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RESEARCH ARTICLE

Structurally defined signaling in neuro-glia units in the enteric nervous system

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Abstract

Coordination of gastrointestinal function relies on joint efforts of enteric neurons and glia, whose crosstalk is vital for the integration of their activity. To investigate the signaling mechanisms and to delineate the spatial aspects of enteric neuron-to-glia communication within enteric ganglia we developed a method to stimulate single enteric neurons while monitoring the activity of neighboring enteric glial cells. We combined cytosolic calcium uncaging of individual enteric neurons with calcium imaging of enteric glial cells expressing a genetically encoded calcium indicator and demonstrate that enteric neurons signal to enteric glial cells through pannexins using paracrine purinergic pathways. Sparse labeling of enteric neurons and high-resolution analysis of the structural relation between neuronal cell bodies, varicose release sites and enteric glia uncovered that this form of neuron-to-glia communication is contained between the cell body of an enteric neuron and its surrounding enteric glial cells. Our results reveal the spatial and functional foundation of neuro-glia units as an operational cellular assembly in the enteric nervous system.

KEYWORDS

Ca²⁺ imaging, Ca²⁺ uncaging, enteric glial cell, enteric neuron, gastrointestinal tract, purinergic signaling, synaptic

1 | INTRODUCTION

Enteric glia comprise a diverse and plastic population of cells in the gastrointestinal tract whose phenotype depends on their location within the gut wall and the physiological status of the enteric nervous system (ENS; Boesmans, Lasrado, Vanden Berghe, & Pachnis, 2015; Hanani & Reichenbach, 1994; Lasrado et al., 2017; Rao et al., 2015). This phenotypic heterogeneity, in all likelihood, underlies the wide

variety of functions that have been ascribed to enteric glia in recent years (Boesmans & Vanden Berghe, 2017). Indeed, together with enteric neurons, enteric glial cells are involved in controlling many ENS functions including mucosal sensation and secretion, gastrointestinal motility, and immune responses (Bohorquez & Liddle, 2015; Chow & Gulbransen, 2017; Neunlist et al., 2014; Sharkey, 2015; Veiga-Fernandes & Pachnis, 2017). Although it was believed that enteric glial cells regulate processes such as epithelial cell proliferation



and barrier function independently from enteric neurons, recent evidence does not support this (Grubišić & Gulbransen, 2017; Rao et al., 2017). The regulation of ENS functions, therefore, appears to be a collaborative effort of enteric neurons and glial cells that warrants fine-tuned communication between both cell types. This crosstalk is fundamental for both signaling within the ENS and for communication between the ENS and other cell types within and outside the gastrointestinal tract (Grubišić & Gulbransen, 2017; Ochoa-Cortes et al., 2016).

Several modes of communication between enteric neurons and glial cells have been described. These range from slower signaling mechanisms such as the release of mediators including nitric oxide (Esposito et al., 2007; MacEachern, Patel, McKay, & Sharkey, 2011) and glutathione (Abdo et al., 2010; Brown & Gulbransen, 2017) to rapid communication in the course of enteric neurotransmission. The latter is embodied by the fact that activity displayed by enteric glial cells during nerve-controlled intestinal motor patterns (Broadhead, Bayguinov, Okamoto, Heredia, & Smith, 2012) is necessary for normal motility (McClain et al., 2014) and that several neurotransmitters involved in ENS signaling can activate enteric glial cells, at least in vitro (Boesmans et al., 2013; Garrido, Segura, Zhang, & Mulholland, 2002; Kimball & Mulholland, 1996; Zhang, Sarosi Jr., Barnhart, & Mulholland, 1998). A well-described form of enteric neuron-to-glia communication involves neuronal release of purines, which activate purinergic receptors present on enteric glia. This was demonstrated in a number of Ca^{2+} imaging studies in which electrical stimulation to specifically activate enteric neurons (Boesmans et al., 2013; Gomes et al., 2009; Gulbransen & Sharkey, 2009) or sympathetic extrinsics (Gulbransen, Bains, & Sharkey, 2010) also elicited secondary Ca^{2+} transients in enteric glial cells. Pharmacological interventions revealed the purinergic nature of this signaling process and led to the concept that adenosine triphosphate (ATP) is co-released with neurotransmitters to engage enteric glial cells during synaptic transmission in the ENS. This mode of neuron-to-glia communication is dependent on nerve conduction and classical neurotransmitter release, and therefore is typically blocked by tetrodotoxin (TTX). However, TTX-insensitive purinergic signaling from enteric neurons to glia has also been demonstrated (Brown, McClain, Watson, Patel, & Gulbransen, 2016; Fung et al., 2017; Gulbransen et al., 2012). A neuro-glia circuit regulated by vasoactive intestinal peptide (VIP) was recently discovered in the mouse submucosal plexus (Fung et al., 2017). Here, VIP acting on vasoactive intestinal peptide receptor-1 (VPAC1) expressed by cholinergic neurons stimulates purine release to evoke Ca^{2+} transients in enteric glia. Interestingly, activation of vasoactive intestinal peptide receptor-2 (VPAC2) receptors inhibited enteric glial Ca^{2+} activity. The exact mechanism and the structural basis of these TTX-insensitive glial responses remains unclear but might involve neuronal ATP release through pannexin 1 (Pannx1) channels. Pannx1 is a member of the pannexin family of channel-forming glycoproteins and plays a pivotal role in many neuron-glia interactions (Dahl & Muller, 2014; Hanstein, Hanani, Scemes, & Spray, 2016; Penuela, Gehi, & Laird, 2013; Sosinsky et al., 2011; Wang & Dahl, 2018). So far, the role of enteric Pannx1 channels has only been studied in the context of neuronal cell death and gastrointestinal inflammation (Brown et al., 2016; Gulbransen et al., 2012). However, pannexin channel mediated signaling is also involved in physiologic information processing and

postsynaptic function (Dolmatova et al., 2012; Huang et al., 2007; Thompson et al., 2008) and, therefore, might contribute to ENS activity associated with normal gut function.

In the current study, to investigate the signaling mechanisms and to delineate the spatial aspects of pannexin-mediated enteric neuron-to-glia communication, we aimed to stimulate single enteric neurons while monitoring activity in neighboring enteric glial cells. Therefore, we developed an experimental setup to combine Ca^{2+} uncaging of individual enteric neurons with Ca^{2+} imaging of enteric glial cells expressing a genetically encoded Ca^{2+} indicator.

2 | MATERIALS AND METHODS

2.1 | Mice

Wnt1::Cre;R26R-GCaMP3 and *Sox10::CreER^{T2};R26R-GCaMP3* mice were generated by mating *R26R-GCaMP3* (The Jackson Laboratory, Bar Harbor, ME; Zariwala et al., 2012) with *Wnt1::Cre* (Danielian, Mucino, Rowitch, Michael, & McMahon, 1998) and *Sox10::CreER^{T2}* (SER26; Laranjeira et al., 2011) transgenic mice respectively, and are hereafter called *Wnt1|GCaMP3* and *Sox10|GCaMP3*. In *Wnt1|GCaMP3* mice, all enteric neurons and glia express the genetically encoded Ca^{2+} indicator, GCaMP3 (Boesmans, Martens, et al., 2013). In vivo labeling of enteric glial cells in *Sox10|GCaMP3* animals was achieved by intraperitoneal injections of 0.1–0.2 mg/g of 4-hydroxy tamoxifen (4-OHT, Sigma-Aldrich, St. Louis, MO) dissolved in an ethanol/sunflower oil (1:9) mixture at 10 mg/ml. For immunohistochemical and viral vector labeling studies wild-type C57Bl6/J mice were also used. Mice of either sex between 6 and 16 weeks of age were used and sacrificed by cervical dislocation. All experiments were approved by the Animal Ethics Committees of the University of Leuven.

2.2 | Mouse enteric nervous system cultures

Primary cultures containing enteric neurons and glial cells were prepared as described previously (Lowette, Tack, & Vanden Berghe, 2014). Briefly, tissue preparations of longitudinal muscle with adherent myenteric plexus were isolated from the ileum of adult *Wnt1|GCaMP3* mice and collected in previously oxygenated Krebs solution (95% O_2 to 5% CO_2 , 4°C). After washing, tissue preparations were digested in a collagenase type II (14.67 mg/ml, Worthington cat#: LS004176)/protease (10 mg/ml, Sigma-Aldrich cat# P4630)/albumin (5% in phosphate buffered saline [PBS], Invitrogen, Carlsbad, CA) mixture for 8–12 min at 37°C. After stopping the enzymatic digestion by adding Krebs solution with 10% foetal bovine serum (FBS) and washing by centrifugation the pellet was resuspended in medium (DMEM F-12) enriched with 10% FBS, 1% glutamine and 0.5% penicillin/streptomycin (Lonza Group Ltd, Basel, Switzerland). The cells were plated on glass coverslips coated with poly-D-lysine hydro bromide (0.5 mg/ml in 0.15 M borate buffer) and laminin (20 µg/ml in PBS; Sigma-Aldrich) and cultured at 37°C (95% O_2 to 5% CO_2). After 24 hr, the medium was replaced by serum-free medium supplemented with nerve growth factor (50 ng/ml, Alomone Laboratories, Jerusalem, Israel), N2 (0.2%), and G5 (0.2%; Invitrogen).

2.3 | Viral vector labeling of enteric neurons

Production and purification of recombinant adeno-associated virus 2/9 vector (rAAV2/9) was performed by the Leuven Viral Vector Core (University of Leuven) as previously described (Van der Perren et al., 2011). Briefly, HEK 293T cells were transfected using a 25-kDa linear polyethylenimine solution using the pAdVDeltaF6 adenoviral helper plasmid, pAAV2/9 serotype and AAV-TF CMV-eGFP-T2A-fLuc (AAV transfer plasmid encoding eGFP and fLuc reporters driven by a CMV promoter) in a ratio of 1:1:1. Viral vector particles collected from the concentrated supernatant were purified using an iodixanol step gradient. The final sample was aliquoted and stored at -80°C . Titers (GC/ml) for AAV stocks were analyzed by real-time PCR. rAAV2/9-CMV-eGFP was delivered to wild type C56Bl6/J adult mice via tail vein injection. Mice were placed under an incandescent lamp for 15–20 min and physically restrained. Ten-microliter vector solution (titer: 8.47×10^{11} GC/ml) in 0.01 M PBS supplemented with 5% sucrose for a total volume of 250 μl was injected into the vein at a slight angle using a 33-gauge needle. Mice were sacrificed 2 weeks after injection and intestinal tissues were fixed, washed and prepared for immunohistochemistry as described below.

2.4 | Calcium imaging, calcium uncaging, and analysis

For in situ experiments, the large intestine was carefully isolated from Sox10|GCaMP3 mice and pinned flat in a Sylgard-lined dish filled with Krebs solution, bubbled with 95% O_2 to 5% CO_2 at room temperature. The mucosal, submucosal, and longitudinal muscle layers were carefully removed to obtain a circular muscle with adherent myenteric plexus preparation which was mounted over a small inox ring, immobilized by a matched rubber O-ring (Vanden Berghe, Kenyon, & Smith, 2002). Myenteric plexus preparations were placed in a recording chamber and constantly superfused with carbogenated Krebs solution at room temperature containing 1 μM nifedipine (Sigma-Aldrich) via a local gravity-fed (± 1 ml/min) perfusion pipette. For experiments on primary cultures, cells were used between 3 and maximum 5 days in vitro.

For agonist and electrical stimulation experiments live imaging was performed on an upright Zeiss Axio Examiner.Z1 microscope equipped with a Poly V xenon monochromator (TILL Photonics, Gräfelfing, Germany) and water dipping lens (20 $\times$, NA 1.0, Zeiss, Oberkochen, Germany). GCaMP3 was excited at 475 nm and captured at 525/50 nm (at 2 Hz) on a Sencam-QE CCD camera (PCO) using TillVisiON (TILL Photonics). Agonists were diluted in Krebs solution and superfused using the perfusion pipette positioned directly adjacent to the field of view (ATP, 10 μM , 20 s and Substance P [subP, 1 μM , 15 s], both from Sigma). Myenteric plexus preparations were stimulated electrically using a train of pulses (20 Hz, 2 s, 300 μs) transmitted from a Grass stimulation unit via a focal electrode (50 μm diameter tungsten wire) placed on an interganglionic connective leading to the selected myenteric ganglia within the field of view.

Ca^{2+} uncaging experiments were performed on an inverted spinning disk confocal microscope (Nikon Ti – Andor Revolution – Yokogawa CSU-X1 Spinning Disk [Andor, Belfast, Northern Ireland]) with a Nikon

40 $\times$ lens (LWD, NA 1.1, WI). GCaMP3 was excited at 488 nm and single planes were recorded at 2–4 Hz. Before imaging, myenteric plexus preparations were loaded with o-nitrophenyl EGTA (NP-EGTA-AM, 1 μM in carbogenated Krebs) for 30 min at room temperature (Burgalossi et al., 2012). After an initial set of experiments (see results section), we chose to maximize the uncaging efficiency by directing (FRAPPA, Andor) 75% of a 100 mW 405 nm laser beam to a photo-stimulated area that covered the whole cell body, while exposing each pixel twice for 100 μs . For the experiments with antagonists the myenteric plexus preparations were incubated in antagonist-containing Krebs solution for 10–15 min prior to and during imaging (TTX, 1 μM ; suramin 200 μM ; pyridoxal phosphate-6-axophenyl-2'-4'-disulfonic acid, PPADS, 30 μM ; probenecid, 1 mM).

Analysis was performed with custom-written routines in Igor Pro (Wavemetrics, Portland, OR; Boesmans, Martens, et al., 2013). Regions of interest were drawn, after which the average Ca^{2+} signal intensity was calculated, normalized to the initial GCaMP3 values and reported as F/F_0 . Cells or varicosities were considered as responders when the GCaMP3 signal rose above baseline plus three times the intrinsic noise (SD) during the recording. The Ca^{2+} transient amplitudes were measured as the maximum increase in $[\text{Ca}^{2+}]_i$ above baseline ($\max F_i/F_0$).

2.5 | Immunofluorescence, confocal microscopy, and image analysis

Immunofluorescent labeling was performed as described previously (Boesmans, Rocha, Reis, Holt, & Vanden Berghe, 2014). Intestinal whole-mount preparations containing the myenteric plexus were dissected in carbogenated ice cold Krebs solution and pinned onto Sylgard Petri dishes and fixed in 4% paraformaldehyde in 0.1 M PBS for 45 min at room temperature. The preparations were washed in PBS, permeabilized in 0.5% Triton X-100 in PBS containing 4% donkey serum (blocking solution) for 2–4 hr at room temperature, and incubated in the following primary antibodies diluted in blocking solution for 24 hr at 4°C : mouse anti-HuCD (1:500, Invitrogen cat#: A21271, RRID: AB_221448), rabbit anti-S100 β (1:500, Dako cat#: Z0311, RRID: AB_10013383, Glostrup, Denmark), goat anti-Sox10 (1:300, Santa Cruz Biotechnologies cat#: sc17342, AB_2195374, Dallas, TX), rat anti-GFP (1:1000, Gentaur cat#: 04404–84, RRID: AB_10013361, Brussels, Belgium) and rabbit anti-synapsin (1:500, Abcam cat#: ab64581, RRID: AB_1281135, Cambridge, United Kingdom). After washing, preparations were incubated in secondary (donkey-hosted) antibodies, also diluted in blocking solution for 2–4 hr at room temperature: anti-rabbit Alexa 488 (cat# A21206, RRID: AB_2535792) and 594 (cat# A21207, RRID: AB_141637), anti-mouse Alexa 594 (cat# A21203, RRID: AB_2535789), anti-goat Alexa 488 (cat# A11055, RRID: AB_2534102) and anti-rat Alexa 488 (cat# A21208, RRID: AB_2535794; all 1:1000, Invitrogen). Preparations were imaged using a Zeiss LSM780 multiphoton and Zeiss LSM880 Airyscan confocal microscope (CIC, University of Leuven) assisted with ZEN software (Zeiss). Fluorescent micrograph panels represent maximum projections of confocal z-stacks and are constructed in ImageJ/Fiji (Schindelin et al., 2012; Schneider, Rasband, & Eliceiri, 2012). Three-dimensional (3D)-reconstructions and contact surface quantifications were obtained with



Imaris 9.02 (Bitplane, Oxford Instruments, Zurich, Switzerland) after using Huygens Professional (Scientific Volume Imaging, Hilversum, The Netherlands) for automatic background fluorescence subtraction and image deconvolution. The Surface Contact Area extension available in Imaris XTensions was used to calculate the contact between the synapsin and HuCD surfaces. The primary surface was based on the HuCD channel, and only those synaptic (synapsin channel) contacts that were in close proximity to that surface ($<1 \mu\text{m}$) were selected. Subsequently they were subdivided and color-coded based on their position along the z-axis, relative to the neuron's center position (Z) and its diameter (D): Yellow: $([>Z + D/2] + [<Z - D/2])$ versus red: $([<Z + D/2] + [>Z - D/2])$.

2.6 | Data collection and statistical analysis

All data was collected from a minimum of three mice for each experimental condition. For the majority of data sets, n represents the number of enteric neurons or glia undergoing Ca^{2+} uncaging. Other n values are specified in the text. To compare Ca^{2+} responses and cell counts, two-tailed t tests or one-way ANOVA with Bonferroni's post-hoc tests were performed in Microsoft Excel or GraphPad Prism. Differences were considered to be statistically significant when $p < .05$. All data represented in graph bars is shown as mean $\pm$ SEM.

3 | RESULTS

3.1 | GCaMP3 expression and function in enteric glial cells

With the purpose of monitoring enteric glia activity specifically, and to avoid interpretation of overlapping neuronal signals (Boesmans, Martens, et al., 2013) we aimed to target the genetically encoded Ca^{2+} indicator GCaMP3 to enteric glial cells only. Therefore, we crossed inducible Sox10-CreERT2 mice with the Cre-dependent R26-LsL-GCaMP3 line (Laranjeira et al., 2011; Zariwala et al., 2012). The offspring, hereafter termed Sox10|GCaMP3, between 2 and 4 months of age, were treated with a single intraperitoneal injection of 4-OHT 2 weeks prior to experiments to induce recombination in enteric glial cells. Antibody labeling for the glial markers S100 β or Sox10 confirmed that the majority of enteric glial cells expressed GCaMP3 (Figure 1a,b). GCaMP3 expression was present in all enteric glia subtypes residing in the myenteric plexus (Boesmans, Lasrado, et al., 2015) (Figure 1a). In addition, a number of enteric neurons were found to be GCaMP3-positive, as shown by labeling with antibodies for HuCD. In a small number of ganglia ($\pm 4\%$), GCaMP3 expression was restricted to enteric neurons.

To validate the functionality of GCaMP3 in enteric glial cells, colonic myenteric plexus preparations were isolated from adult Sox10|GCaMP3 mice and processed for live Ca^{2+} imaging (Figure 1c). Local and brief perfusion of agonists known to activate enteric glial cells (Boesmans, Cirillo, et al., 2013; Boesmans, Lasrado, et al., 2015; Broadhead et al., 2012) induced increases in glial GCaMP3 fluorescence (Figure 1d,e). Both ATP (10 μM , 20 s, Supporting Information

Movie S1) and subP (1 μM , 15 s, Supporting Information Movie S2) evoked rapid and robust Ca^{2+} transients throughout the ganglionic enteric glia network. Next, we confirmed that GCaMP3 expressing enteric glia were also responsive when only enteric neurons were stimulated. Purinergic neuron-to-glia signaling has previously been demonstrated by transmitting trains of electrical pulses via interganglionic connectives in both guinea pig and mouse tissue loaded with synthetic Ca^{2+} indicators (Boesmans, Martens, et al., 2013; Gomes et al., 2009; Gulbransen & Sharkey, 2009). Likewise, stimulation of interganglionic connectives via a focal electrode (50 μm diameter platinum wire) induced Ca^{2+} transients in enteric glial cells expressing GCaMP3 (Figure 1f). These experiments demonstrate that Sox10|GCaMP3 mice are an excellent tool to monitor enteric glial cell activity and to study enteric neuron-to-glia communication.

3.2 | Calcium uncaging in single enteric neurons and glia in vitro

To induce intracellular Ca^{2+} increases at the single cell level, we loaded primary ENS cultures derived from Wnt1|GCaMP3 mice, where GCaMP3 is expressed by all enteric neurons and glia (Boesmans, Martens, et al., 2013) with the cell permeable Ca^{2+} uncaging probe o-nitrophenyl EGTA (NP-EGTA-AM, 1 μM) (Figure 2a). This non-fluorescent photolabile Ca^{2+} chelator increases its K_d from 80 nM to more than 1 mM upon ultraviolet light illumination (Ellis-Davies & Kaplan, 1994; Ellis-Davies, Kaplan, & Barsotti, 1996). Because the spinning disc confocal microscope used for our experiments is equipped with a laser beam delivery system (FRAPPA, Andor) that allows the targeting of user-defined regions on the specimen, we were able to photo-stimulate single GCaMP3-positive cells grown in culture (Figure 2b,e, Supporting Information Movie S3). The amount of uncaged Ca^{2+} , as measured by the relative increase in GCaMP3 fluorescence, could be controlled by adjusting the 405 nm laser power (Figure 2g) and by adapting the area and duration of exposure. Upon NP-EGTA photolysis enteric neuronal cell bodies showed an immediate rise in cytosolic Ca^{2+} (Figure 2f). Similarly, Ca^{2+} uncaging in single enteric glial cells induced transient increases in GCaMP3 fluorescence (Figure 2c,g). Thus, photo-stimulation by means of Ca^{2+} uncaging can be used to evoke increases in intracellular Ca^{2+} concentration in individual ENS cells in vitro.

3.3 | Photo-stimulation of individual enteric neurons and glia in myenteric plexus preparations

We next tested our novel Ca^{2+} uncaging approach in freshly dissected colonic myenteric plexus preparations isolated from Sox10|GCaMP3 mice and loaded with NP-EGTA-AM (2 μM) (Figure 3a). First, we took advantage of the sparse off-target neuronal expression pattern to test NP-EGTA photolysis on single enteric neuronal cell bodies. Similar to our in vitro findings, ganglionic enteric neurons displayed an immediate rise in cytosolic Ca^{2+} upon Ca^{2+} uncaging (Figure 3b,d). Ca^{2+} uncaging in individual enteric glial cells also induced transient increases in GCaMP3 fluorescence (Figure 3c,d). To confirm the specificity of NP-EGTA in our experimental approach a number of cells (neurons and glia) were photo-stimulated twice, separated by a 5 min interval. Here, Ca^{2+} transients could only be detected at the first round of

stimulation suggesting that the vast majority of caged Ca^{2+} was released during the initial photolysis (Figure 3e). To ensure cells were still viable after successful photo-stimulation, myenteric plexus preparations were subsequently challenged by 75 mM K^+ depolarization. All cells tested ($n = 11$) displayed significant increases in GCaMP3 fluorescence upon superfusion with 75 mM K^+ Krebs solution (data

not shown). Finally, performing the photo-stimulation procedure in GCaMP3-positive cells that were not loaded with NP-EGTA caused no Ca^{2+} transients. Although in some cases a marginal drop in GCaMP3 intensity could be observed (<5%), this indicates that exposure to the 405 nm laser alone had no considerable effect on the GCaMP3 molecule or on cellular Ca^{2+} homeostasis.

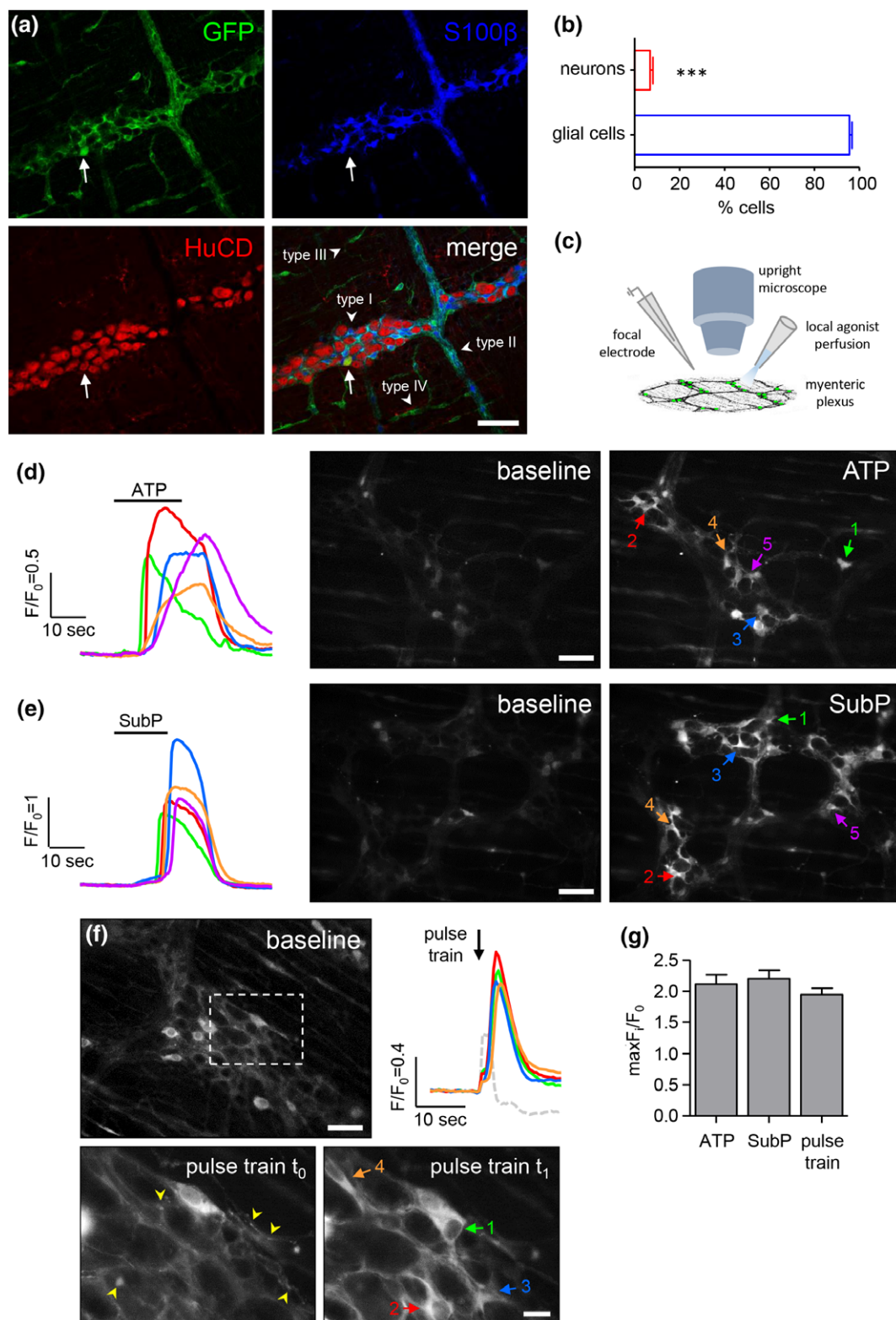


FIGURE 1 Legend on next page.

3.4 | Calcium increases induced in enteric neurons cause calcium responses in neighboring glia

To investigate enteric neuron-to-glia communication at the level of single cells, we photo-stimulated cell bodies of enteric neurons negative for GCaMP3 and monitored Ca^{2+} levels in GCaMP3+ enteric glial cells within the same ganglion of the targeted neuronal cell body in freshly dissected myenteric plexus preparations. Neuronal Ca^{2+} uncaging induced robust Ca^{2+} transients in neighboring enteric glial cells (Figure 4a, Supplementary movie S4). Because of our strategy to direct Ca^{2+} indicator expression to enteric glial cells specifically using Sox10|GCaMP3 mice, we were able to monitor the Ca^{2+} responses of individual cells and their processes in more detail. This helped to allocate the restricted Ca^{2+} responses to individual glial cells, which enabled us to calculate the total number of glial cells responding to neuronal Ca^{2+} uncaging. On average 1.9 ± 0.2 glial cells responded with a GCaMP3 transient (max F_i/F_0 : 2.3 ± 0.2) after uncaging of a single enteric neuron. These cells were always directly surrounding and adjacent to the stimulated neuronal cell body (Figure 4a). Since most neurotransmitter release machinery of enteric neurons locates to varicosities in neuronal processes (Vanden Berghe & Klingauf, 2007) this suggests that nerve conduction is not necessary for this mode of neuro-to-glia crosstalk.

3.5 | Enteric neuro-glia units and their relation with neurotransmitter release sites

To investigate the spatial relationship between enteric neurons and glia within myenteric ganglia we combined viral vector mediated GFP transduction of enteric neurons with immunolabeling of enteric glial cells. Adult C57BL/6J mice were transduced via tail vein injection with AAV2/9-CMV-eGFP in order to sparsely label enteric neurons. After immunostaining for S100 β , confocal microscopy and image deconvolution, the number of enteric glia associated with a single

GFP+ neuronal cell body was determined. We found that within myenteric ganglia, on average 2.3 enteric glial cells associate with an enteric neuron cell body (Figure 4b,c). Considering the inclusion of a number of non-responders in the live imaging experiments (where neuronal Ca^{2+} uncaging did not induce Ca^{2+} transients in glia) this matches well with the, on average, 1.9 enteric glia responding to neuronal Ca^{2+} uncaging and suggests that the structural relationship between enteric neurons and glia underpins their spatially restricted crosstalk.

3.6 | Enteric neurons use pannexins to signal to glial neighbors via purinergic mechanisms

Purinergic signaling has previously been shown to be the main mechanism by which enteric neurons signal to enteric glia (Gomes et al., 2009; Gulbransen & Sharkey, 2009). This is the case for ENS activity involving neuronal firing but also in TTX insensitive neuron-to-glia signaling. Here, blocking P2 receptors with Suramin (200 μM) reduced glial Ca^{2+} transients (Figure 5a,b). Also pyridoxal phosphate-6-axophenyl-2'-4'-disulfonic acid (PPADS, 30 μM), another antagonist of P2 receptors, inhibited both the number of enteric glial cells responding to neuronal Ca^{2+} uncaging and the amplitude of glial Ca^{2+} rises (Figure 5a,b). This confirms earlier findings and indicates that the stimulation of neurons via Ca^{2+} uncaging also activates enteric glial cells via purinergic receptors. Although a lower number of glia responded to neuronal Ca^{2+} uncaging when tissues were incubated with TTX prior to stimulation (1 μM), this did not affect the amplitude of glial Ca^{2+} transients (Figure 5a,b). While this does not exclude the possibility that classic nerve conduction contributes to this mode of enteric neuron-to-glia communication, the response of only closely apposing enteric glial cells suggests that neuronal processes are not activated to release purines after Ca^{2+} uncaging of their cell bodies. It also suggests that purine release is not occurring in a vesicular fashion. Indeed,

FIGURE 1 Calcium imaging using Sox10::CreER^{T2};R26R-GCaMP3 mice. (a) Confocal maximum projection of a colonic myenteric plexus preparation obtained from a Sox10::CreER^{T2};R26R-GCaMP3 mouse 2 weeks after administration of 4-hydroxy tamoxifen. Antibody labeling for GFP (GCaMP3+ cells, green), S100 β (enteric glia, blue) and HuCD (enteric neurons, red) shows that the majority of enteric glial cells within myenteric ganglia express GCaMP3. Note that also a few enteric neurons express GCaMP3 (arrow) and that GCaMP3 is expressed by all enteric glia subtypes (arrowheads in merged panel). Scale bar: 50 μm . (b) Quantification of the proportion of GCaMP3 expressing enteric neurons (HuCD+/GCaMP3+, $n = 101$) and enteric glial cells (S100+/GCaMP3+, $n = 1,351$) (***) ($p < .0001$ two-tailed t test). (c) Experimental setup for in situ Ca^{2+} imaging of enteric glial cells using an upright wide-field microscope. (d) Adenosine triphosphate (ATP) activation of enteric glial cells. Left: Ca^{2+} transients of individual enteric glial cells (depicted by color-coded numbers shown in the right panel) induced by local perfusion with ATP. Middle: baseline GCaMP3 fluorescence in a myenteric ganglion before stimulation. Right: peak GCaMP3 fluorescence of the same ganglion upon ATP stimulation. Scale bar: 50 μm . (e) Substance P (subP) activation of enteric glial cells. Left: Ca^{2+} transients of individual enteric glial cells (depicted by color-coded numbers shown in the right panel) induced by local perfusion with subP. Middle: baseline GCaMP3 fluorescence in a myenteric ganglion before stimulation. Right: peak GCaMP3 fluorescence of the same ganglion upon subP stimulation. Scale bar: 50 μm . (f) Electrical stimulation of enteric neurons evokes Ca^{2+} transients in enteric glial cells, as shown by live imaging of Sox10::CreER^{T2};R26R-GCaMP3 myenteric plexus in situ. The gray scale images display a colonic myenteric plexus preparation in which enteric neurons were stimulated (upper panel shows baseline before stimulation) by an electrical pulse train (2 s, 20 Hz) via a focal electrode positioned on an interganglionic connective (linked to the myenteric ganglion but adjacent to the field of view). A magnification of the inset is shown in the lower panels with left: GCaMP3 fluorescence at the start of the pulse train (t_0) showing activation of neuronal varicosities (yellow arrowheads); and right: GCaMP3 fluorescence 1 s after termination of the electrical stimulation (t_1) showing activation of enteric glial cells. The Ca^{2+} transients of the individual enteric glial cells depicted by the color-coded numbers (lower panels) show that enteric glia respond secondarily to neuronal stimulation. The gray-colored GCaMP3 tracing represents the average Ca^{2+} transient of the neuronal varicosities depicted by the yellow arrowheads—note that the neuronal Ca^{2+} tracing, which is calculated from relatively small regions of interest (selected varicosities), deflects below baseline as a result of tissue contraction. Scale bar upper panel: 50 μm , lower panels: 10 μm . (g) Comparison of the average maximal GCaMP3 fluorescence amplitudes of enteric glial Ca^{2+} responses upon ATP and SubP perfusion or electrical stimulation of enteric neurons [Color figure can be viewed at wileyonlinelibrary.com]

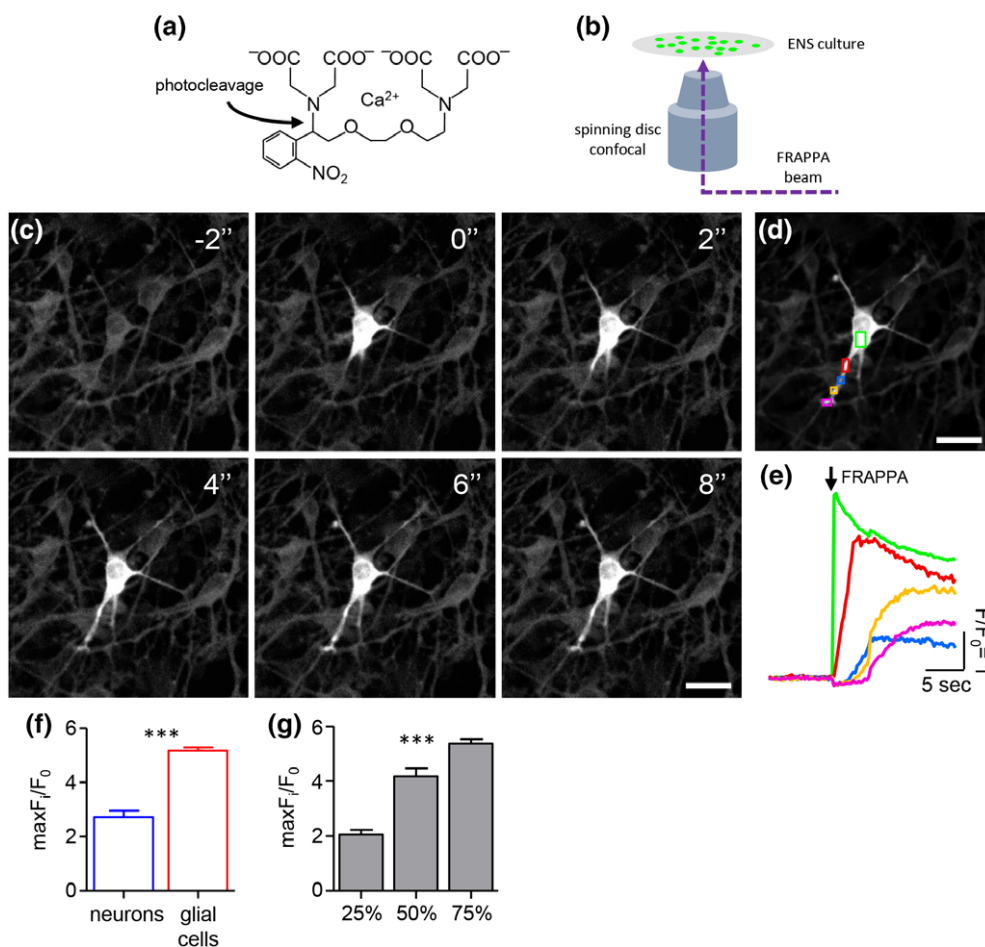


FIGURE 2 Calcium uncaging in enteric neurons and glia in vitro. (a) Chemical structure of o-nitrophenyl EGTA (NP-EGTA) and its site for photocleavage to release caged Ca²⁺. (b) Experimental setup for simultaneous Ca²⁺ uncaging and imaging of cultured enteric neurons and glia using an inverted spinning disc confocal microscope equipped with a galvano-driven point-scanner (FRAPPA, Andor) that allows delivering the laser beam to user-defined regions on the specimen. (c) Time series images (every 2 s) of the GCaMP3 response induced by Ca²⁺ uncaging (FRAPPA was executed in a square region confined to the soma of the enteric glial cell in the center of the panel) of a single enteric glial cell. (d) Maximum projection of time series shown in c and color-coded regions-of-interest (ROIs) used to calculate the GCaMP3 tracings shown in e. (e) Ca²⁺ transients within the ROIs depicted in d, showing that [Ca²⁺]_i in the cell soma rises immediately (green), while it increases gradually toward the terminal of the glial cell process. (f) Average maximal GCaMP3 fluorescence amplitudes of neuronal and glial Ca²⁺ responses upon Ca²⁺ uncaging ($n = 18, 19$; *** $p < .0001$ two-tailed t test). (g) Average maximal GCaMP3 fluorescence amplitudes induced by Ca²⁺ uncaging at increasing laser powers (% of 100 mW 405 nm laser; $n = 18, 20, 14$; *** $p < .0001$ one-way ANOVA). Scale bars: 20 μm [Color figure can be viewed at wileyonlinelibrary.com]

incubation with probenecid (1 mM), a blocker of pannexin channels (Gulbransen et al., 2012; Silverman, Locovei, & Dahl, 2008), significantly inhibited Ca²⁺ transients in enteric glial cells (Figure 5a,b).

To further investigate the possible contribution of purinergic release from neuronal varicosities onto neighboring enteric glial cells, we examined how the apposition between neurotransmitter release sites and neuronal cell bodies within myenteric ganglia of the mouse colon is spatially organized. Therefore, we performed immunofluorescence stainings for synapsin and HuCD to label neuronal varicosities and neuronal cell bodies, respectively. 3D reconstruction and surface rendering revealed that only $14.8\% \pm 3.5\%$ of the total contact between synapsin+ and HuCD+ surface areas surrounds neuronal cell bodies perpendicularly to the plane of the myenteric plexus (Figure 5c,d). Thus, the majority of neuronal varicosities encircle neuronal cell bodies parallel to the myenteric plexus plane and therefore, are not photo-stimulated during our Ca²⁺ uncaging experiments. Together, these data

indicate that enteric neurons communicate to adjacent enteric glial cells in a purinergic fashion via pannexin channels expressed on their cell bodies.

4 | DISCUSSION

Communication from enteric neurons to glia is paramount for ENS signaling and essential for normal gut function. In the current study, we explored the structural and functional relationship between individual myenteric neurons and surrounding type-I enteric glial cells. Using a combination of single-cell photo-stimulation, selective genetically encoded Ca²⁺ imaging and sparse viral vector labeling, we demonstrate that enteric neurons employ pannexins to signal to enteric glial cells in a paracrine purinergic fashion. We are the first to show that pannexin mediated purinergic signaling in the ENS occurs within spatially restricted neuro-glia units.

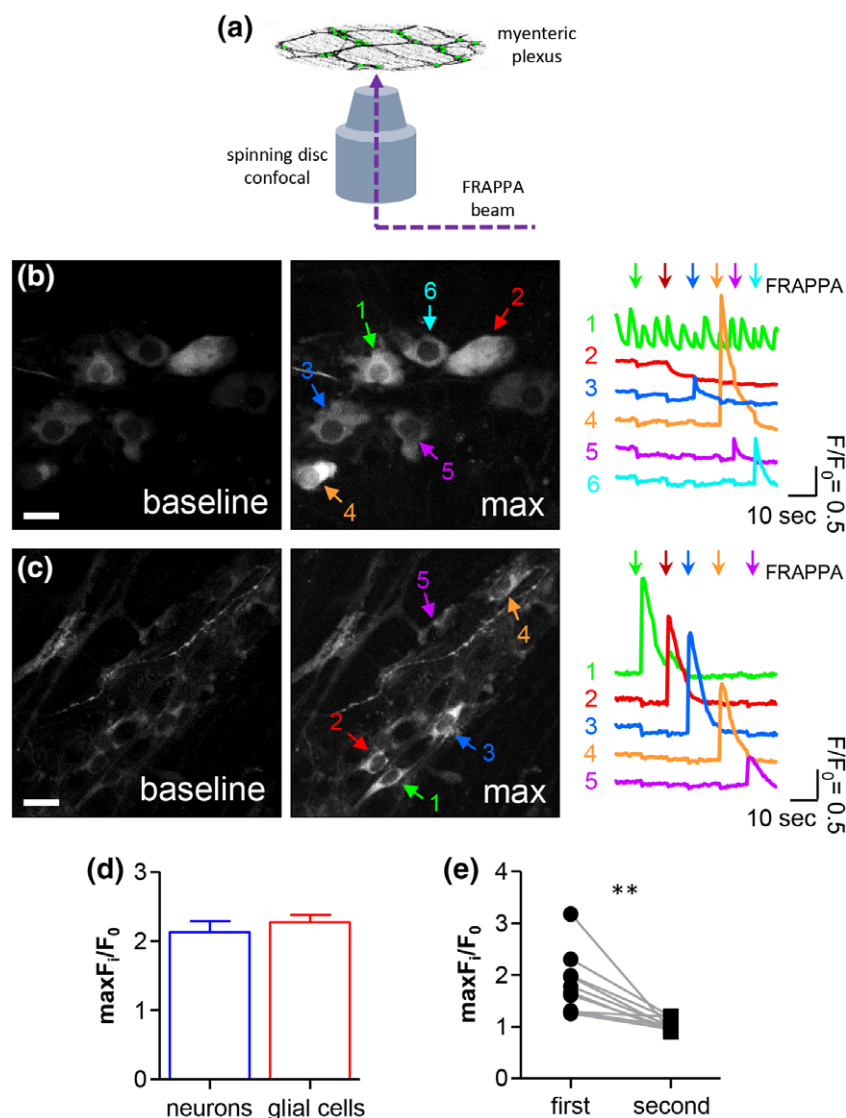


FIGURE 3 Calcium uncaging in enteric neurons and glia in situ. (a) Experimental setup for simultaneous Ca²⁺ uncaging and imaging of enteric neurons and glia in myenteric plexus preparations of mouse colon. GCaMP3 is visualized on an inverted spinning disc confocal microscope equipped with a galvano-driven point-scanner (FRAPPA, Andor) that allows delivering the laser beam to user-defined regions on the specimen. (b) Ca²⁺ uncaging of GCaMP3+ enteric neurons in situ. Left: baseline GCaMP3 fluorescence in a myenteric ganglion containing GCaMP3+ enteric neurons before stimulation. Middle: Maximum projection of the same ganglion, which was recorded (60 s in total) with a series of consecutive photo-stimulations directed at individual GCaMP3+ enteric neurons. Right: GCaMP3 tracings of individual enteric neurons (depicted by color-coded numbers shown in the middle panel) showing Ca²⁺ transients induced by Ca²⁺ uncaging (cells 3–6). Note that cell 1 (spontaneously active) and cell 2 are not affected by the photo-stimulation. Color-coded arrows depict the time of Ca²⁺ uncaging of the corresponding cells. (c) Ca²⁺ uncaging of GCaMP3+ enteric glial cells in situ. Left: baseline GCaMP3 fluorescence in a myenteric ganglion before stimulation. Middle: Maximum projection of the same ganglion, which was recorded (60 s in total) with a series of consecutive photo-stimulations directed at individual GCaMP3+ enteric glial cells. Right: GCaMP3 tracings of individual enteric glia (depicted by color-coded numbers shown in the middle panel) showing Ca²⁺ transients induced by Ca²⁺ uncaging. (d) Average maximal GCaMP3 fluorescence amplitudes of neuronal and glial Ca²⁺ responses upon Ca²⁺ uncaging in situ ($n = 29, 19$; $p = .52$ two-tailed t test). (e) Average maximal GCaMP3 fluorescence amplitudes after a first and second round of Ca²⁺ uncaging in situ ($n = 10$; $**p = .002$ paired t test). Scale bars: 20 μm [Color figure can be viewed at wileyonlinelibrary.com]

For monitoring enteric glial cell activity, we induced expression of the genetically encoded Ca²⁺ indicator GCaMP3 in enteric glial cells specifically by using a Sox10::CreERT2 driver line. Although faster and more sensitive genetically encoded indicators are currently available (Boesmans, Hao, & Vanden Berghe, 2018), GCaMP3 targeted to enteric glia via inducible Sox10 regulatory elements (Laranjeira et al., 2011) performed extremely well in combination with the chosen stimuli and microscopic imaging platform. Purinergic and neurokinin receptor stimulation evoked robust Ca²⁺ transients in enteric glial cells in situ within

seconds after starting local agonist administration. In addition, electrical stimulation of enteric neurons resulted in prompt GCaMP3 rises in enteric glial cells within myenteric ganglia. These results are in line with what we observed using Wnt1|GCaMP3 mice (Fung et al., 2017) and with the Ca²⁺ indicator dye Fluo-4 (Boesmans, Martens, et al., 2013) and are comparable to other studies using GCaMP3 (Hennig et al., 2015) and GCaMP5G-tdT (McClain & Gulbransen, 2017). Notably, the differences with other studies in terms of expression pattern and response kinetics could be due to the

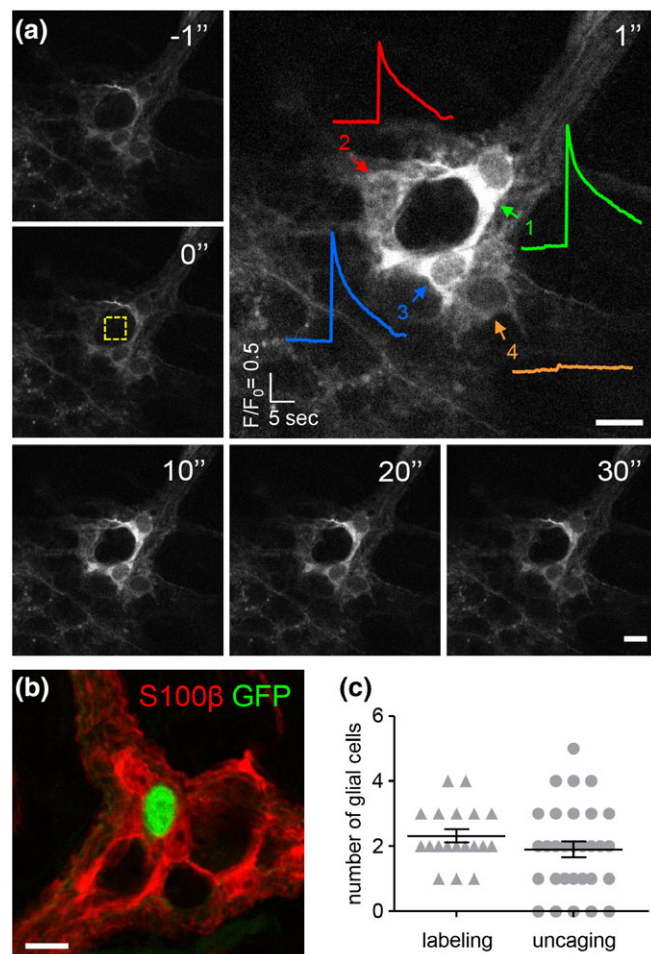


FIGURE 4 Neuronal calcium uncaging induces calcium transients in neighboring enteric glial cells. (a) Time series images showing a typical example of a Ca^{2+} uncaging event in a GCaMP3⁻ enteric neuron and subsequent activation of surrounding GCaMP3⁺ enteric glial cells. The Ca^{2+} transient profiles of four neighboring enteric glial cells are shown (cells 1–3 respond, enteric glial cell 4 does not). The yellow square indicates the region of interest that was photo-stimulated (FRAPPA, Andor). Scale bars: 20 μm . (b) Confocal maximum projection of an immunofluorescence labeling of S100 β ⁺ enteric glial cells (red) that surround a GFP transduced enteric neuron (green). Scale bar: 10 μm . (c) Quantification of the number of enteric glial cells that surround a single enteric neuronal cell body observed after immunofluorescence labeling as compared with the number of enteric glial cells responding after neuronal Ca^{2+} uncaging ($n = 19, 30$; $p = .2402$ two-tailed t test) [Color figure can be viewed at wileyonlinelibrary.com]

distinct genetic systems, different stimulus supply or alternative imaging methodology.

In addition to the novel combination of transgenic mouse lines to monitor enteric glial cell activity, we introduced photolysis of caged Ca^{2+} as a unique approach to elevate the cytosolic Ca^{2+} concentration in the cell body of single enteric neurons or glia. To validate the use of the photolabile Ca^{2+} -uncaging compound NP-EGTA we first performed a set of experiments on NP-EGTA-AM loaded primary cultured enteric neurons and glia expressing GCaMP3. Although Ca^{2+} is ideally uncaged from NP-EGTA using ultraviolet light flash photolysis, we found that the 405 nm laser light used in our experiments also reproducibly triggered Ca^{2+} transients when targeted to the cell body of cultured ENS

cells. This is in agreement with other studies that have used near-UV light for NP-EGTA Ca^{2+} uncaging (Doerner, Delling, & Clapham, 2015; Thyssen et al., 2010). Based on our in situ experiments we conclude that photolysis of NP-EGTA in enteric neurons is ideally suited to induce a sudden cytosolic Ca^{2+} concentration increase, which is a trigger for the release of mediators through membrane channels such as pannexins.

To selectively evoke neuronal Ca^{2+} transients, others have successfully used application of 2'/(3')-O-(4-benzoylbenzoyl) ATP (BzATP), an agonist of P2X7 receptors exclusively expressed by enteric neurons (Brown et al., 2016; Gulbransen et al., 2012). This approach results in the stimulation of the overall population of enteric neurons evoking purine release through Panx1, which secondarily activates P2Y1 receptors on enteric glial cells. However, because of the bulk administration of the neuronal agonist and the simultaneous activation of multiple enteric neurons, the spatial extend of this neuron-to-glia communication is difficult to examine. Here, owing to our single-cell stimulation methodology, we show that this neuron-to-glia communication is restricted between neuronal cell bodies and surrounding enteric glial cells. Moreover, this tight functional relationship is mirrored by the fact that about 2–3 enteric glial cells are associated with a single neuronal cell body, a ratio that we observed after viral vector and immunofluorescence labeling. The activation of only 2–3 enteric glial cells also implies that further spreading via connexin-43 hemichannels (McClain et al., 2014) to involve other enteric glial cells does not occur after Ca^{2+} uncaging of a single enteric neuron. This suggests that enteric glial cells are not spreading activity by default but that under certain conditions (e.g., single neuron activity) glial activation remains spatially restricted. Whether these neuro-glia ensembles form computational units within enteric ganglia, when they are established and become functional during ENS development (Hao et al., 2011; Hao et al., 2013), and how these relate to the clonal composition of the ENS (Lasrado et al., 2017), are intriguing questions that are subject to our future research.

Our findings support previous reports showing that enteric neuron-to-glia communication can occur independent of nerve conduction (Brown et al., 2016; Fung et al., 2017; Gulbransen et al., 2012). It is possible that the glial responses that remain after incubation with TTX are due to activation of TTX resistant Na_v channels (Padilla et al., 2007; Rugiero et al., 2003), which in turn, could make specific neurons signal to enteric glial cells in a synaptic fashion. However, this seems unlikely as only glial cells in close proximity of the stimulated enteric neuron display Ca^{2+} transients upon Ca^{2+} uncaging. Furthermore, the high-resolution analysis of the spatial distribution of the synaptic apposition between synapsin punctae and neuronal cell bodies shows that the vast majority of varicose release sites within myenteric ganglia is organized as a halo encircling the neuronal cell bodies parallel with the plexus plane running along the longitudinal axis of the gut. Despite this preferential ring like organization and our observation that only a very small fraction of varicosities is located above and below the neuron of interest, we cannot fully exclude the possibility that cell body-directed Ca^{2+} uncaging stimulates transmitter release from a presynaptic terminal located above or below the targeted neurons and adds to the activation of neighboring enteric glial cells. In future experiments, genetically encoded photolabile Ca^{2+}

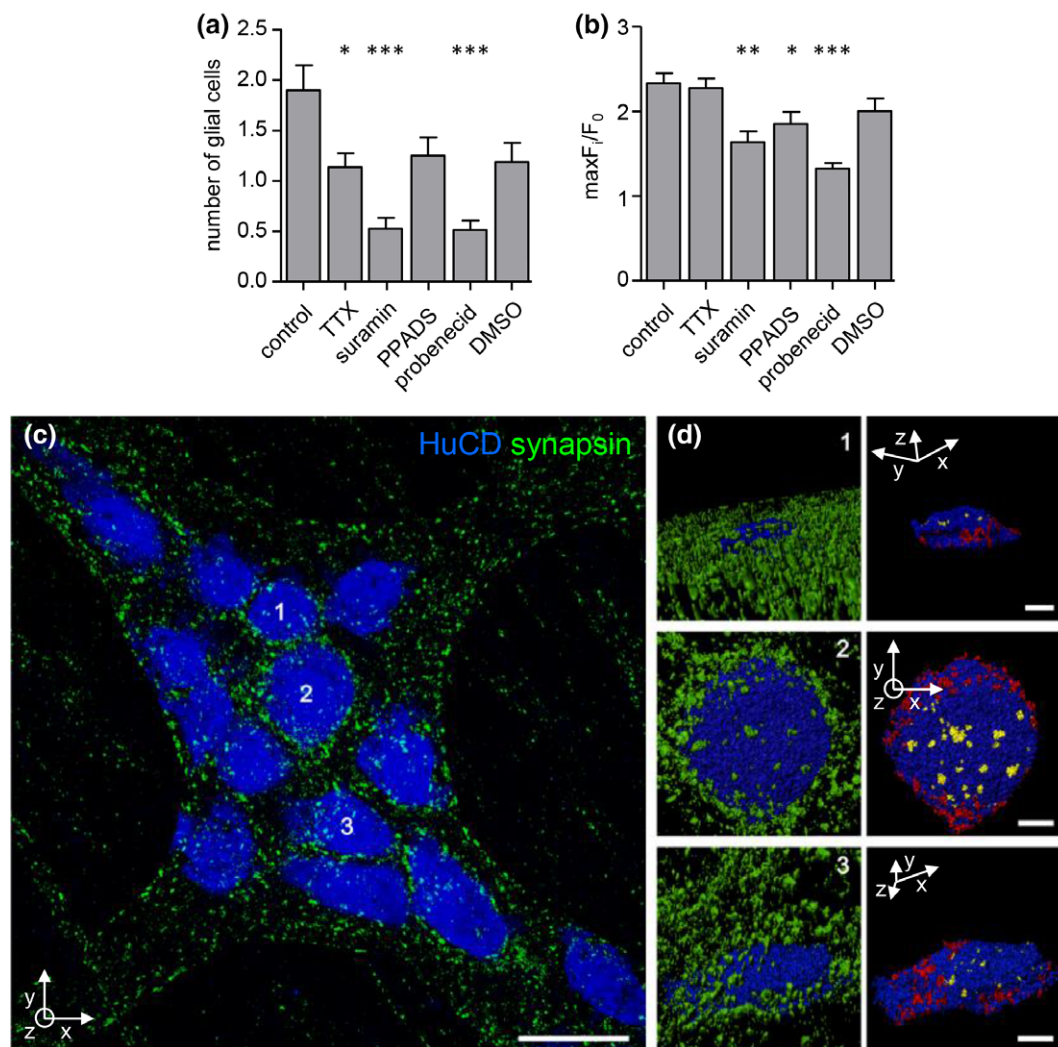


FIGURE 5 Neuronal calcium uncaging evokes enteric neurons to signal to enteric glial cells by releasing purines via pannexin channels.

(a) Quantification of the number of glial cells responding after Ca^{2+} uncaging of enteric neurons in the presence of drugs (or vehicle) as compared with control ($n = 30$; $p < .0001$ one-way ANOVA). Tetrodotoxin (TTX, $n = 22$), suramin ($n = 40$), and probenecid ($n = 43$) significantly reduced the number of responding glial cells, whilst pyridoxal phosphate-6-axophenyl-2'-4'-disulfonic acid (PPADS, $n = 34$) and dimethylsulfoxide (DMSO, $n = 16$) did not. Bonferroni's post hoc test: * $p < .05$, *** $p < .001$. (b) Average Ca^{2+} transient amplitudes in enteric glial cells induced by Ca^{2+} uncaging of enteric neurons in the presence of drugs (or vehicle) as compared with control ($p < .0001$ one-way ANOVA). Suramin, PPADS and probenecid significantly reduced the maximal glial Ca^{2+} rises, whilst TTX and DMSO did not. Bonferroni's post-hoc test: * $p < .05$, ** $p < .01$, *** $p < .001$. (c) Example of a confocal maximum projection of a colonic myenteric ganglion after immunofluorescence labeling for HuCD (blue) and synapsin (green) used for the quantification and localization of the surface contact area between neuronal cell bodies and neurotransmitter release sites. Scale bar: 25 μm . (d) 3D views (left panels) of the cells 1–3 (depicted in c) shown at different angles of orientation (white xyz axes). The synapsin+/HuCD+ contact surfaces were determined and color-coded (right panels) along their position in the z-axis (yellow: located on top or bottom (= perpendicular, relative to the imaging and plexus plane); red: adjacent to neuronal cell bodies (= parallel, relative to the imaging and plexus plane). Scale bars: 5 μm [Color figure can be viewed at wileyonlinelibrary.com]

chelators and other optogenetic constructs should enable us to rule out the contribution of such signaling mechanisms (Boesmans et al., 2018; Boesmans, Hao, & Vanden Berghe, 2015).

A largely unresolved question is how enteric glial cells interpret and process the signals they receive from enteric neurons to eventually execute their presumptive roles in the ENS and in gastrointestinal function. Enteric glial cells displaying transient increases in intracellular Ca^{2+} concentration are considered "active" and although the exact transmitters remain unknown, these Ca^{2+} transients probably trigger different modes of gliotransmission such as Ca^{2+} -dependent exocytosis or mediator release via connexin 43 hemichannels (Grubisic & Parpura, 2017). Although enteric glia signaling and communication between enteric glial

cells were not directly examined in the current study, we anticipate that different modes of gliotransmission are initiated when enteric glial cells are activated through neuronal pannexin channels as opposed to their employment during synaptic communication between enteric neurons.

In conclusion, in this study we have been able to stimulate single enteric neurons by means of Ca^{2+} uncaging while monitoring the activity of enteric glial cells in situ. We demonstrate that enteric neurons communicate with adjacent enteric glial cells by releasing purines through pannexin channels. Our results uncover a structural and functional basis for neuro-glia units as a novel cellular assembly in the ENS.

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CONFLICTS OF INTEREST

The authors disclose no conflicts.

AUTHOR CONTRIBUTIONS

W.B. performed experiments and wrote the manuscript; W.B. and P.V.B. designed the study; P.V.B. led the project and provided intellectual input; C.F. performed experiments; M.M.H., C.V.d.H., J.T. and V.P. provided intellectual input; Z.L. performed 3D and contact surface analysis; C.V.d.H. provided viral vector; V.P. provided Sox10::CreER^{T2} (SER26) mice.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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