than with the Neuro-CCD system ($283 \mu\text{m}^2$ with $\times 40$ objective), we usually achieved single-cell resolution. Acquisition parameters were the same as with the Neuro-CCD system. This setup was additionally equipped with a conventional video camera (Mod. 4910, CoHU Inc., SL Microtest) to obtain high-resolution fluorescence images of the stained ganglia (Scion Image, Scion Corp.). Both setups measured the fluorescence intensity in arbitrary units and calculated the relative change in fluorescence ($\Delta F/F$) which is linearly related to changes in the membrane potential V_m (Neunlist et al., 1999; Kao et al., 2001). Interganglionic fiber tracts were stimulated

electrically with a Teflon coated platinum electrode (25 μm diameter, Medwire 10Ir1T, Science Products) connected to a stimulator (S88, Grass-Telefactor) with a constant voltage isolation unit (Grass SIU-5). Electrical pulses had durations of 300 μs and amplitudes varying between 1 and 6 V. Drugs could be applied to single ganglia by pressure ejection (2 psi) from microejection pipettes which were placed close to the ganglion (approximately 200 μm) with the help of a micromanipulator (ejection speed: $55 \pm 27 \text{ nl s}^{-1}$ (mean \pm standard deviation, $n = 5$)). With these parameters, the drug was diluted by a factor of approximately 10 before it reached the ganglion.⁶ The following drugs were used for pressure application: Dimethylphenylpiperazinium (DMPP, Sigma-Aldrich), (-)-Nicotine Hydrogen Tartrate (Sigma-Aldrich), N-Methyl-D-aspartic acid (NMDA, Sigma-Aldrich), (R,S)-AMPA (AMPA) and Kainic acid (RBI). DMPP was used at a concentration of 10 or 100 μM (in Krebs solution) in the ejection pipette. (-)-Nicotine Hydrogen Tartrate (Sigma-Aldrich) was prepared at a final concentration of 100 μM in Krebs solution. With this concentration, the pH of the Krebs solution remained stable at 7.4. The NMDA solution was used at a concentration of 10 mM in a Krebs solution that contained 5 μM glycine and no magnesium that was also used for the experiments. AMPA was dissolved at a concentration of 1 mM also in the Krebs solution that was also used for the experiments and contained 25 μM cyclothiazide (Biomol) to prevent receptor desensitization.^{19,20} Kainic acid was used at a concentration of 1 mM. All solutions were prepared daily fresh from a stock solution (nicotine) or directly from the substance. For the solutions of nicotine, AMPA, and kainic acid we tested routinely the pH of the final solution and always found it close to 7.4. The following antagonists were applied to the preparation with the superfusing Krebs solution in the final concentrations indicated: Hexamethonium chloride (Sigma-Aldrich), 200 μM , (+)-MK-801 hydrogen maleate (Dizocilpine, (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate, Sigma-RBI, Germany), 20 μM , D-APV (D-(-)-2-Amino-5-phosphonopentanoic acid, Sigma-RBI), 20–40 μM , glycine (Sigma-Aldrich), 5–10 μM , DNQX disodium salt (6,7-Dinitroquinoxaline-2,3-dione disodium salt, Tocris, Germany), 40 μM .

To analyze the signals, we counted the dye-labeled neuronal cell bodies per ganglion. Individual cells can be visualized since the dye incorporates into the membrane revealing the outline of individual cell bodies. The overlay of signals and ganglion image allowed us to analyze the response of individual cells.

2.4 | Calcium imaging

Preparation of the tissue samples for the calcium imaging experiments was identical to the preparation procedure for voltage-sensitive dye imaging. Each specimen was mounted onto Sylgard rings and stained for 60 min in Krebs solution that contained Fluo-4AM (Invitrogen, Karlsruhe, Germany, now ThermoFisher Scientific) at a concentration of 10 μM and probenecid (0.5 mM, Sigma-Aldrich). Each specimen

was then transferred to the recording chamber, mounted on an inverted microscope (IX 50, Olympus) and superfused with Carbogen bubbled Krebs solution at 37°C. Calcium signals were recorded for 5 s at frame rates of 40 or 125 Hz with the NeuroCCD system and a FITC filter set (excitation: HC482/35, dichroic: BS506, emission: HC536/40, AHF Analysentechnik) at magnifications 20×–40×.

2.5 | Ussing chamber experiments

To test the effect of the nicotinic antagonist hexamethonium and the NMDA antagonist D-APV on electrically-evoked chloride secretion in intact submucosa/mucosa preparations from the human intestine we used the Ussing chamber technique as previously described in detail (Krueger et al., 2016). The preparations were mounted in Ussing chambers with a recording area of 1.08 cm². Mucosal and serosal sides were bathed separately in Krebs solution. The solution was maintained at 37°C and continuously bubbled with 95% O₂ and 5% CO₂. The trans-epithelial potential difference was measured by Krebs-agar bridges on each side of the tissue which were connected to calomel half-cells. A pair of Ag/AgCl electrodes were connected to a voltage clamp apparatus (VCC 600, Physiologic Instruments) that compensated for the solution resistance between the electrodes. Data were recorded with a PowerLab System and LabChart (version 4.2–8.1.16) software (ADInstruments Ltd). The tissue could be stimulated electrically by electrodes made from aluminum foil which were placed on either side of the tissue. The stimulating electrodes were connected to a constant voltage stimulator (Grass SD-9). Stimulation was achieved by delivering a train of pulses to the tissue with the following stimulation parameters: pulse amplitude: 20 V, pulse frequency: 10 Hz, pulse duration 1 ms, train duration 10 s. Drugs were added to the serosal side of the preparation. Before starting the actual measurements, the tissues were allowed to equilibrate for at least 30 min.

2.6 | Immunohistochemistry

Tissue specimens were fixed overnight at room temperature in a solution containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer and then washed (3 × 10 min) in phosphate buffer. Until further processing, the samples were stored at 4°C in phosphate-buffered saline (PBS) containing 0.1% NaN₃. Wholmount preparations containing the inner submucosal plexus were first incubated (1 h, room temperature) in PBS plus Triton-X-100 (TX100; 0.5%) and horse serum (4%). Then the preparations were incubated at room temperature in PBS/0.5% TX100/4% horse serum/0.1% NaN₃ for 40 h with the primary antisera (mouse anti Hu C/D biotin conjugated 1:50 (Molecular Probes) and rat anti nAChR 1:500 (mAB210, BabCo) which is specific for muscle α1 and neuronal α3 and α5 subunits of nicotinic acetylcholine receptors. Other antibodies against the α4 subunit (mAB299, BabCo) and the α7

(mAB306, BabCo) subunit showed no reliable staining in the human submucosa. After washing (3 × 10 min in PBS) the preparations were incubated for 6 h with the secondary antibodies (donkey anti-rat Cy5 1:200 (Dianova) and streptavidin Cy2 1:200 (Dianova)).

After the final washing, the tissues were mounted with Citifluor AF1 (Science Services, Munich, Germany) on poly-l-lysine coated coverslips. Tissues were finally inspected with an Olympus Microscope BX61 WI (Olympus, Hamburg, Germany) with appropriate filter blocks. The microscope was equipped with a SIS Fview II CCD-camera and the analysis version 3.1 software (Soft Imaging System GmbH, Münster, Germany) for image acquisition and analysis. The panel for the presentation of immunohistochemistry pictures was constructed with EZFig 1.0 beta 12.²¹

2.7 | Data analysis and statistics

The total number of neurons for each ganglion was determined by visual inspection of images from the Di-8-ANEPPS stained ganglion.¹⁴ These images were taken from the ganglion during the experiment with the CCD-camera at the highest possible resolution (70 × 70 pixels) (Neuro-CCD system) or with the video camera (Neuro-PDA system). During the analysis of the optical data, traces of all detectors of the CCD-camera were projected onto the corresponding image of the ganglion. Signals from individual nerve cells were then obtained by manually binning the detectors which recorded activity from that particular cell. Thus, we were able to determine the number of neurons in each ganglion that responded to electrical stimulation or the application of a substance via pressure application. Neuron numbers in immunohistochemistry preparations were determined manually in representative pictures from each preparation.

Statistical analyses were performed using SigmaPlot 12.5 (Systat, Erkrath, Germany) and statistical tests are indicated in the text. Data are given as mean ± standard deviation or median (25% percentile/75% percentile) depending on the normal or non-normal distribution of the data. A *p*-value ≤ 0.05 was considered as statistically significant.

3 | RESULTS

3.1 | Neuroimaging

We performed neuroimaging experiments (voltage-sensitive dye imaging and calcium imaging) in 48 preparations from 47 patients (mean age 63.5 ± 13.7 years, 16 female, small intestine (si): 11, large intestine (li): 31, rectum: 5 (re), caecum: 1). Electrical stimulation of interganglionic fiber tracts led to signals in ganglionic areas which consisted of one or both of the following components as previously reported (Schemann et al., 2002): Initially, a signal with a sharp onset which represents either an axonal compound or an antidromic action potential. This was followed by a second signal

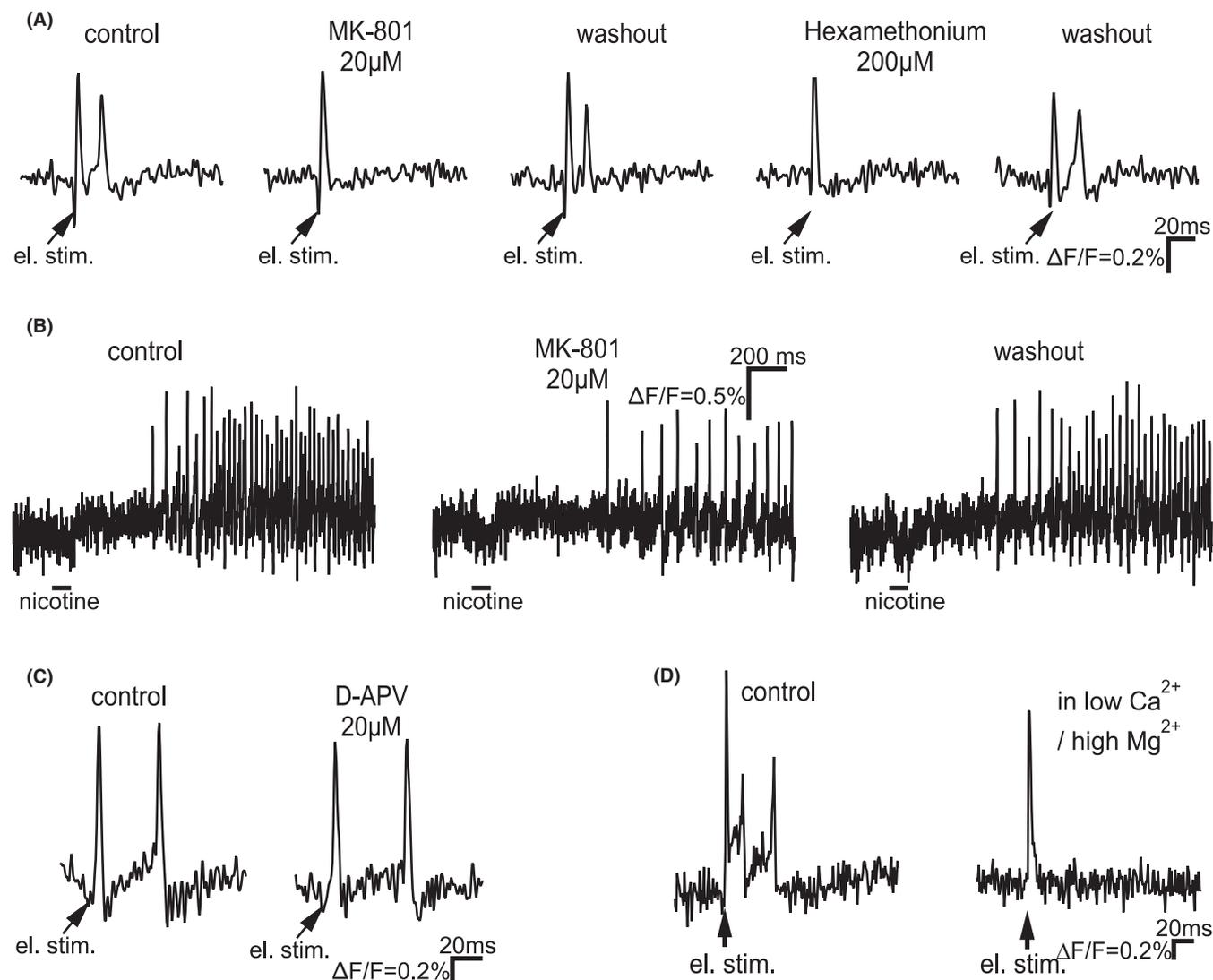


FIGURE 1 (A) The action potential that is fired by a human submucous neuron in response to electrical stimulation of an interganglionic fiber tract (arrow) is blocked by the NMDA antagonist MK-801 (second trace) and is restored upon washout (third trace). The nicotinic blocker hexamethonium also blocks the action potential in the same neuron (fourth trace). Also, the action of hexamethonium is reversed upon washout (fifth trace). (B) The NMDA antagonist MK-801 reduces the response of human submucous neurons to nicotine. Under control conditions (left trace), a neuron fires action potentials at a high frequency in response to a brief application of nicotine (black bar) directly onto the ganglion. The response is greatly reduced during perfusion with MK-801 (middle trace) and restored upon washout (right trace). (C) The specific NMDA antagonist D-APV is not able to block synaptic action potentials. The signals in response to electrical stimulation under control conditions (left trace) are not changed in the presence of D-AP5 (right trace). (D) Electrical stimulation (arrow) of interganglionic fiber tracts evokes two types of signals in the human submucous plexus: A fast component that follows immediately the stimulation is of non-synaptic origin because it persists during a block of the synaptic release of neurotransmitters under low Ca²⁺/high Mg²⁺ perfusion (right trace). A slower component that consisted in this neuron of two action potentials (left trace) is of synaptic origin because it is blocked in low Ca²⁺/high Mg²⁺ perfusion (right trace)

that was inhibited in a low calcium/high magnesium solution and was therefore of synaptic origin (Figure 1D). The fast time course of the second component suggested the involvement of ionotropic receptors in the generation of these signals. In order to test the nature of the fast synaptic component, we used the nicotinic blocker hexamethonium and the NMDA receptor antagonists MK-801 and D-APV. Hexamethonium at a concentration of 200 μM blocked synaptically evoked fEPSPs and fEPSP triggered spikes in 29 out of 29 neurons in six ganglia (6 preparations, 6 patients, si: 1, li: 2,

re: 3) (Figure 1A). MK-801 (20 μM) blocked fEPSP triggered spikes (Figure 1A) in 14 out of 22 neurons (6 ganglia, 6 preparations, 6 patients, si: 0, li: 6, re: 0). The percentage of neurons per ganglion in which the synaptic input was blocked by MK-801 ranged from 0% to 100% (68 ± 42%). However, in an experiment in which we first perfused MK-801 (20 μM) and, after a washout period, hexamethonium (200 μM), we showed that MK-801 was almost as effective as hexamethonium in blocking electrically-evoked synaptic APs (Figure 1A). MK-801 blocked 81% of the responses to

electrical stimulations of interganglionic fiber tracts while hexamethonium blocked 100% (6 neurons, 1 ganglion, 1 patient). This would either indicate the simultaneous presence of the respective receptors on these cells or non-specific effects of the antagonists. An effect of hexamethonium on the NMDA receptor can be ruled out.²² On the other hand, an action of MK-801 on nicotinic receptors as described in the guinea pig ENS²³ was a possibility. We, therefore, used the more specific NMDA antagonist D-APV (20 and 40 μ M). This antagonist blocked the synaptic signal only in 1 out of 40 neurons (2.5%) (12 ganglia, 9 preparations, 8 patients, si: 1, li: 4, re: 2, caecum: 1) (Figure 1C).

The effect of the nicotinic agonist DMPP was tested by pressure application of the substance onto individual ganglia. The concentration of DMPP in the ejection pipette was 10 or 100 μ M and ejection times of 20–200 ms was used. Pressure application of DMPP led to a rapid discharge of multiple action potentials in 56 out of 73 neurons (corresponding to $79.9 \pm 18.7\%$ of the neurons per ganglion, 13 ganglia, 8 patients, si: 3, li: 5, range 46–100%). MK-801 (20 μ M) had no significant effect on the discharge of action potentials after spritz application of DMPP (100 μ M) (action potential frequency: before MK-801: 13.7 ± 9.0 Hz, in MK-801: 13.3 ± 8.3 Hz, $p = 0.85$, 5 neurons, 2 ganglia, 2 patients, si: 1, re: 1). However, MK-801 reduced the number of action potentials in response to pressure application of (-)-nicotine (1 and 100 μ M) significantly to $32.7 \pm 22\%$ of the control value (range 0–65%). After a washout period, the response to the application of (-)-nicotine recovered completely (action potential frequency before MK-801: 16.6 ± 8.0 Hz, in MK-801: 6.2 ± 4.3 Hz, after washout: 15.0 ± 7.3 Hz, $p < 0.001$, 9 neurons, 3 ganglia, 3 preparations, 3 patients, li: 3) (Figure 1B).

As a further test for the presence of functional NMDA receptors, we used pressure application of NMDA (10 mM) directly to Di-8-ANEPPS stained ganglia in the human SMP. For these experiments, we used Mg^{2+} -free Krebs solution which contained 5–10 μ M glycine to facilitate potential responses.²⁴ NMDA was applied for 200–800 ms to 34 neurons (5 ganglia, 3 patients, si: 1, li: 2). In two neurons (6%) we detected discharge of action potentials after NMDA application, which, however, was not reproducible.

Because the calcium imaging technique could be more sensitive in detecting subthreshold activation of neurons, we tested the effect of pressure applied NMDA (10 mM) in 10 ganglia (3 patients, si: 1, li: 2). As before, the experiments were done with Mg^{2+} -free Krebs solution with added glycine (10 μ M). We could distinguish 29 neurons that responded to the application of 50 mM KCl with a mean increase in fluorescence of $51.9 \pm 33.8\%$. Of these, three neurons (10.3%) showed an increase in fluorescence ($10.6 \pm 6.1\%$) in response to the application of NMDA while all other neurons only showed signals that were not discernible from background fluctuations ($\leq 1\%$).

We also tested agonists for the two other ionotropic glutamate receptors (AMPA and kainate) in voltage-sensitive dye imaging experiments. The experiments with AMPA were done with 25 μ M cyclothiazide in the perfusing Krebs solution to prevent AMPA receptor desensitization.^{19,20} Pressure application of AMPA at a concentration of 1 mM had no excitatory effect in 41 neurons (10 ganglia, 5 patients, li: 5). Pressure application of kainic acid (1 mM, 200–800 ms) had an excitatory effect in one out of 42 neurons that also responded to the application of nicotine (Figure 2) (2.4%, 13 ganglia, 6 patients, si: 3, li: 3). The response consisted of a discharge of action potentials and could be blocked by perfusion of DNQX (40 μ M), a blocker of non-NMDA ionotropic glutamate receptors. The response returned after a washout period (Figure 2).

3.2 | Ussing chamber experiments

Ussing chamber measurements were performed in muscle-stripped mucosa-submucosa preparations from 40 patients (20 female, mean age: 67.6 ± 12.8 years, from one patient we received small and large intestine). Activation of the submucosal plexus by electrical field stimulation led to a transient rise in short circuit current (I_{sc}). This rise is abolished by the blocker of fast sodium channels TTX (1 μ M) and therefore mediated by neurogenic mechanisms.¹¹ Addition of hexamethonium (200 μ M) to the serosal solution had no effect on basal chloride secretion. However,

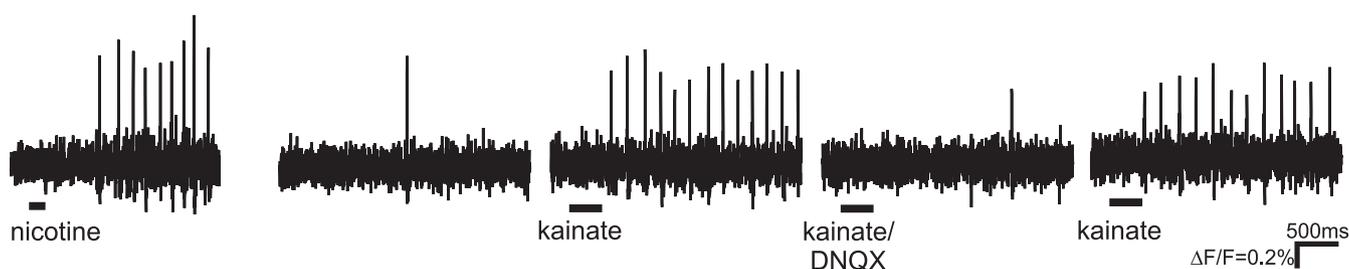


FIGURE 2 Response of one neuron to pressure application of nicotine (100 μ M) and kainic acid (kainate, 1 mM). From left to right: First trace: The neuron fires a train of action potentials in response to the application of nicotine (200 ms, black bar below trace). Note the shorter duration of this trace in comparison to the other traces. Second trace: With no substance applied, the neuron fires only one spontaneous action potential during the recording period. Third trace: The application of kainate also evokes a train of action potentials in the same neuron. Fourth trace: During the perfusion of the AMPA/Kainate blocker (40 μ M, 6 min in the bath) DNQX, the response of the neuron to the application of kainate is blocked. The neuron fires only one action potential as in the second trace. Fifth trace: After a washout period of 15 min, the response to kainate is restored. Scale bar under the fifth trace applies to all traces

FIGURE 3 Mucosal secretion in human preparations is mediated by nicotinic receptors. (A) Two example traces from an Ussing chamber experiment show the response of a human mucosa/submucosa preparation to electrical field stimulation (EFS). The tissue shows an immediate and long-lasting rise in short circuit current I_{sc} (black trace) that is reduced after the block of nicotinic receptors with hexamethonium (grey trace). (B) The response to EFS is reduced significantly by hexamethonium ($n = 14$, $p < 0.001$, Wilcoxon Signed Rank Test) while D-AP5 has no statistically significant effect ($n = 15$, $p = 0.073$, Wilcoxon Signed Rank Test)

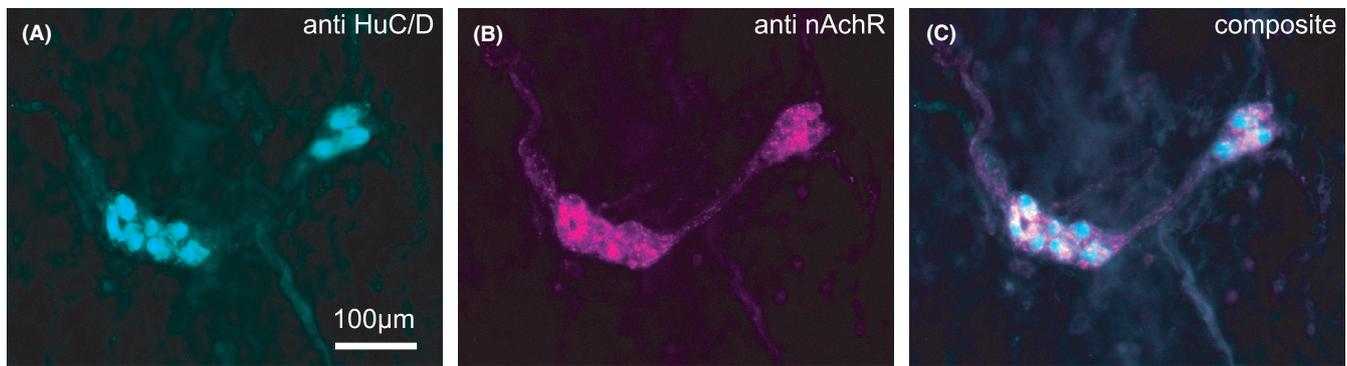
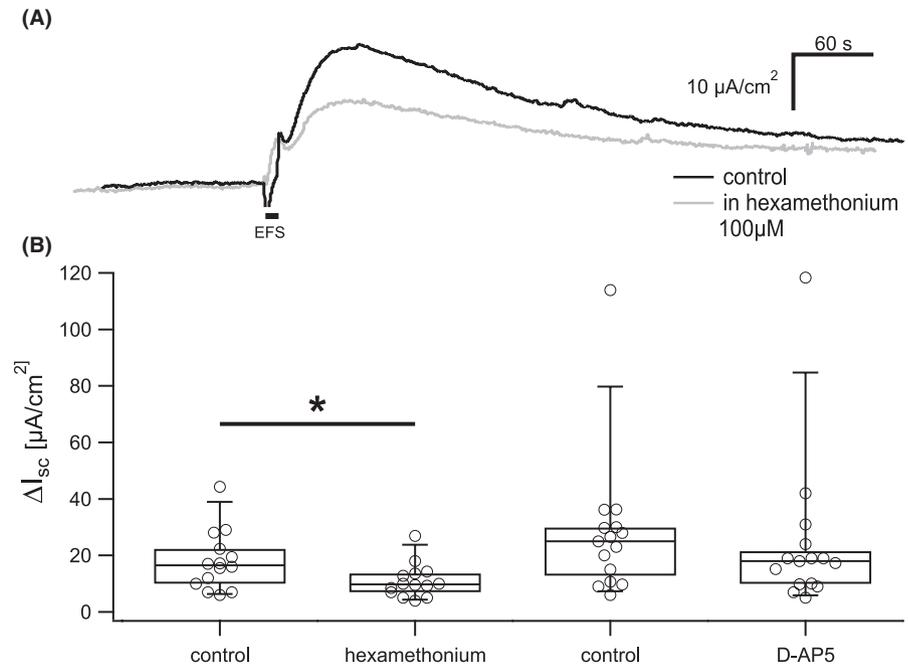


FIGURE 4 Virtually all neurons in the human submucous plexus express nicotinic receptors. (A) Neurons in a ganglion are stained with an antibody against the pan-neuronal marker HuC/D, shown in cyan. (B) All neurons in the same ganglion are also stained with an antibody against nicotinic acetylcholine receptors, shown in magenta. (C) Composite picture of A and B. Scale bar in A applies to all pictures

hexamethonium reduced the response to field stimulation significantly by 37.7(–43.6/–18.8)% (from 16.5 (9.3/23.7) $\mu\text{A cm}^{-2}$ (control stimulation) to 9.8 (6.5/13.7) $\mu\text{A cm}^{-2}$ (in hexamethonium) ($p < 0.001$, Wilcoxon Signed Rank Test, $n = 14$ patients, si: 2, li: 9, re: 3) (Figure 3). We have already shown that the nicotinic agonist DMPP markedly increases the basal I_{sc} in human intestinal tissue.¹³ To test if this increase could be due to the presence of nicotinic receptors directly on epithelial cells, we compared the effects of nicotine (10 μM) in the absence and presence of TTX (1 μM). TTX abolished the increase in I_{sc} by nicotine (control: 8.6 (6.3/25.8) $\mu\text{A cm}^{-2}$ vs TTX: 0.4 (0.2/1.9) $\mu\text{A cm}^{-2}$, $p = 0.004$, Wilcoxon Signed Rank Test, $n = 6$ patients, si: 1, li: 5).

Addition of D-APV (100 μM) to the serosal solution had no effect on basal chloride secretion. In contrast to the effect of hexamethonium, D-APV had no significant effect on the response to field stimulation. Five preparations showed an increase while ten preparations showed a decrease in ΔI_{sc} resulting in a non-significant reduction by –15.2(–35.7/4.3)% (control response: 25.0 (10.7/30.0)

$\mu\text{A cm}^{-2}$, after D-APV: 18.0 (9.8/24.0) $\mu\text{A cm}^{-2}$, $p = 0.073$, Wilcoxon Signed Rank Test, $n = 15$ patients, si: 1, li: 10, re: 4) (Figure 3). The variability of this data is comparable to similar experiments that showed the reproducibility of responses to electrical field stimulation in a large number of human specimens.¹¹ As a further test for the involvement of NMDA receptors, we applied NMDA directly in Ussing chamber experiments. These experiments were performed in the presence of a Mg^{2+} -free Krebs solution containing glycine (10 μM).²⁴ NMDA (100 μM) had no significant effect on basal I_{sc} (control: $80.0 \pm 32.7 \mu\text{A cm}^{-2}$ vs $82.7 \pm 30.9 \mu\text{A cm}^{-2}$, $p = 0.28$, paired t-Test, $n = 5$ patients, li: 4, re: 1).

3.3 | Immunohistochemistry

Double labeling with antibodies against the neuronal proteins HuC/HuD to label all neurons and against the nicotinic receptor was performed in six preparations from the colonic submucosal plexus from

six patients (three female, mean age 66.7 ± 13.5 years). On average 18 ± 2 ganglia per preparation were examined, giving a total of 108 ganglia with 1003 neurons. The number of neurons per ganglion was 9.4 ± 1.9 . Single neurons lying outside of ganglia in nerve strands were not included in the calculations. Almost all submucosal neurons ($98.7 \pm 2.4\%$, $n = 6$ preparations) stained positive for the nicotinic receptor (Figure 4).

4 | DISCUSSION

The present study revealed that acetylcholine acting on nicotinic receptors mediated fEPSPs in the human enteric nervous system. This conclusion is based on the findings that fEPSPs were blocked by the nicotinic antagonist hexamethonium while the nicotinic agonists DMPP and (-)-nicotine mimicked them. Functional studies revealed that the epithelial response to electrical field stimulation was reduced to 58% by hexamethonium. The presence of nicotinic receptors was demonstrated by immunohistochemistry in nearly 100% of human submucosal neurons. The fact that the number of neurons that responded to DMPP in the MSORT experiments (79.9%) was lower than the number of neurons that stained positive for the nicotinic receptor can be explained in several ways: First, it is possible that the number of neurons responding to DMPP which was determined in the MSORT experiments was an underestimation: Because subthreshold activations have a much lower amplitude, we may have missed these signals in some neurons with suboptimal voltage-sensitive dye staining. Secondly, a functional nicotinic receptor is composed of five α or β subunits.¹ The antibodies that we used were directed against subunits α_1 , α_2 , or α_3 , but not all of the stained neurons might express functional nicotinic receptors. Additionally, receptors will have different pharmacological profiles depending on the species and receptor subunit composition.²⁵

Because the vast majority, if not all, of submucosal neurons express nicotinic receptors it can be concluded that this receptor is not restricted to a specific functional class of neurons. Pathological changes in nicotinic neurotransmission might therefore lead to more general gastrointestinal symptoms.²⁶ Indeed, patients with high titers for antibodies against ganglionic acetylcholine receptors may develop autoimmune autonomic ganglionopathy (AAG) with gastrointestinal symptoms (among other symptoms of autonomic dysfunction).^{27,28} This, together with our data indicates that nicotinic receptors are important for the normal function of the human gut. The results from our Ussing experiments further support this conclusion: The rise in chloride secretion that was evoked by electrical stimulation of the submucosal plexus was decreased significantly by blocking nicotinic receptors. This is also in line with our previous study that showed a clear-cut rise of the short circuit current in human colonic mucosa preparations after the addition of a nicotinic agonist to the serosal side of the preparations.¹³ The secretory epithelium in human tissue does not possess nicotinic receptors because we could abolish nicotine-evoked secretion with tetrodotoxin. This demonstrates that the hexamethonium sensitive component of

the secretory response is due to the activation of cholinergic interneurons which then activate secretomotor neurons. Human tissue shows here a strong difference to findings in rat colon where an effect of epithelial nicotinic receptors on chloride secretion has been demonstrated.^{29,30}

Hexamethonium blocked all neuronal responses to the stimulation of interganglionic fiber tracts. This does not necessarily mean that acetylcholine is the only neurotransmitter that is responsible for fEPSPs in the human submucous plexus. Other neurotransmitters could have subthreshold effects that were not detectable with our method. In a calcium imaging study of preparations of the human jejunal submucosal plexus, only 50% of the response to electrical field stimulation was blocked by hexamethonium.³¹ The lower potency of hexamethonium compared to our study was likely due to train stimulation that releases a mixture of acetylcholine and other neurotransmitters which cause fast and slow excitatory postsynaptic potentials. Additionally, it is possible that some neurons in this study were activated antidromically. Although antagonism of nicotinic receptors blocked fEPSPs in our study, ATP may contribute to fEPSPs. There is indirect evidence as a blocker of ionotropic ATP receptors decreases Ca-transients to a 25 Hz stimulus train in human submucous plexus neurons.¹²

Another possible mediator of fast synaptic transmission in the human ENS would be glutamate acting on NMDA, AMPA, or kainate receptors as has already been shown for the guinea pig (see¹). Although the marked effect of the NMDA-antagonist MK-801 (Dizocilpine) in our experiments first supported the hypothesis of an excitatory role of NMDA receptors, the highly selective NMDA-antagonist D-APV (at concentrations which have already been used in the human enteric nervous system to block NMDA mediated responses³² had only an effect in one out of 40 neurons. We attribute the different effects of the two drugs to the nonspecific inhibitory action of MK-801 on nicotinic receptors which has already been shown in guinea-pig myenteric neurons²³ and peripheral and central neurons of other species.³³⁻³⁵ The IC_{50} value for this effect has been determined for cloned human nicotinic receptors (2.7 μ M for receptors of the $\alpha_3\beta_4$ subtype to 36 μ M for receptors of the $\alpha_2\beta_2$ subtype,³⁵ and was well in the range of the concentrations of MK-801 used in the present study. The most prevalent nicotinic receptor subunits in the enteric nervous system are $\alpha_3\beta_4$, and this could explain the potent blocking effect of MK-801 on nicotinic receptors in our experiments.^{1,36} The fact that MK-801 was not able to block DMPP effects can possibly be explained by the greater sensitivity of receptors of the $\alpha_3\beta_4$ subtype for DMPP than for nicotine.²⁵ as has been shown for neurons in the guinea-pig submucosal plexus.³⁷ It is also possible that nicotinic receptors with different subunit composition are present in the human submucosal plexus. Experiments with subtype-specific agonists and antagonists would be necessary to prove this.

The more specific NMDA antagonist D-APV had only marginal effects on fEPSPs as well as on nerve-evoked secretion. Additionally, NMDA had no significant effect on baseline secretion when we applied it directly in the Ussing chamber. This indicates a minor role for

fast synaptic transmission through NMDA receptors in the human submucous plexus. These findings are consistent with the lack of ionotropic glutamate receptors in the submucosal plexus of the guinea pig and piglet.^{20,38} However, other studies indicate strong species and/or region dependency of the expression of NMDA receptors in the gut. A calcium imaging study in the myenteric plexus of the mouse colon was able to demonstrate functional NMDA receptors but no indication of glutamate-mediated fast synaptic transmission.³⁹ On the other hand, an organ bath study with preparations from the guinea pig ileum found a clear indication of an excitatory action of NMDA receptors in the myenteric plexus.⁴⁰ Another study found NMDA mediated acetylcholine release from preparations of the human colon.³² The responsible NMDA receptor-expressing neurons were likely located in the myenteric plexus. In addition, we rarely found effects of the two other ionotropic glutamate receptors of the AMPA- and kainate-subtype in the human submucous plexus. The presence of ionotropic glutamate receptors in the ENS remains controversial: Liu et al. were able to demonstrate excitatory effects of NMDA and AMPA that could be blocked by the respective antagonists in the guinea pig myenteric plexus.¹⁹ On the other hand, Ren et al. were not able to reproduce these results in the same preparation.²⁴ Although they found also excitatory responses, these could be attributed to pH-effects of the applied substances. In our present study, we used lower concentrations of AMPA and kainate and checked the pH of the applied substances. In addition, we facilitated potential excitatory effects by using a 0 mM Mg⁺⁺ solution with 25 μ M glycine for the NMDA experiments and 25 μ M cyclothiazide solution for the AMPA experiments.²⁴ Regardless of these measures, we did not record consistent responses mediated through ionotropic glutamatergic receptors in the human submucous plexus. This does not completely rule out a role for glutamatergic neurotransmission in the human ENS: The focus of the current study was on ionotropic and not on metabotropic receptors. In addition, glutamatergic receptors might also be present in the myenteric plexus or in other regions of the gut. However, a study by Wang et al. found no neurogenic effects of L-Glutamate in human small bowel on chloride secretion or motility.⁴¹

Most of our experiments were done in samples from the large intestine. The number of samples from other regions of the gastrointestinal tract (small bowel, rectum, and cecum) was distributed randomly between the experimental groups and thus was too small to allow statistical comparison. However, during analysis of the data, we found no indications of regional differences. The good agreement between findings of our neuropharmacology in the inner submucous plexus (that is closest to the mucosa) and the pharmacology of the secretory responses in Ussing chamber experiments (where the preparations included also the outer submucous plexus) argues also against significant differences between the different layers of the submucous plexus.

The present data suggest acetylcholine as a major neurotransmitter mediating fast excitatory synaptic neurotransmission in the human submucosal plexus. We conclude that fEPSPs in the human

submucous plexus are mediated mainly by acetylcholine (present study) with a small contribution of serotonin (5-HT) and ATP.^{12,13} Ionotropic glutamate receptors (NMDA, AMPA, and kainate) seem to play only a minor role. Future studies should exercise caution when interpreting the functional role of glutamatergic ionotropic receptors in fast synaptic transmission of human submucosal plexus.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the excellent technical help from Birgit Kuch.

CONFLICT OF INTERESTS

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: KM and MS. Performed the experiments: KM, DK and SS. Analyzed the data: KM, DK, and SS. FZ, IED, and JT contributed materials. KM and MS wrote the manuscript. All authors contributed to editing and revising the manuscript. All authors read and approved the final manuscript.

ORCID

Klaus Michel  <https://orcid.org/0000-0003-2359-6428>

Ihsan Ekin Demir  <https://orcid.org/0000-0001-8241-1871>

Michael Schemann  <https://orcid.org/0000-0003-1007-9843>

REFERENCES

- Galligan JJ. Ligand-gated ion channels in the enteric nervous system. *Neurogastroenterol Motil.* 2002;14(6):611-623.
- Vanner S, Surprenant A. Effects of 5-HT₃ receptor antagonists on 5-HT and nicotinic depolarizations in guinea-pig submucosal neurons. *Br J Pharmacol.* 1990;99(4):840-844.
- Moore BA, Vanner S. Organization of intrinsic cholinergic neurons projecting within submucosal plexus of guinea pig ileum. *Am J Physiol.* 1998;275(3):G490-G497.
- Moore BA, Vanner S. Properties of synaptic inputs from myenteric neurons innervating submucosal S neurons in guinea pig ileum. *Am J Physiol Gastrointest Liver Physiol.* 2000;278(2):G273-G280.
- Monro RL, Bertrand PP, Bornstein JC. ATP participates in three excitatory postsynaptic potentials in the submucous plexus of the guinea pig ileum. *J Physiol.* 2004;556(Pt 2):571-584.
- Breunig E, Michel K, Zeller F, Seidl S, Weyhern CWHV, Schemann M. Histamine excites neurones in the human submucous plexus through activation of H₁, H₂, H₃ and H₄ receptors. *J Physiol.* 2007;583(2):731-742.
- Brown DR, Timmermans J-P. Lessons from the porcine enteric nervous system. *Neurogastroenterol Motil.* 2004;16(s1):50-54.
- Cornelissen W, Laet AD, Kroese ABA, van Bogaert P-P, Scheuermann DW, Timmermans J-P. Excitatory synaptic inputs on myenteric Dogiel type II neurones of the pig ileum. *J Comp Neurol.* 2001;432(2):137-154.
- Cornelissen W, De Laet A, Kroese ABA, Van Bogaert P-P, Scheuermann DW, Timmermans J-P. Electrophysiological features of morphological Dogiel Type II neurons in the myenteric plexus of pig small intestine. *J Neurophysiol.* 2000;84(1):102-111.
- Cornelissen WB, de Laet AB, Kroese AB, et al. Species-dependent features of Dogiel type II neurones in the mammalian enteric nervous system. *Eur J Morphol.* 1999;37(4-5):241-249.

11. Krueger D, Michel K, Zeller F, et al. Neural influences on human intestinal epithelium in vitro. *J Physiol*. 2016;594(2):357-372.
12. Liñán-Rico A, Wunderlich JE, Enneking JT, et al. Neuropharmacology of purinergic receptors in human submucous plexus: Involvement of P2X₁, P2X₂, P2X₃ channels, P2Y and A₃ metabotropic receptors in neurotransmission. *Neuropharmacology*. 2015;95:83-99.
13. Michel K, Zeller F, Langer R, et al. Serotonin excites neurons in the human submucous plexus via 5-HT₃ receptors. *Gastroenterology*. 2005;128(5):1317-1326.
14. Schemann M, Michel K, Peters S, Bischoff SC, Neunlist M. Cutting-edge technology. III. Imaging and the gastrointestinal tract: mapping the human enteric nervous system. *Am J Physiol Gastrointest Liver Physiol*. 2002;282(6):G919-G925.
15. Timmermans J-P, Hens J, Adriaensen D. Outer submucous plexus: An intrinsic nerve network involved in both secretory and motility processes in the intestine of large mammals and humans. *Anat Rec*. 2001;262(1):71-78.
16. Brookes SJ, Ewart WR, Wingate DL. Intracellular recordings from myenteric neurones in the human colon. *J Physiol*. 1987;390(1):305-318.
17. Carbone SE, Jovanovska V, Nurgali K, Brookes SJH. Human enteric neurons: morphological, electrophysiological, and neurochemical identification. *Neurogastroenterol Motil*. 2014;26(12):1812-1816.
18. Neunlist M, Peters S, Schemann M. Multisite optical recording of excitability in the enteric nervous system. *Neurogastroenterol Motil*. 1999;11(5):393-402.
19. Liu M-T, Rothstein JD, Gershon MD, Kirchgessner AL. Glutamatergic enteric neurons. *J Neurosci*. 1997;17(12):4764-4784.
20. Giaroni C, Zanetti E, Marino F, et al. Glutamate receptors of the AMPA type modulate neurotransmitter release and peristalsis in the guinea-pig isolated colon. *Life Sci*. 2000;67(14):1747-1757.
21. Aigouy B, Mirouse V. SciencifiG: a tool to build publication-ready scientific figures. *Nat Methods*. 2013;10(11):1048.
22. Chen H-SV, Lipton SA. Pharmacological implications of two distinct mechanisms of interaction of memantine with N-methyl-D-aspartate-gated channels. *J Pharmacol Exp Ther*. 2005;314(3):961-971.
23. Galligan JJ, North RA. MK-801 blocks nicotinic depolarizations of guinea pig myenteric neurons. *Neurosci Lett*. 1990;108(1-2):105-109.
24. Ren J, Hu H-Z, Liu S, Xia Y, Wood JD. Glutamate receptors in the enteric nervous system: ionotropic or metabotropic? *Neurogastroenterol Motil*. 2000;12(3):257-264.
25. Chavez-Noriega LE, Crona JH, Washburn MS, Urrutia A, Elliott KJ, Johnson EC. Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors h alpha 2 beta 2, h alpha 2 beta 4, h alpha 3 beta 2, h alpha 3 beta 4, h alpha 4 beta 2, h alpha 4 beta 4 and h alpha 7 expressed in *Xenopus* oocytes. *J Pharmacol Exp Ther*. 1997;280(1):346-356.
26. Carbone SE, Jovanovska V, Brookes SJH, Nurgali K. Electrophysiological and morphological changes in colonic myenteric neurons from chemotherapy-treated patients: A pilot study. *Neurogastroenterol Motil*. 2016;28(7):975-984.
27. Vernino S, Low PA, Fealey RD, Stewart JD, Farrugia G, Lennon VA. Autoantibodies to ganglionic acetylcholine receptors in autoimmune autonomic neuropathies. *N Engl J Med*. 2000;343(12):847-855.
28. Wang Z, Low PA, Jordan J, et al. Autoimmune autonomic ganglionopathy: IgG effects on ganglionic acetylcholine receptor current. *Neurology*. 2007;68(22):1917-1921.
29. Bader S, Diener M. Novel aspects of cholinergic regulation of colonic ion transport. *Pharmacol Res Perspect*. 2015;3(3):e00139.
30. Lottig L, Bader S, Jimenez M, Diener M. Evidence for metabotropic function of epithelial nicotinic cholinergic receptors in rat colon. *Br J Pharmacol*. 2019;176(9):1328-1340.
31. Wunderlich JE, Needleman BJ, Chen Z, et al. Dual purinergic synaptic transmission in the human enteric nervous system. *Am J Physiol Gastrointest Liver Physiol*. 2008;294(2):G554-G566.
32. Giaroni C, Zanetti E, Chiaravalli AM, et al. Evidence for a glutamatergic modulation of the cholinergic function in the human enteric nervous system via NMDA receptors. *Eur J Pharmacol*. 2003;476(1):63-69.
33. Arias HR, McCardy EA, Blanton MP. Characterization of the dizocilpine binding site on the nicotinic acetylcholine receptor. *Mol Pharmacol*. 2001;59(5):1051-1060.
34. Buisson B, Bertrand D. Open-channel blockers at the human $\alpha 4 \beta 2$ neuronal nicotinic acetylcholine receptor. *Mol Pharmacol*. 1998;53(3):555-563.
35. Yamakura T, Chavez-Noriega LE, Harris RA. Subunit-dependent inhibition of human neuronal nicotinic acetylcholine receptors and other ligand-gated ion channels by dissociative anesthetics ketamine and dizocilpine. *Anesthesiology*. 2000;92(4):1144-1153.
36. Galligan JJ, North RA. Pharmacology and function of nicotinic acetylcholine and P2X receptors in the enteric nervous system. *Neurogastroenterol Motil*. 2004;16(Suppl):164-170.
37. Zhou X, Ren J, Brown E, Schneider D, Caraballo-Lopez Y, Galligan JJ. Pharmacological properties of nicotinic acetylcholine receptors expressed by guinea pig small intestinal myenteric neurons. *J Pharmacol Exp Ther*. 2002;302(3):889-897.
38. Rhoads JM, Argenzio RA, Chen W, Gomez GG. Asparagine stimulates piglet intestinal Cl⁻ secretion by a mechanism requiring a submucosal glutamate receptor and nitric oxide. *J Pharmacol Exp Ther*. 1995;274(1):404-412.
39. Swaminathan M, Hill-Yardin EL, Bornstein JC, Foong JPP. Endogenous glutamate excites myenteric calbindin neurons by activating group I metabotropic glutamate receptors in the mouse colon. *Front Neurosci*. 2019;13:426.
40. Moroni F, Luzzi S, Franchi-Micheli S, Zilletti L. The presence of N-methyl-d-aspartate-type receptors for glutamic acid in the guinea pig myenteric plexus. *Neurosci Lett*. 1986;68(1):57-62.
41. Wang G-D, Wang X-Y, Xia Y, Wood JD. Dietary glutamate: Interactions with the enteric nervous system. *J Neurogastroenterol Motil*. 2014;20(1):41-53.

How to cite this article: Michel K, Krüger D, Schäuffele S, et al. Fast synaptic excitatory neurotransmission in the human submucosal plexus. *Neurogastroenterology & Motility*. 2021;33:e14164. <https://doi.org/10.1111/nmo.14164>