Supplementary Information

Title: **Activation of neuronal P2X7 receptor-Pannexin-1 mediates death of enteric neurons during colitis**

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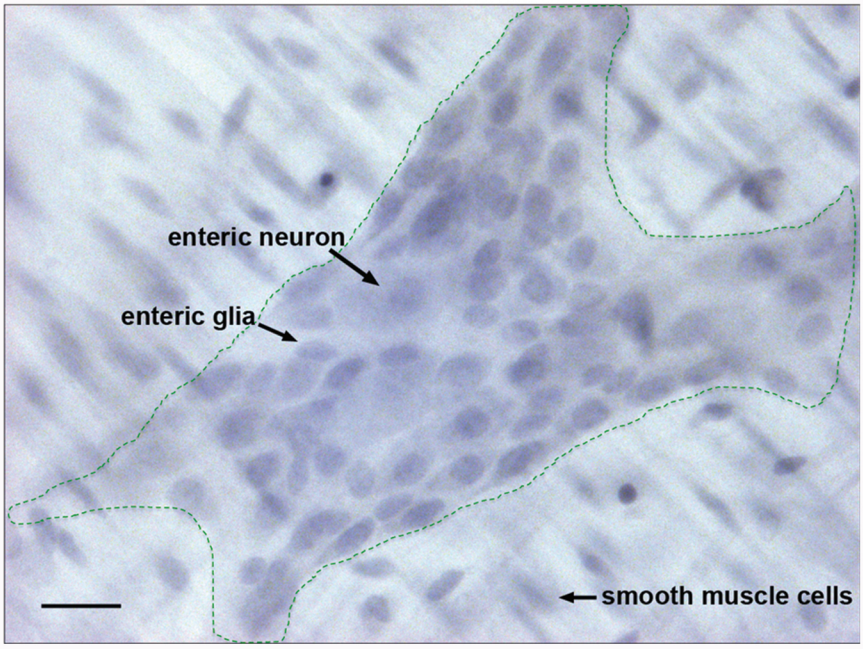
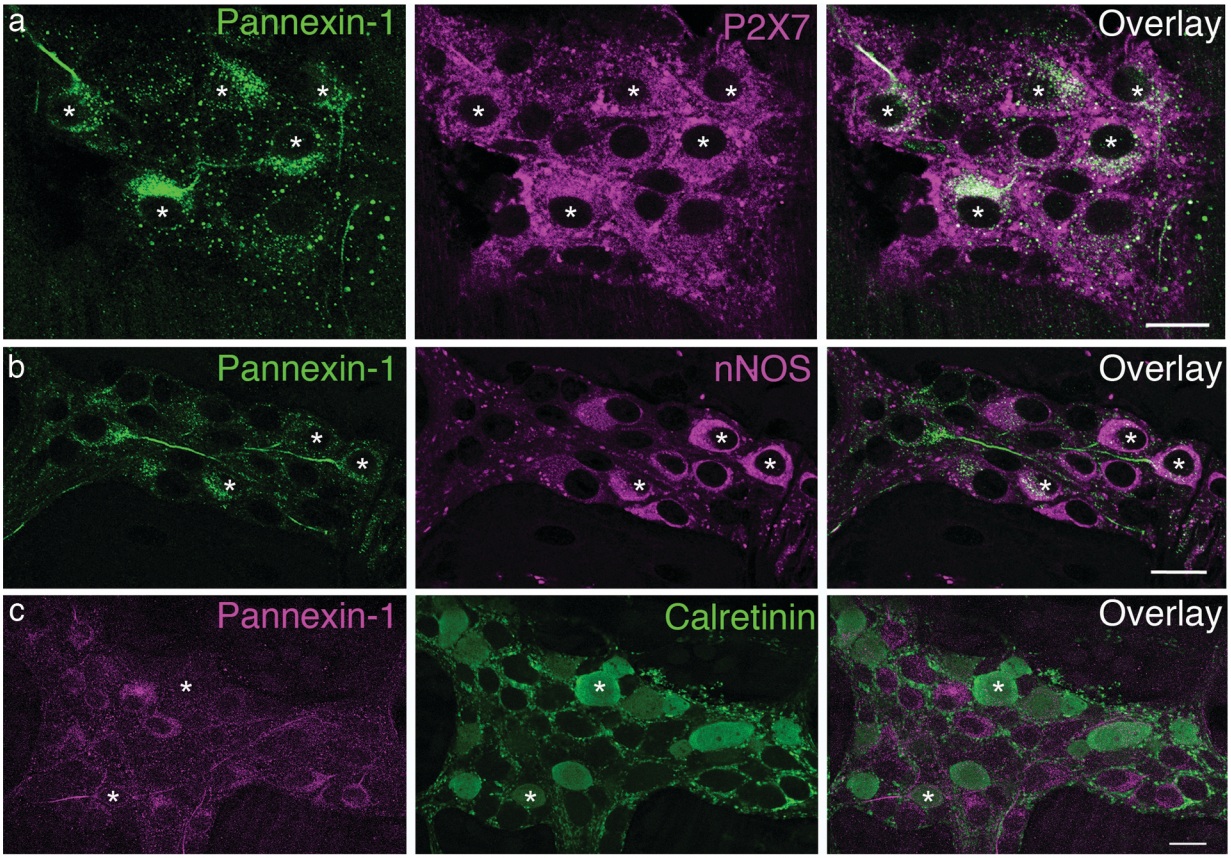
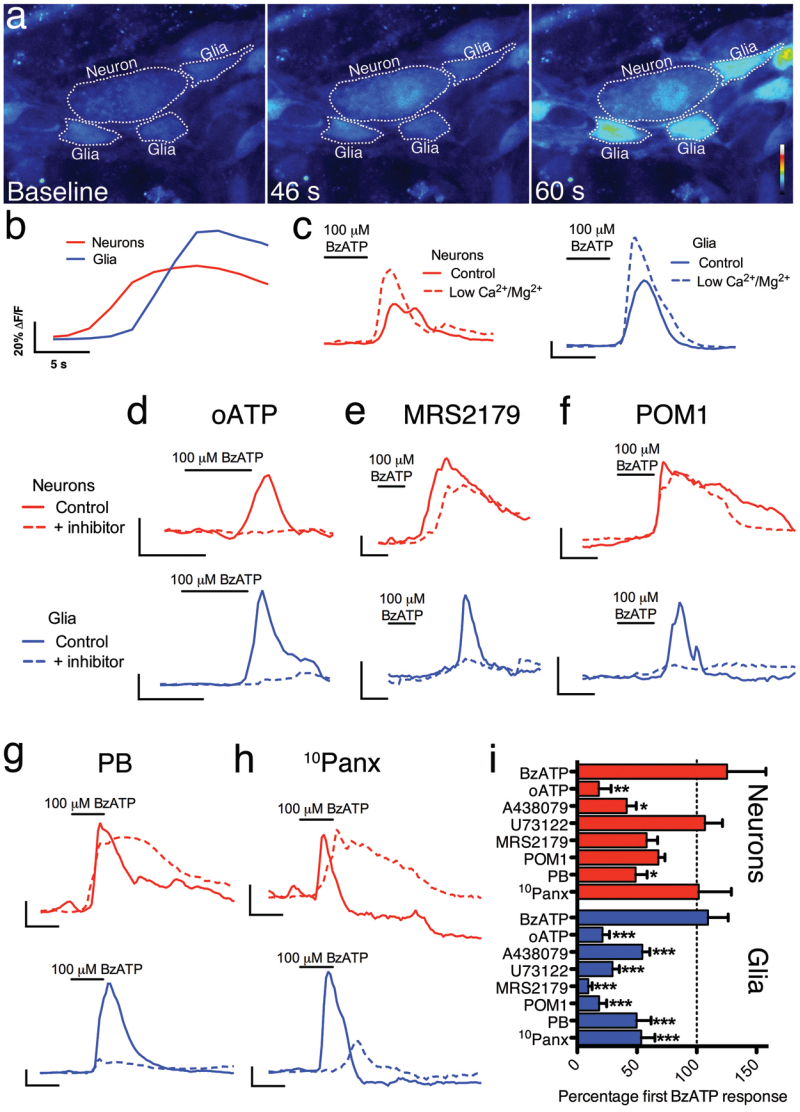


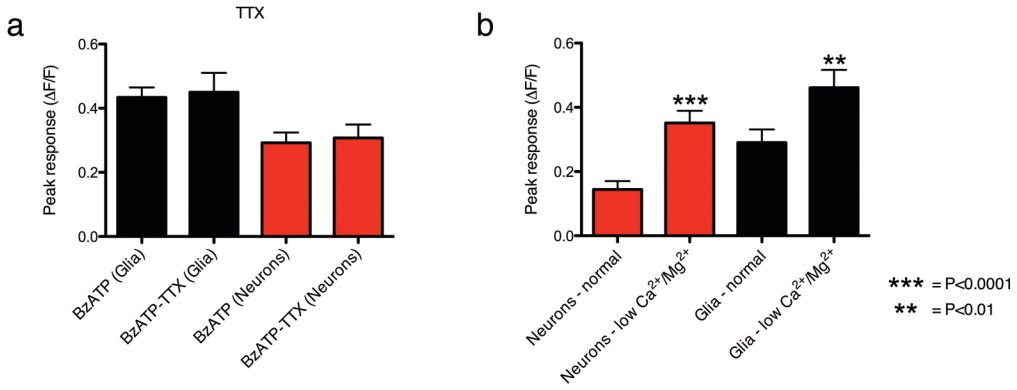
Supplementary Figure 1. Representative whole-mount preparation used for an in vitro cell death assay stained with hematoxylin and eosin (H&E). Myenteric ganglia (outlined by green dashed line) display normal morphology and are filled with neurons and glia (labeled). Longitudinal muscle cells (labeled) lie beneath the myenteric plexus. Immune cells are not found adjacent to, or infiltrating, myenteric ganglia in these preparations. Scale bar = 20 µm.



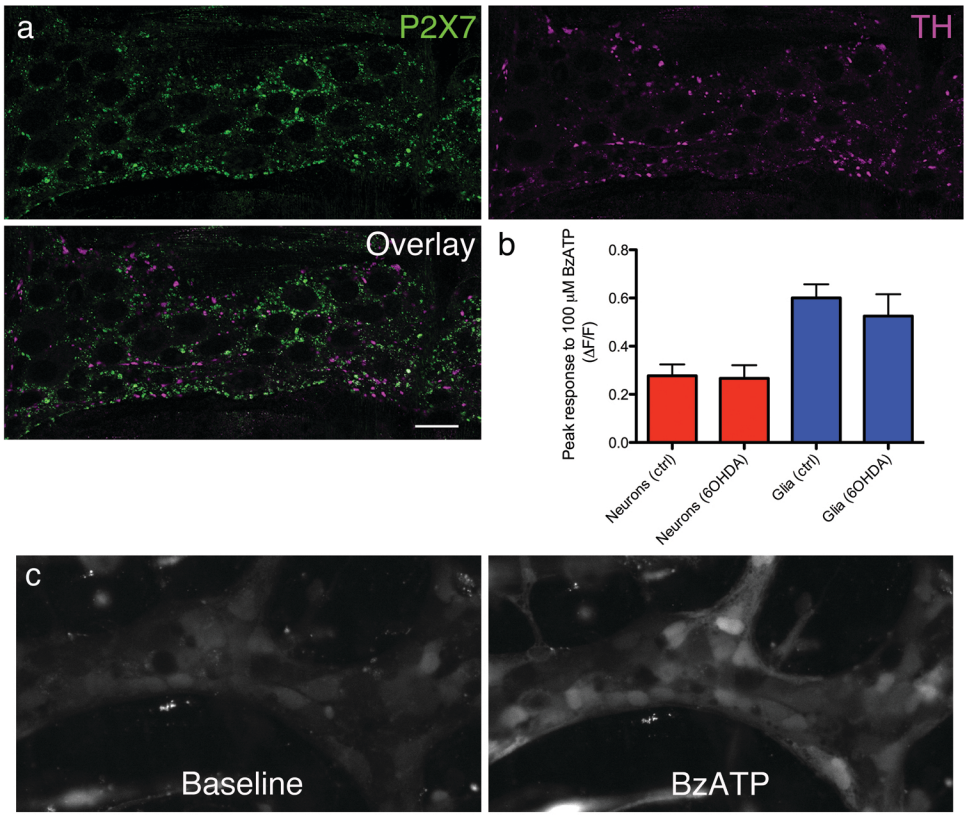
Supplementary Figure 2. Dogiel type I and II myenteric neurons co–express pannexin–1 and P2X7. (**a**) Dual immunohistochemical labeling of pannexin–1 (green) and P2X7 (magenta) in the mouse colon myenteric plexus. Asterices highlight five pannexin–1–P2X7 dual-labeled neurons. Scale bars = 20 µm. (**b**) Pannexin–1 (green) and nNOS (magenta) immunohistochemistry in the mouse colon myenteric plexus. Dogiel type I neurons identified by immunoreactivity to nNOS (magenta) are highly immunoreactive for pannexin-1 (green). Asterices denote three pannexin–1–nNOS dual labeled neurons. (**c**) Dual–labeling for pannexin–1 (magenta) and calretinin (green) in the mouse colon myenteric plexus. Dogiel type II neurons (identified by immunoreactivity to calretinin, green) also express pannexin-1 (magenta) but at a lower intensity than Dogiel type I neurons. Asterices identify two pannexin–1–calretinin dual–labeled neurons.



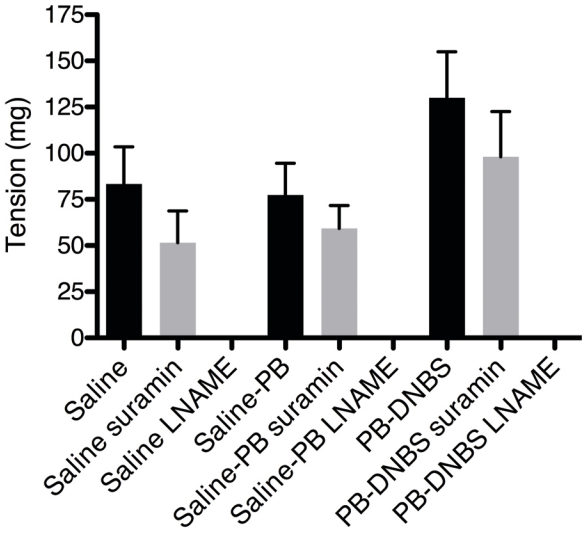
Supplementary Figure 3. Enteric neurons release ATP through pannexin-1 following activation of P2X7Rs. (**a**) Representative images (pseudocolored) from a Ca^{2+} imaging experiment where a whole-mount preparation of the myenteric plexus was challenged with the P2X7R agonist BzATP (100 μM, 30 s). (**b**) Average glial (blue) and neuronal (red) responses within a single myenteric ganglia upon application of BzATP. Time from BzATP application to 20% response in neurons precedes glia on average by 6.75 s, *n* = 16 ganglia). (**c**) Neuronal and glial responses to BzATP in normal buffer (solid lines) and in buffer containing low Ca^{2+} and Mg^{2+} (dashed lines). (**d** – **h)** Traces depict average responses to BzATP in neurons (red) and glia (blue) before (solid lines) and after (dashed lines) addition of various inhibitors. Scale bars = 10% ΔF/F (y axis) and 30 s (x axis). (**i**) Summary data of mean (±s.e.m.) peak Ca^{2+} responses (normalized to initial BzATP response magnitude within the same ganglion) in neurons (red) or glia (blue) in response to sham block and BzATP alone or in combination with various inhibitors. [oATP, 100 μM, *n* = 8 ganglia; A438079, 30 μM, *n* = 10 ganglia; MRS2179, 10 μM, *n* = 12 ganglia; POM1, 50 μM, neurons *n* = 11 ganglia, glia *n* = 10 ganglia; probenecid (PB), 2 mM, *n* = 12 ganglia; ^{10}Panx, 100 μM, *n* = 12 ganalia] Significance determined by ANOVA with **P*< 0.05, ***P*< 0.001, ****P*< 0.0001 versus sham block and BzATP alone.



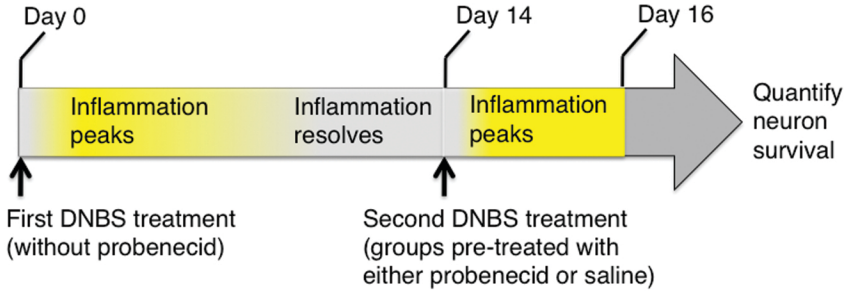
Supplementary Figure 4. Effect of TTX and low extracellular divalent cations on neuronal and glial responses to BzATP. (**a**) Mean peak Ca^{2+} response to BzATP in neurons (red) and glia (black) in normal buffer or in the presence of tetrodotoxin (TTX; 1 µM)(*n* = 21 ganglia BzATP glia, 14 BzATP-TTX glia, 20 BzATP neurons, and 15 BzATP-TTX neurons). (**b**)Mean peak Ca^{2+} response to BzATP in neurons (red) and glia (black) in normal buffer or in buffer containing low Ca^{2+} and Mg^{2+} (**P<0.01 or ***P<0.0001 as compared to response in normal buffer, two-tailed t-test, n = 15 ganglia per group).



Supplementary Figure 5. Enteric neuron and glial responses to BzATP are not mediated by extrinsic nerves. (**a**) Immunohistochemistry for P2X7 (green) and tyrosine hydroxylase (TH; magenta; a marker of sympathetic nerves) in the mouse colon myenteric plexus. Images of a single optical plane (1 µm) demonstrate that P2X7-ir (green) does not co-localize with TH (magenta). Scale bar = 20 µm. (**b**) Mean peak responses to BzATP in neurons (red) and glia (blue) are not altered by acute sympathectomy with 6-hydroxydopamine (6OHDA). (**c**) Images of Fluo-4 fluorescence in myenteric plexus-longitudinal muscle preparations maintained in culture for three days to sever all extrinsic connections at baseline (left) and when stimulated with BzATP (right). Note that neurons and glia still respond to the P2X7 agonist in the absence of extrinsic innervation.



Supplementary Figure 6. Relaxations in the mouse colon elicited by electrical field stimulation (EFS) are mediated by nitrergic innervation. Relaxations are not significantly affected by suramin (200 μM) but are completely absent in the presence of L-NAME (100 μM) in all treatment groups ($n = 8$ animals Saline, 3 Saline suramin, 5 Saline L-NAME, 7 Saline-PB, 3 Saline-PB suramin, 4 Saline-PB L-NAME, 6 PB-DNBS, 3 PB-DNBS suramin, and 4 PB-DNBS L-NAME).



Supplementary Figure 7. Experimental scheme used to replicate the relapsing–remitting nature of colitis.

Antibody	Source	Dilution
Primary Antibodies		
biotinylated mouse anti-HuC/D	Invitrogen Canada Inc, Burlington, ON	1:200
rabbit anti-pannexin1	Invitrogen Canada Inc, Burlington, ON	1:100
rabbit anti-P2X7R	Alomone Labs, Ltd., Jerusalem, Israel	1:200
rabbit anti-P2X7R-ATTO550	Alomone Labs, Ltd., Jerusalem, Israel	1:100
chicken anti-GFAP	Abcam, Cambridge, MA	1:1000
sheep anti-nNOS	Chemicon, Millipore, Billerica, MA	1:400
goat anti-calretinin	Swant, Marly, Switzerland	1:1000
mouse anti-Nlrp3	AdipoGen, San Diego, CA	1:250
rabbit anti-Asc	Enzo Life Sciences, Plymouth Meeting, PA	1:250
Sheep anti-tyrosine hydroxylase	Chemicon, Millipore, Billerica, MA	1:1000
Secondary Antibodies		
streptavidin Alexa Fluor 568	Invitrogen Canada Inc, Burlington, ON	1:200
goat anti-rabbit Alexa Fluor 488	Invitrogen Canada Inc, Burlington, ON	1:200
goat anti-chicken Alexa Fluor 488	Invitrogen Canada Inc, Burlington, ON	1:200
goat anti-chicken Alexa Fluor 568	Invitrogen Canada Inc, Burlington, ON	1:200
goat anti-mouse Alexa Fluor 488	Invitrogen Canada Inc, Burlington, ON	1:200
donkey anti-rabbit CY3	Jackson Labs, BioCAN Scientific Inc, Mississauga, ON	1:300
donkey anti-sheep Cy3	Jackson Labs, BioCAN Scientific Inc, Mississauga, ON	1:200
donkey anti-goat FITC	Jackson Labs, BioCAN Scientific Inc, Mississauga, ON	1:100

Supplementary Table 1. Primary and secondary antibodies, suppliers, and dilutions used in the present study.

Supplementary Methods

Calcium imaging data analysis Ca^{2+} imaging data was analyzed as described by Gulbransen et al. (2010). Regions of interest (ROIs) were drawn and relative fluorescence measured using Imaging Workbench 6 (INDEC BioSystems). Analysis and generation of traces was performed using Prism 5 software (Graphpad Software Inc.). Traces show the change in fluorescence (ΔF/F) over time represent the mean response of all glial or neuronal RIOs within a single ganglion. Responses (magnitude of peak ΔF/F over baseline) were determined as increases in intracellular Ca^{2+} >3 standard deviations from baseline lasting more than 3 s. Responses are reported as the percentage of an initial response to 100 μM BzATP in the same ganglion.

Colonic contractility and relaxation studies Segments of distal colon were removed from mice in each treatment group, submerged in ice-cold oxygenated Kreb's solution and gently flushed to remove luminal contents. Each colon was divided into four 1 cm colon segments and suspended longitudinally in an organ bath by 5-0 silk ligated at each end (Krebs's solution, 37°C, aerated with 95% O_2, 5%CO_2). These preparations were positioned between a pair of parallel electrodes attached to an isometric force transducer (Harvard Apparatus, Kent, UK) coupled to a transducer amplifier relayed to a bioelectric amplifier. Mechanical activity of the muscle was recorded after a 15 min equilibration period under basal tension of 500 mg. Muscle contractility and relaxation was stimulated with EFS (4Hz for 10 s, 60V, 0.5 ms pulse duration, 50 s intervals)

and values recorded and presented as mg contractions or mg relaxations. EFS induced contractions and relaxations were completely abolished by tetrodotoxin (TTX; 2μM).

In a subset of experiments, sodium nitroprusside (SNP; 100 μM) or bethanechol (10 μM) was added to the organ bath and the maximum relaxation produced by SNP or maximum contraction produced by bethanechol was recorded and presented as mg relaxation or tension relative to steady-state levels, respectively.

To determine if EFS induced relaxations are nitrergic or purinergic, the preparations were incubated with L-NAME (100 μM) or suramin (200 μM) respectively.

6–Hydroxydopamine (6-OHDA) chemical sympathectomy 6–OHDA treatment was performed as described by Gulbransen et al. (2010) with minor modifications for mice. Briefly, mice received one subcutaneous injection of 100 mg kg^{-1} 6–OHDA (Sigma) dissolved in H_2O with 0.1% sodium metabisulfite as a stabilizing agent. Tissue was taken two days after injection. Loss of tyrosine hydroxylase (TH)-immunoreactive nerve fibers in the myenteric plexus confirmed the effectiveness of the 6–OHDA treatment.

Longitudinal muscle – myenteric plexus (LMMP) cultures LMMP whole-mount preparations were dissected from the mouse colon as described for Ca^{2+} imaging but in Medium 199 supplemented with 25 mM HEPES, 5 mM $NaHCO_3$

and 2 mM Glutamine (pH 7.4). Following dissection and enzyme treatment, LMMPs were rinsed with, and maintained in sterile media with 10% normal bovine serum (Sigma), penicillin (1000 U mL^{-1}, Sigma) and streptomycin (1 mg mL^{-1}, Sigma) in an incubator (37°C, 50% CO_2) for three days. Media was exchanged after two days in culture. Following the culture period, LMMPs were rinsed in normal buffer and processed for Ca^{2+} imaging as with acutely dissected preparations.