



# Purinergic P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors control enteric nervous system activity through neuro-glia-macrophage crosstalk

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## Abstract

Purines are important mediators of intercellular communication in the enteric nervous system (ENS) that participate in physiological gut functions and disease. Purinergic transmission is prominent in mechanisms of crosstalk between enteric neurons and glia where enteric glia exhibit high responsiveness to adenosine diphosphate (ADP) through P2Y<sub>1</sub> receptors and neurons to adenosine triphosphate (ATP) through P2X<sub>3</sub> receptors. Despite functional data suggesting that enteric glia are the primary site of P2Y<sub>1</sub> expression in the ENS, gene sequencing suggests that P2Y<sub>1</sub> expression is more enriched in neurons than glia. The reason for the mismatch between genomic and functional data is unclear but could involve co-expression of inhibitory P2Y<sub>12</sub> receptors in neurons. We addressed this issue by studying the expression and function of P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors in the mouse ENS using live immunolabeling and calcium imaging techniques. The data show that ADP drives activity among enteric glia and neurons in the myenteric plexus. Interestingly, inhibiting P2Y<sub>12</sub> activity increased neuron responses to ADP and overall spontaneous activity among enteric neurons and glia while decreasing the magnitude of glial responses to ADP. Investigating the location of the receptors involved revealed P2Y<sub>1</sub> receptor expression by both neurons and glia, while P2Y<sub>12</sub> receptor expression was minimal in the ENS. Instead, P2Y<sub>12</sub> expression was enriched in the surrounding muscularis macrophages. Macrophages positive for P2Y<sub>12</sub> overlapped with CD163 positive subsets that have known inhibitory influences over myenteric neurocircuits. Together, these data suggest that macrophage P2Y<sub>12</sub> pathways act to constrain activity in the ENS, which could have implications in mechanisms that contribute to enteric hyperexcitability following disease.

**Keywords** Purinergic signaling · Enteric glia · Enteric nervous system · Intestine

## Introduction

Purines are important mediators of intercellular communication in the enteric nervous system (ENS) and of gut inflammation in disease. These effects are transduced through actions on multiple metabotropic (P2Y and P1) and ionotropic (P2X) purinergic receptors expressed by diverse cell types throughout the intestinal wall [1–3]. Within the myenteric plexus, adenosine triphosphate (ATP) contributes to fast synaptic communication among myenteric neurons through P2X<sub>2</sub> and P2X<sub>3</sub> receptor subtypes [4–7] while slow

excitatory effects of ATP on neurons are mediated by degradation products such as adenosine diphosphate (ADP) acting at P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>6</sub>, and P2Y<sub>12</sub> receptors [2, 8–11]. The fast inhibitory component of enteric neuromuscular transmission also involves purines acting at P2Y<sub>1</sub> receptors expressed in the SIP syncytium [12–14]. Purine levels within the ENS are regulated by several ectonucleotidases expressed by enteric neurons and glial cells which contribute to the diversity of purinergic responses in the ENS by generating breakdown products including ADP, AMP, and adenosine [15, 16]. Shifts in purine concentrations are important drivers of gut inflammation through mechanisms that involve actions at neuronal P2X<sub>7</sub> receptors and associated pathways [17–19].

The enteric glia which surround enteric neurons are particularly sensitive to purines and mount large calcium transients in response to purines generated by neurons [17, 20–22]. The receptor subtype responsible for this effect is believed to be P2Y<sub>1</sub> in mice and P2Y<sub>4</sub> in guinea pigs

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[18, 20–22]. In support, cell culture calcium imaging data show that enteric glia exhibit robust responses to ADP that are sensitive to blockade by MRS2179 while the adjacent enteric neurons exhibit limited responsiveness to ADP [22]. Similarly, enteric glia in intact preparations of mouse myenteric plexus exhibit large calcium responses to ADP that are sensitive to MRS2179 [17, 23]. These observations would suggest that enteric glia are the primary site of P2Y<sub>1</sub> receptor expression in the myenteric plexus. However, single cell sequencing data show *P2ry1* expression in both enteric neurons and glia with an apparent enrichment in neurons compared to glia [24–26]. Electrophysiological data show that guinea pig myenteric neurons in intact tissue do exhibit responses to ADP so it is possible that neurons do express functional P2Y<sub>1</sub> receptors [10, 11]. The reason for this disconnect between gene expression and functional calcium imaging data is unclear.

A potential explanation for the apparent conflicting functional and gene data is that co-expression of P2Y<sub>12</sub> in neurons obscures neuronal calcium responses mediated by P2Y<sub>1</sub>. ADP is an agonist at both P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors but promotes G<sub>q</sub> pathways that stimulate calcium through P2Y<sub>1</sub> and G<sub>i</sub> pathways that inhibit activity through P2Y<sub>12</sub> [27]. Co-expression of P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors is well defined in platelet biology [28, 29] but interestingly, interactions between P2Y<sub>1</sub> and P2Y<sub>12</sub> have synergistic effects on platelet responses [30–32]. Complementary effects of P2Y<sub>1</sub> and P2Y<sub>12</sub> on calcium signaling have also been observed in glioma cells while opposing actions on cell survival have been reported in astrocytoma cells [33, 34]. Gene expression data and immunolabeling support the concept that enteric neurons co-express P2Y<sub>12</sub> while glia do not [9, 35, 36] but potential implications of receptor interactions in neurons are unexplored in the ENS.

Our goal in this study was to test the hypothesis that co-expression of P2Y<sub>1</sub> and P2Y<sub>12</sub> underlies the low apparent responsiveness of enteric neurons to ADP while the sole expression of P2Y<sub>1</sub> in enteric glia permits large calcium responses to ADP. We approached this question using a combination of live immunolabeling techniques and functional imaging of enteric neuron and glial activity in mice expressing genetically encoded calcium reporters. As expected, P2Y<sub>1</sub> receptor labeling was observed in both neurons and glia in the myenteric plexus but to our surprise, P2Y<sub>12</sub> labeling was minimal in the plexus and primarily expressed by muscularis macrophages. Inhibiting P2Y<sub>12</sub> activity increased neuron responses to ADP and overall spontaneous activity among enteric neurons and glia while decreasing the magnitude of glial responses to ADP. Together, these data suggest that macrophage P2Y<sub>12</sub> pathways act to constrain activity in the ENS.

## Materials and methods

### Animals

All protocols were approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC) and mice were housed in specific pathogen-free conditions in facilities accredited by the Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC) International. Mice of both sexes were used for experiments at 8–12 weeks of age. Mice were maintained in a temperature-controlled environment (Optimice® cage system; Animal Care Systems Inc., Centennial, CO) on a 12 h light: dark cycle with access to water and a minimal phytoestrogen diet (Diet Number 2919; Envigo, Indianapolis, IN) ad libitum.

Transgenic mice expressing the genetically encoded Ca<sup>2+</sup> indicator GCaMP5g-tdT in neurons and glia (*Wnt1*<sup>Cre2</sup>;GCaMP5g-tdT) or glia (*Sox10*<sup>CreERT2</sup>;GCaMP5g-tdT) were bred in-house and generated, as described previously [37, 38], by crossing *Wnt1*<sup>Cre2</sup> mice [B6.Cg-*E2f1*<sup>Tg(Wnt1-cre)2Sor/J</sup>; Jackson Laboratory, Bay Harbor, ME; RRID:IMSR\_JAX:022501] or *Sox10*<sup>CreERT2</sup> mice [39], a gift from Dr. Vassilis Pachnis (The Francis Crick Institute, London, UK) with *PC::G5-tdT* mice [B6;129S6-*Polr2a*<sup>tm1(CAG-GCaMP5g,-tdTomato)Tvr/J</sup>; Jackson Laboratory; RRID:IMSR\_JAX:024477]. Transgenic mice expressing the genetically encoded Ca<sup>2+</sup> indicator Lck-GCaMP6f in neurons (*BAF53b*<sup>Cre</sup>;GCaMP6 mice) were bred in-house and generated by crossing *BAF53b-Cre* mice [40] [Tg(Actl6b-Cre)4092Jiwu/J; Jackson Laboratory; RRID:IMSR\_JAX:027826] with *Lck-GCaMP6f* mice [C57BL/6N-Gt(ROSA)26Sor<sup>tm1(CAG-GCaMP6f)Khakh/J</sup>; Jackson Laboratory; RRID:IMSR\_JAX:029626]. Background control strains were used as wild-type (WT) mice. Mice were genotyped by Transnetyx, Inc. (Cordova, TN, USA) and CreER<sup>T2</sup> activity was induced in *Sox10*<sup>CreERT2</sup>;GCaMP5g-tdT by intraperitoneal injections of 4-hydroxytamoxifen (0.1 mg/g) for 2 consecutive days, 1 week before conducting experiments.

### Mouse colonic tissue isolation and processing

Distal colonic tissue was collected in ice-cold Kreb's buffer consisting of 121 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM HEPES, 21.2 mM NaHCO<sub>3</sub>, 1 mM pyruvic acid, and 8 mM glucose (pH adjusted to 7.4 with NaOH) before further processing. Isolated colonic tissue was longitudinally slit open along the mesenteric border and pinned out using insect pins in a Sylgard coated petri dish. Whole-mount circular

muscle-myenteric plexus (CMMP) preparations were generated by removing the mucosa and flipping the tissue before removing the longitudinal muscle layer by microdissection as described in prior work [23].

### Live whole-mount immunohistochemistry (IHC)

Live whole-mount CMMP preparations from WT and *BAF53b<sup>Cre</sup>;GCaMP6* mice were pinned out in 35 mm Sylgard coated petri dishes. Antibody details are supplied in Table 1. Briefly, primary antibodies were diluted in Krebs's buffer and applied for 4 h at 4° C. CMMPs were rinsed five times with PBS. Images were acquired through the 20x (W Plan-Apochromat, 1.0 n.a.) DIC VIS-IR M27 75 mm objective of a Zeiss LSM880 confocal microscope (Carl Zeiss, White Plains, NY).

### Ca<sup>2+</sup> imaging

Live whole-mount CMMP preparations of colon myenteric plexus from *Wnt1<sup>Cre2</sup>;GCaMP5g-tdT* and *Sox10<sup>CreERT2</sup>;GCaMP5g-tdT* mice were prepared for Ca<sup>2+</sup> imaging as previously described [41]. Preparations were continuously perfused with fresh, prewarmed (37° C) Krebs's buffer at a flow rate of 2–3 mL min<sup>-1</sup>. Baseline activity was recorded for 30–60 s prior to challenging samples with ADP applied via the bath solution (100 µM) or a focal micropipette (1 mM). Glass micropipettes were fabricated from capillary glass (0.85 mm ID/ 1.25 mm OD, end-opening 60–80 µm of diameter) using a pipette puller (P-87 Flaming-Brown Micropipette Puller, Sutter Instruments Corporation) and were back-filled with ADP dissolved in Krebs's Buffer. Drugs were then applied using gentle positive pressure applied with a 5 mL syringe connected to a pipette holder. This approach delivered microliter volumes of drugs and affected only the ganglion of interest as described previously [37]. PSB0739 (25 µM) was applied to samples for 10 min to antagonize P2Y<sub>12</sub> receptors [42] and subsequent challenges with ADP were conducted in the presence of PSB0739.

Images were acquired at a rate of 1 Hz through the 20× wide-field water-immersion objective lens (Olympus

XLUMPLFLN20xW, 1.0 n.a.) of an upright Olympus BX51WI fixed-stage microscope (Olympus, Tokyo, Japan) using MetaMorph (Molecular Devices, San Jose, CA) software and a Neo sCMOS camera (Andor, South Windsor, CT). Illumination for fluorescence imaging was provided by a DG4 Xenon light source (Sutter Instrument, Novato, CA).

### Data analysis

Raw TIFF files from Ca<sup>2+</sup> imaging experiments were imported into Fiji (ImageJ 2 V: 2.14.0/1.54f) where they were processed by smoothing, subtracting background (rolling balls), corrected for movement using StackReg, and cropped. Processed files were analyzed with SparkAn 5.5.6.0 (Adrian D. Boven and Mark T. Nelson, Department of Pharmacology, College of Medicine, University of Vermont, Burlington, VT). Regions of interest (ROIs) were drawn around glia and neurons identified in *Wnt1<sup>Cre2</sup>;GCaMP5g-tdT* mice and glia in *Sox10<sup>CreERT2</sup>;GCaMP5g-tdT* mice by tdTomato expression and morphology as described previously [37]. Analysis and generation of traces were performed using GraphPad Prism 10 (GraphPad Software, La Jolla, CA). Traces represent the change in fluorescence ( $\Delta F/F_0$ ) over time [43] for cells or show the averaged response of all cells within a ganglion (where indicated). Heatmaps of cellular responses were generated in GraphPad Prism and temporal color-coded images were generated in Fiji using the temporal color code function. Amplitude was assessed by measuring the maximal response ( $\Delta F/F_0$ ) at least 3 SD above baseline. Area under the curve (AUC;  $\Delta F/F_0$ \*s) was calculated to measure total peak area. Spontaneous activity was assessed as activity during the period preceding stimulation with ADP. Ca<sup>2+</sup> imaging experiments were conducted in 4 mice (mixed sex) and N values represent individual cells for quantification of amplitude and AUF. N values represent ganglia (% cells per ganglion) in assessments of spontaneous activity.

Co-expression of cells were quantified from 135 cells within field of view from n = 3 mice.

Data were analyzed using Prism 10 and are shown as mean ± SEM. Comparisons of responses pre/post PSB0739

**Table 1** Primary antibodies and isotype controls used in this study

| Antibody                | Source                      | Catalog No  | Resource ID | Dilution |
|-------------------------|-----------------------------|-------------|-------------|----------|
| ACSA-2 APC              | Miltenyi Biotec, Auburn, CA | 130–117-535 | AB_2727978  | 1:50     |
| PDGFRα/CD140a APC       | Thermo Fisher, Carlsbad, CA | 17–1401-81  | AB_529482   | 1:100    |
| CD163 FITC              | Thermo Fisher, Carlsbad, CA | 11–1631-82  | AB_2815035  | 1:200    |
| MHC-II Super Bright 436 | Thermo Fisher, Carlsbad, CA | 62–5231-82  | AB_2688070  | 1:100    |
| P2Y <sub>1</sub> FITC   | Alomone, Jerusalem, Israel  | APR-021-F   | AB_2925128  | 1:200    |
| P2Y <sub>12</sub> PE    | Alomone, Jerusalem, Israel  | APR-020-PE  | AB_2876814  | 1:200    |

ACSA-2 astrocyte cell surface antigen-2; *PDGFRα* platelet derived growth factor receptor alpha MHC-II, major histocompatibility complex type II

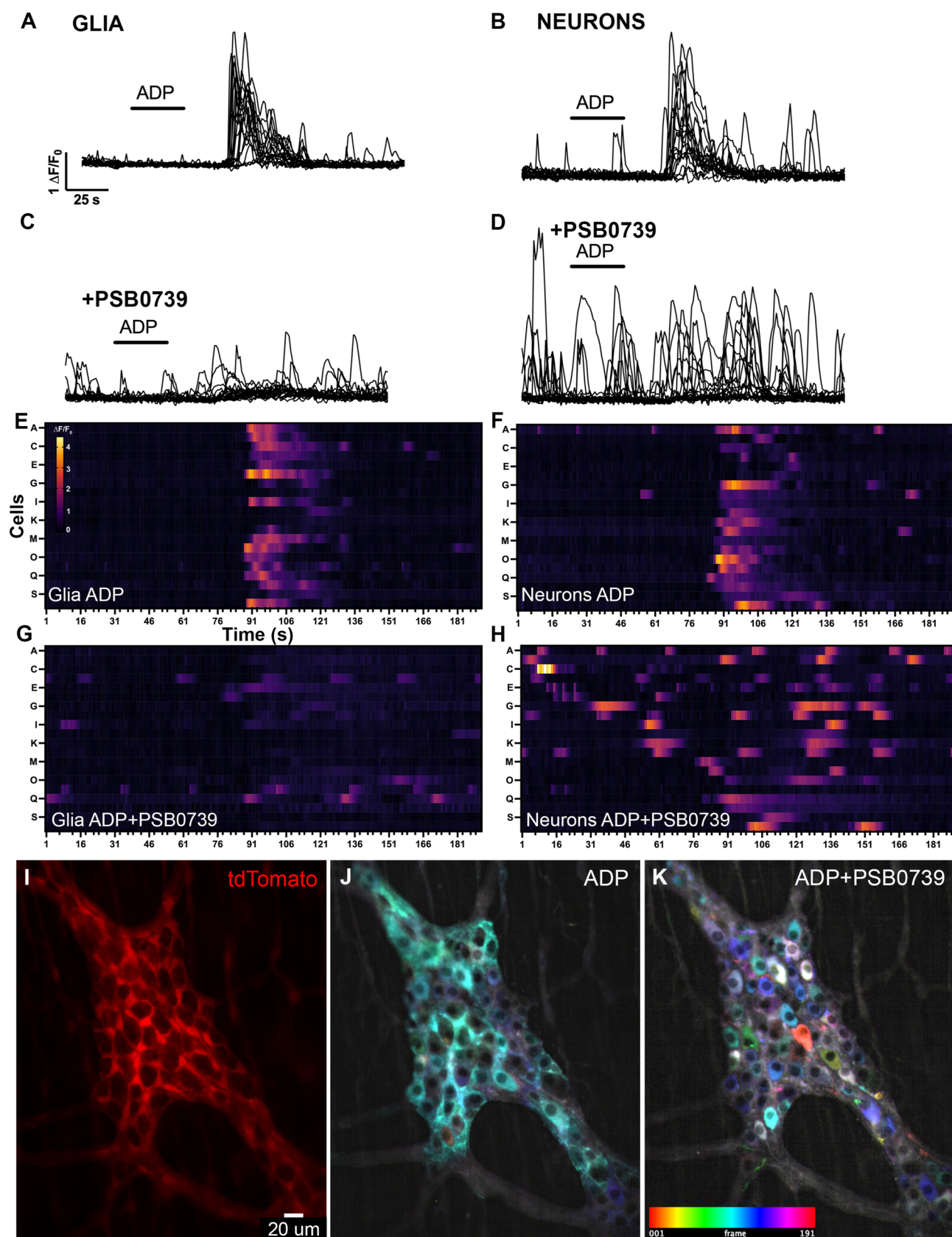


**Fig. 1** Effects of P2Y<sub>12</sub> inhibition on enteric glial and neuronal responses to ADP in *Wnt1<sup>Cre2</sup>;GCaMP5g-tdT* mice. (A, B) Representative traces showing calcium responses evoked by ADP (100  $\mu$ M bath application) in enteric glia (A;  $n=20$ ) and neurons (B;  $n=20$ ) within a single myenteric ganglion from the colon of a *Wnt1<sup>Cre2</sup>;GCaMP5g-tdT* mouse. Note that glia **A** exhibited low spontaneous activity and responded to ADP with uniform large magnitude calcium responses. **B** Spontaneous activity was more common in neurons, which exhibited variable responses to ADP. **C** Following incubation with the P2Y<sub>12</sub> antagonist PSB0739 (25  $\mu$ M), enteric glia exhibited spontaneous activity and suppressed responses to ADP. **D** In neurons, PSB0739 increased spontaneous activity and decreased coordination of the response to ADP. **E–H** Heatmaps of individual cell responses from the ganglion shown in the traces in A–D, respectively. Note the loss of coordinated ADP responses and increase in spontaneous activity. **I** tdTomato expression in this ganglion was used in combination with morphology to identify glia (high tdTomato) and neurons (low tdTomato). **J, K** Images showing normal GCaMP responses in this ganglion to ADP and following incubation with PSB0739 (J and K, respectively) are represented as temporal color-coded images in which cell color corresponds to the time of response in the experiment. Note that the normal ADP response occurs in a narrow time window and is dominated by glia, while activity following PSB0739 is primarily neuronal and occurs stochastically throughout the experiment

were made using two-tailed paired t tests and two-tailed Wilcoxon matched pairs signed rank tests following assessment of normality. A P-value less than 0.05 was considered significant.

## Chemicals and reagents

PSB0739 was purchased from Tocris Bioscience (Bio-Techne, Minneapolis, MN). All other chemicals and reagents were purchased from Millipore Sigma (Burlington, MA, USA), unless otherwise stated.

## Results

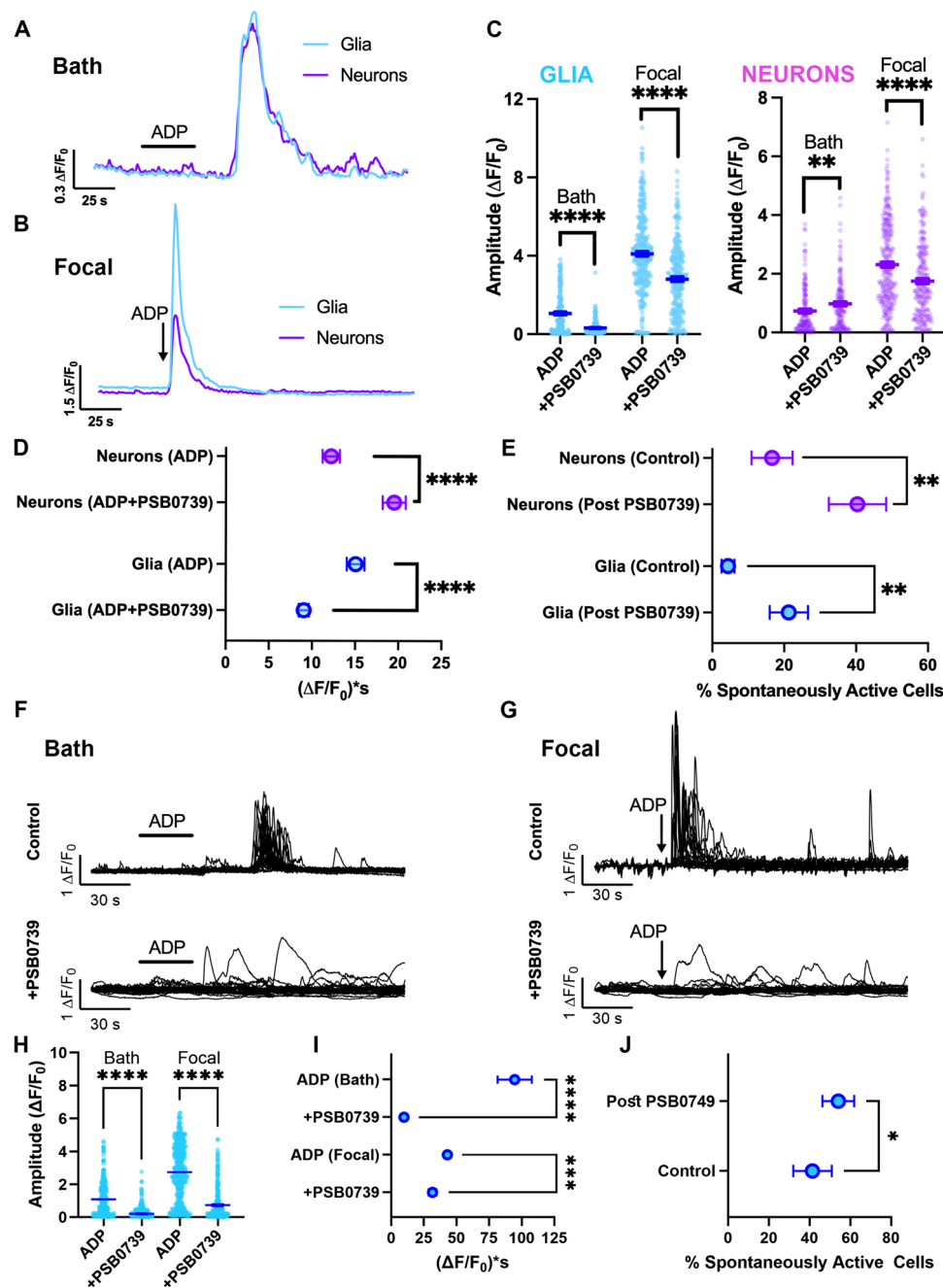
### ADP drives robust activity among myenteric glia and neurons

Enteric glia in the mouse colon myenteric plexus are highly responsive to ADP in intact tissue and in primary culture [22, 44, 45]. In contrast, myenteric neurons display comparatively minor direct responses to ADP and neuronal ADP responses involve mechanisms downstream of glial signaling. In agreement with earlier studies, we observed large magnitude Ca<sup>2+</sup> responses in enteric glia when challenged with ADP applied either in the bath solution or via a focal micropipette to a single ganglion (Figs. 1A, E, J and 2). Myenteric neurons also exhibited responses to ADP; however, neuronal responses tended to be smaller and more variable than those in glia (Fig. 1B, F, J). Glial responses to ADP were 45% greater in amplitude than those evoked in

neurons when stimulated via ADP in the bath solution (glia  $1.06 \pm 0.08 \Delta F/F_0$  vs neurons  $0.73 \pm 0.07 \Delta F/F_0$ ) and 77% greater in amplitude when stimulated via focal application of ADP to a single ganglion (glia  $4.10 \pm 0.03 \Delta F/F_0$  vs neurons  $2.31 \pm 0.09 \Delta F/F_0$ ) (Fig. 2C). Glial responses profiles were also 23.8% broader than neuronal responses as reflected by measurements of area under the curve (AUC, Fig. 2D). Glial responses to ADP typically preceded those of neurons and this was most evident when ADP application was controlled by focal microinjection (Fig. 2B). However, neurons exhibited nearly three times the amount of spontaneous activity under control conditions than glia (neurons 16.6%, glia 4.4%; Fig. 2E), which likely reflects normal ongoing synaptic signaling in myenteric neurocircuits.

### Blocking P2Y<sub>12</sub> receptors shifts ADP responses from glia to neurons and increases spontaneous activity

The cellular imaging results above suggest that glia are the main ADP responsive cells in myenteric ganglia. These observations are difficult to reconcile with single cell sequencing data that suggest extensive neuronal *P2ry1* gene expression in the ENS [26]. To explain this disconnect, we hypothesized that co-expression of P2Y<sub>12</sub> receptors masks P2Y<sub>1</sub> mediated responses in neurons. Therefore, we reasoned that blocking P2Y<sub>12</sub> receptors with the selective antagonist PSB0739 [46] would reveal neuronal responses to ADP. In support, neurons were more active following PSB0739 and exhibited 33.5% larger amplitude responses to ADP applied in the bath solution (Figs. 1D,H,K and 2C,D). AUC measurements for neuronal responses following PSB0739 were also increased by 59.8% (Fig. 2D). However, PSB0739 decreased neuronal responses evoked by focal application of ADP by 24.3%. Interestingly, both neurons and glia exhibited increased spontaneous activity following incubation with PSB0739 (Figs. 1C,D,G,H and 2E). On average, bath application of PSB0739 increased spontaneous neuronal activity two-fold and spontaneous glial activity by over four-fold (Fig. 2E). Despite the increase in spontaneous glial activity, PSB0739 suppressed glial responses to ADP overall and ganglionic responses to ADP were dominated by neurons (Fig. 1K). PSB0739 nearly obliterated glial responses to ADP applied via bath solution (Figs. 1C,G and 2C) and decreased those evoked by focal application to ganglia by 31.5% (Fig. 2C). Glial response AUC measures were also decreased by 59.6% following PSB0739, indicating shorter and lower magnitude responses. To rule out the possibility that apparent changes in glial activity were due to responses in neuron processes that overlapped with glial regions of interest in *Wnt1<sup>Cre2</sup>;GCaMP5g-tdT* mice, we repeated these experiments in samples from



*Sox10<sup>CreERT2</sup>;GCaMP5g-tdT* mice that have glial-restricted GCaMP expression [45]. Data from these recordings showed similar effects of PSB0739 on enteric glial  $\text{Ca}^{2+}$  responses (Fig. 2F,G). PSB0739 again decreased overall glial responses to ADP (bath and focal) and reduced both peak amplitude and AUC (Fig. 2H and I, respectively). PSB0739 also increased glial spontaneous activity by 22.1% (Fig. 2J). Together, these results indicate that  $\text{P2Y}_{12}$  receptors exert inhibitory actions on enteric neurons that when released, potentiate neuronal responses to ADP while inhibiting glial activity.

### Cellular distribution of $\text{P2Y}_1$ and $\text{P2Y}_{12}$ receptors in the myenteric plexus

To understand which cells might be responsible for the effects observed following ADP stimulation and  $\text{P2Y}_{12}$  blockade, we studied the expression of  $\text{P2Y}_1$  and  $\text{P2Y}_{12}$  receptors in the myenteric plexus. We initially evaluated potential cell types expressing  $\text{P2Y}_1$  receptors by screening gene expression in published single-cell and bulk RNAseq datasets [26, 35, 36]. Based on gene expression, both enteric neurons and glia have the potential to express

**Fig. 2** Summary data showing effects of P2Y<sub>12</sub> inhibition on enteric neurons and glial calcium responses to ADP in *Wnt1<sup>Cre2</sup>;GCaMP5g-tdT* mice and *Sox10<sup>CreERT2</sup>;GCaMP5g-tdT* mice. **A, B** Representative traces showing the mean calcium response of neurons ( $n=20$ ) and glia ( $n=20$ ) within a single ganglion from the colon of a *Wnt1<sup>Cre2</sup>;GCaMP5g-tdT* mouse challenged with ADP applied via the bath solution (**A**; 100  $\mu$ M) or a focal micropipette (**B**; 1 mM). **C** Summary data showing effects of PSB0739 on the amplitude (max  $\Delta F/F_0$ ) of glial (Bath;  $n=184$  cells, 10 ganglia; Focal:  $n=249$  cells, 15 ganglia) and neuronal (Bath;  $n=152$  cells, 10 ganglia; Focal:  $n=224$  cells, 15 ganglia) calcium responses evoked by bath or focal application of ADP. Two-tailed paired t test \*\*\*\*  $P<0.0001$ ; \*\*  $P=0.0039$ . **D** Quantification of area under the curve ( $\Delta F/F_0$ \*) of neuronal and glial responses evoked by ADP (bath) in the presence or absence of PSB0739. Two-tailed paired t test \*\*\*\*  $P<0.0001$ . **E** Quantification of the percentage of spontaneously active cells in  $n=11$  ganglia in Krebs's buffer (control) and in the presence of PSB0739 (25  $\mu$ M). Two-tailed Wilcoxon matched pairs signed rank test: Neurons, \*\*  $P=0.0059$ ; Glia, \*\*  $P=0.0078$ . **F, G** Representative traces showing calcium responses evoked by ADP via bath solution (**F**, 100  $\mu$ M) or a focal micropipette (**G**, 1 mM) pre and post-PSB0739 (top and bottom, respectively) in enteric glia ( $n=25$ ) within a single myenteric ganglion from the colon of a *Sox10<sup>CreERT2</sup>;GCaMP5g-tdT* mouse. **H** Summary data showing effects of PSB0739 on the amplitude (max  $\Delta F/F_0$ ) of glial ( $n=435$  cells, 6 ganglia) calcium responses evoked by bath or focal application of ADP. Two-tailed paired t test \*\*\*\*  $P<0.0001$ ; \*\*\*\*  $P<0.0001$ . **I** Quantification of area under the curve ( $\Delta F/F_0$ \*) of glial responses evoked by bath or focal application of ADP in the presence or absence of PSB0739. Two-tailed paired t test \*\*\*\*  $P<0.0001$ ; \*\*\*  $P<0.001$ . **J** Quantification of the percentage of spontaneously active cells in  $n=6$  ganglia in Krebs's buffer (control) and in the presence of PSB0739. Two-tailed Wilcoxon matched pairs signed rank test: Glia, \*  $P=0.0469$

*P2ry1* in mice and humans with an apparent enrichment of *P2ry1* in neurons (Fig. 3A). To determine if protein expression matches gene expression profiles, we performed immunofluorescence labeling in live whole mount preparations of myenteric plexus using a P2Y<sub>1</sub> antibody that targets an extracellular domain of P2Y<sub>1</sub> receptors (Fig. 3B,D). Neurons and glia both displayed positive labeling for P2Y<sub>1</sub> in the mouse colon myenteric plexus. Neuron cell bodies positive for P2Y<sub>1</sub> were evident based on morphology and position within myenteric ganglia (Fig. 3D; asterisks); however, glial P2Y<sub>1</sub> expression was less clear based on morphology alone. Co-labeling with the enteric glial marker astrocyte cell surface antigen 2 (ASCA-2) [47] revealed broad P2Y<sub>1</sub> expression among enteric glia where receptors were localized to both cell bodies and processes (Fig. 3D; arrows). As expected, strong P2Y<sub>1</sub> labeling was also observed in extraganglionic cells that were presumably PDGFR $\alpha$  + cells (Fig. 4) that have well described P2Y<sub>1</sub> expression [12, 13, 48]. Indeed, PDGFR $\alpha$  + (CD140a) cells exhibit P2Y<sub>1</sub> expression, which are involved in purinergic inhibitory neurotransmission to the smooth muscle in the colon [49]. These data support the concept that P2Y<sub>1</sub> receptors are expressed by both enteric neurons and glia within myenteric ganglia.

To understand which cells might underlie the effects of P2Y<sub>12</sub> inhibition, we began by screening enteric neuron and glia gene expression in published RNAseq datasets [26, 35, 36]. *P2ry12* gene expression levels were generally lower in both neurons and glia than what was observed for *P2ry1*; however, gene expression was detected in cells of the mouse and human ENS (Fig. 5A). Immunolabeling in live whole mount preparations of mouse myenteric plexus with P2Y<sub>12</sub> antibodies targeting extracellular domains of the receptor failed to detect expression in enteric ganglia (Fig. 5B). Subsets of these experiments were performed in mice that express the indicator Lck-GCaMP6f in enteric neurons (*BAF53b<sup>Cre</sup>;GCaMP6*) to visualize P2Y<sub>12</sub> labeling in relation to neuron membranes (Fig. 5B,C). Despite an apparent lack of P2Y<sub>12</sub> expression in the myenteric plexus, cells surrounding myenteric ganglia displayed robust P2Y<sub>12</sub> immunoreactivity. We speculated that these cells might be muscularis macrophages based on their morphology and known similarities to microglia, which are enriched in P2Y<sub>12</sub> expression [50]. Co-labeling with antibodies against major histocompatibility complex II (MHC-II) confirmed suspicions that extraganglionic cells were macrophages (Fig. 5C,D). However, macrophages co-expressed P2Y<sub>12</sub> and MHC-II to varying degrees with some MHC-II-positive macrophages displaying robust protein expression while others had little to no P2Y<sub>12</sub> expression.

### CD163 + muscularis macrophages express P2Y<sub>12</sub> receptors in the colon

Muscularis macrophages surrounding myenteric ganglia include both resident and infiltrating subpopulations that have differential effects on the ENS. Resident muscularis macrophages express CD163 and exert an inhibitory influence over myenteric neurocircuits by secreting bone morphogenetic protein 2 (BMP2) [51, 52]. In contrast, markers such as Iba-1 and MHC-II identify broader subsets of macrophages that encompass infiltrating cells that have lesser roles in regulating ENS physiology but greater roles in disease. To understand which macrophage subset expresses P2Y<sub>12</sub>, we co-labeled samples with MHC-II as a marker of pro-inflammatory “M1” macrophages and CD163 as a marker of anti-inflammatory “M2” resident macrophages [53]. Cells within fields of view were quantified, yielding 137 total cells (see Table 2) Multiple subtypes existed within the cell population. CD163 and MHC-II were the most prevalent subtypes, both being expressed in 114 of the 137 cells, but not necessarily being co-expressed in all of those cells (83.2%). Further analysis of those subtypes yielded 85 of the 137 as both CD163 and MHC-II positive (62.0%) and P2Y<sub>12</sub> was expressed in 99 of the 137 cells (72.3%). To understand the expression of P2Y<sub>12</sub> with the expression of the other markers, individual analysis of the

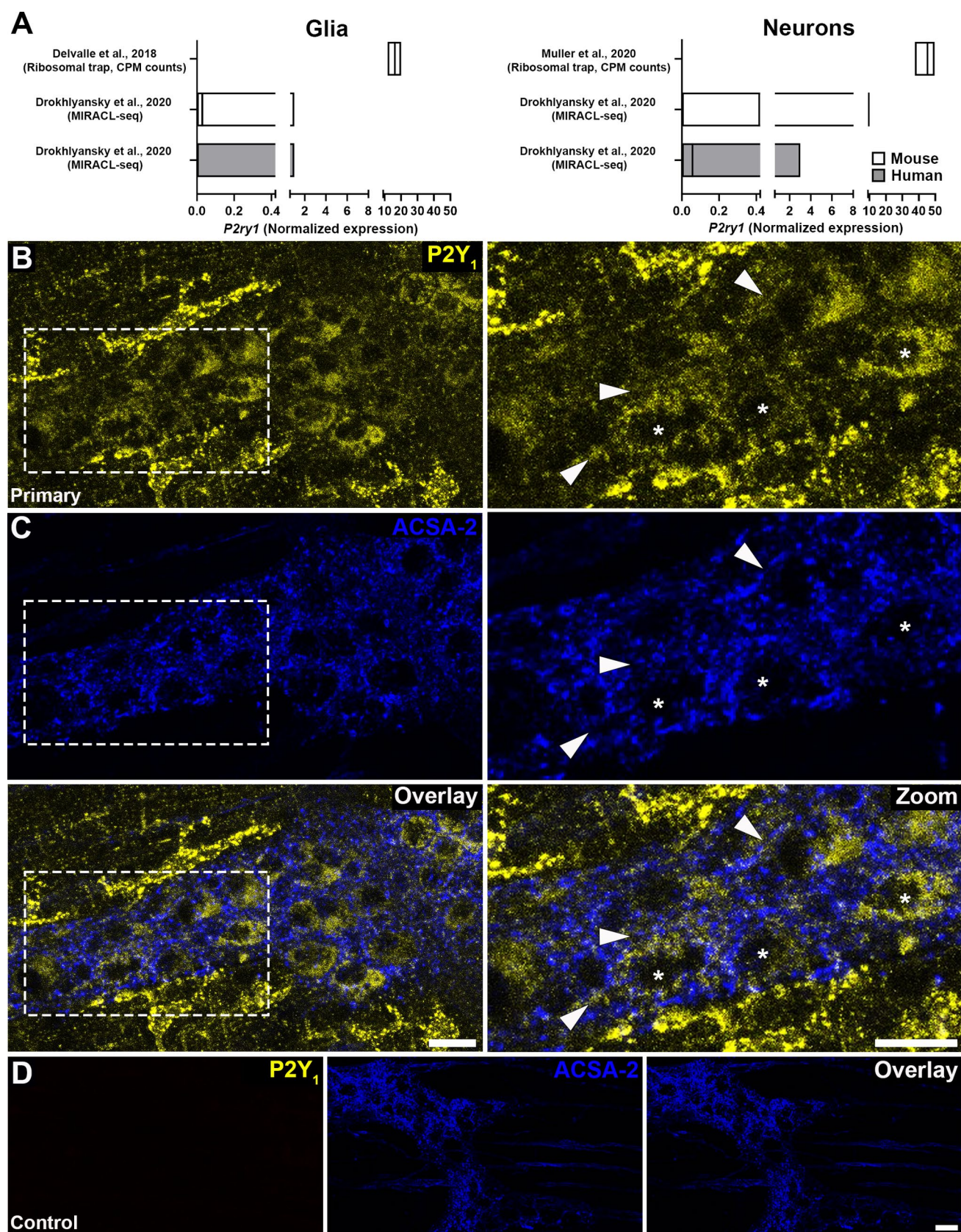


**Fig. 3** Cellular distribution of P2Y<sub>1</sub> receptors in the colonic myenteric plexus. **A** *P2ry1* cell-specific transcript expression in combined glial and neuronal subpopulations (left and right, respectively) from mouse and human colon prepared from published data sets. Graphs are prepared from the published datasets by [26, 35, 36] and data show normalized expression levels (mean ± range). **B–D** Representative confocal images showing P2Y<sub>1</sub> immunoreactivity (yellow) and astrocyte cell surface antigen-2 (ACSA-2) immunoreactivity (blue) in the myenteric plexus of a wild-type (WT) mouse. (B and D) P2Y<sub>1</sub> immunoreactivity is localized to glia and neurons [D; arrowheads and asterisks (\*) in zoom, respectively]. **E** Isotype control for P2Y<sub>1</sub> antibody. Scale bars in B–E = 20 µm. *n* = 3 animals

P2Y<sub>12</sub> population was done. Interestingly, only one cell was identified as being solely P2Y<sub>12</sub> positive. There were slightly higher levels of co-expression observed in P2Y<sub>12</sub> and MHC-II positive cells only as compared to P2Y<sub>12</sub> and CD163 positive cells only (24 cells, 24.2% and 17 cells, 17.2%; respectively). The final subtype observed in the P2Y<sub>12</sub> population were cells positive for all three markers (57 cells, 57.6%). P2Y<sub>12</sub> labeling was observed among both MHC-II+ and CD163+ macrophages (Fig. 6A–D) and a subset of P2Y<sub>12</sub>-expressing cells were negative for both MHC-II and CD163. Further analysis (Table 2) revealed that P2Y<sub>12</sub> expression within CD163+ macrophages was distributed among two populations: P2Y<sub>12</sub>-positive macrophages expressing CD163 (CD163+/MHC-II-, 17.2%, Fig. 6E) and P2Y<sub>12</sub>-positive macrophages expressing both CD163 and MHC-II (CD163+/MHC-II+, 57.6%; Fig. 6E and G, respectively). In contrast, P2Y<sub>12</sub> expression was observed in macrophages expressing MHC-II and not CD163 (CD163-/MHC-II+, 24.2%) and was also absent in a subset of CD163+/MHC-II+ cells (62%, Fig. 6F).

## Discussion

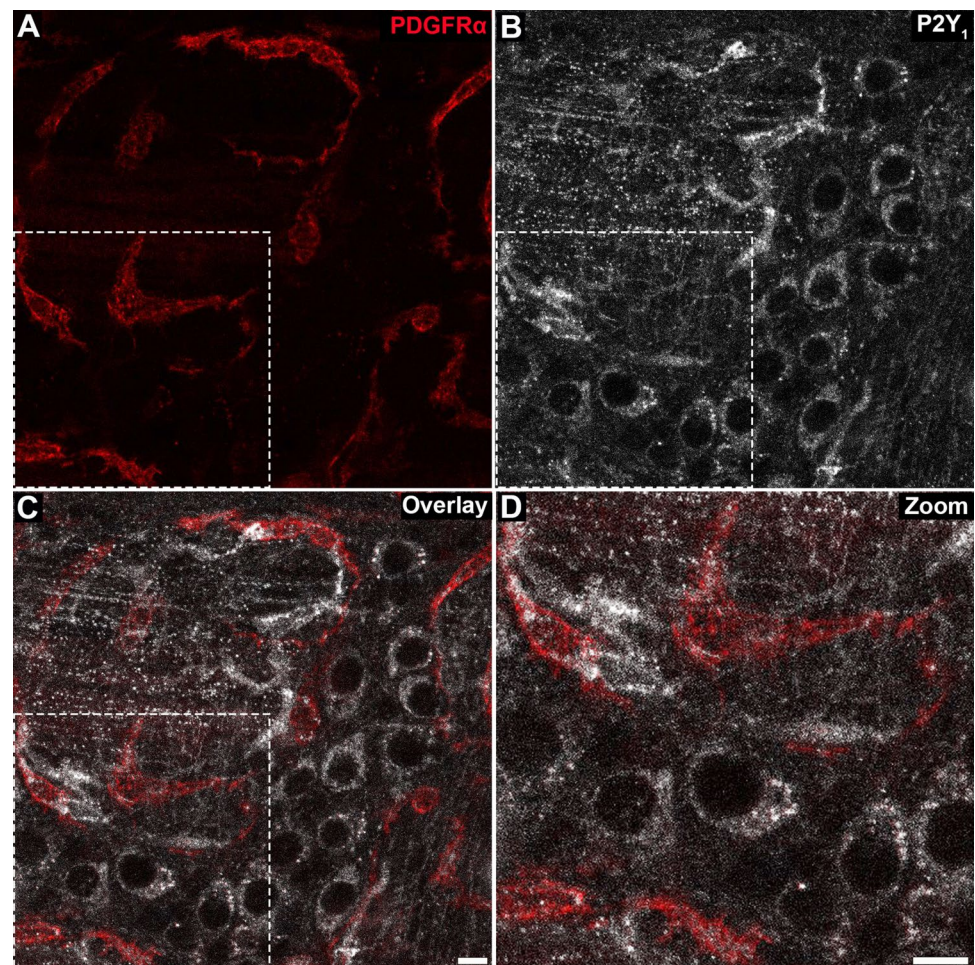
Purines are important mediators of intercellular communication in the ENS and changes in purine levels contribute to neuroplasticity during inflammation. The ATP metabolite ADP is particularly important in mechanisms regulating glial activity and inhibitory neuromuscular transmission in the gut. Within myenteric ganglia, ADP mainly excites enteric glia despite data suggesting that both enteric neurons and glia express P2Y<sub>1</sub> receptors. We studied whether co-expression of P2Y<sub>12</sub> receptors offers a potential explanation for this discrepancy by masking neuronal responses to ADP through P2Y<sub>1</sub>. Our data agree with prior reports suggesting that P2Y<sub>1</sub> receptors are broadly expressed among enteric neurons and glia in the myenteric plexus and that ganglionic responses to ADP are normally dominated by glia. Blocking P2Y<sub>12</sub> receptors switched ganglionic ADP responses to primarily neuronal by enhancing neuron responses and decreasing those in glia while increasing spontaneous activity in both enteric neurons and glia. Surprisingly, assessing

the cellular location of P2Y<sub>12</sub> receptors revealed minimal expression in the myenteric plexus and strong expression by muscularis macrophages. P2Y<sub>12</sub> expressing macrophages co-expressed markers of anti-inflammatory resident muscularis macrophages that exert inhibitory influences over ENS activity. Together, these data suggest that macrophage P2Y<sub>12</sub> pathways act to constrain activity in the ENS and at least partially explain the limited responses of neurons to ADP.

P2Y<sub>1</sub> receptors have received a great deal of attention for their role in nerve-mediated relaxation of the gastrointestinal tract. The main area of focus here has been at the level of the enteric neuroeffector junction where P2Y<sub>1</sub> receptors are expressed by PDGFRα+ fibroblast-like cells [12–14]. Purines such as ATP, b-Nicotinamide Adenine Dinucleotide (b-NAD), and uridine adenosine tetraphosphate (Up4A) are released from the nerve terminals of enteric inhibitory motor neurons and act on P2Y<sub>1</sub> receptors expressed by PDGFRα+ cells to drive the fast component of inhibitory junction potentials in intestinal smooth muscle [12, 54–57]. Extrganglionic cells with a morphology consistent with PDGFRα+ cells exhibited strong P2Y<sub>1</sub> immunoreactivity in our analysis. This observation agrees with the known role of P2Y<sub>1</sub> signaling in these cells and provides additional assurance in the specificity of the antibody labeling strategy used here. P2Y<sub>1</sub> is enriched in PDGFRα+ cells associated with the human colon myenteric plexuses in comparison to smooth muscle cells and interstitial cells of Cajal, which is consistent with the role of P2Y<sub>1</sub> in inhibitory neurotransmission [48]. Within the ganglia, P2Y<sub>1</sub> labeling was observed in both enteric glia and neurons, which agrees with published gene sequencing data. P2Y<sub>1</sub> labeling intensity varied between individual cells but often appeared stronger in neurons than glial cells. This apparent difference could be due to differing locations of P2Y<sub>1</sub> receptors in the two cell types where somatic P2Y<sub>1</sub> expression in neurons appears stronger than labeling in fine glial processes. However, functional Ca<sup>2+</sup> imaging data show that glial responses to ADP in the distal colon myenteric plexus do involve extensive crosstalk with neurons and that glial ADP responses are blunted when neuron activity is reduced by tetrodotoxin [45]. Taking this observation into consideration with the pattern of labeling shown in this study would suggest that both enteric neurons and glia have the potential to express functional P2Y<sub>1</sub> receptors and that cellular crosstalk may act to amplify responses to ADP. Why enteric neurons in cell culture seem relatively insensitive to ADP is not clear but may indicate an immature phenotype of these cells or a lack of factors needed to promote P2Y<sub>1</sub> expression [22].

Relatively little is known regarding enteric P2Y<sub>12</sub> expression beyond isolated reports of potential expression in neurons based on immunolabeling data [9]. The results from these studies suggested extensive P2Y<sub>12</sub> expression among calbindin immunoreactive neurons in the myenteric plexus

**Fig. 4** Cellular distribution of P2Y<sub>1</sub> receptors in PDGFR $\alpha$ + cells near the mouse colonic myenteric plexus. **A–D** Representative confocal images showing the immunoreactivity of PDGFR $\alpha$ /CD140a (A, red), P2Y<sub>1</sub> immunoreactivity (B, gray), overlay and zoom (C and D, respectively) near the myenteric plexus of a wild-type (WT) mouse. Scale bars in C–D = 20  $\mu$ m. *n* = 4 animals



of the guinea pig stomach and intestine. We were unable to replicate these results and observed minimal P2Y<sub>12</sub> labeling in the mouse myenteric plexus. This difference could indicate species difference in receptor expression but more likely reflect the different labeling strategies used. Here, we used a live labeling and imaging approach that targets the functional pool of cell surface receptors while prior observations relied on labeling in fixed samples that identified intracellular targets with unclear relevance to functional cell surface receptors. Nearly all P2Y<sub>12</sub> labeling observed in our study was in muscularis macrophages that surround the myenteric plexus. This pool of muscularis macrophages are long-lived tissue resident cells that fulfill functional roles shared by microglia in the brain [58]. Interestingly, P2Y<sub>12</sub> expression is robust in microglia but is not typically expressed by peripheral macrophages [59]. Similar P2Y<sub>12</sub> receptor expression by muscularis macrophages as microglia adds to the notion of their microglial-like phenotype. In microglia, signaling through P2Y<sub>12</sub> receptors controls their ability to polarize, migrate and extend processes toward sites of damage. Given the similar functional niches occupied by microglia and muscularis macrophages, it is likely that

P2Y<sub>12</sub> receptors fulfill similar roles in both cell types. Most P2Y<sub>12</sub>-expressing macrophages observed in the mouse colon also expressed CD163, which is a marker of tissue-resident, “M2-like” anti-inflammatory macrophages. Fewer MHC-II+, “M1-like” pro-inflammatory macrophages expressed P2Y<sub>12</sub>. This difference agrees with P2Y<sub>12</sub> expression in microglia where inflammation decreases P2Y<sub>12</sub> expression and the ability of microglia to extend their processes toward areas of injury. Understanding how P2Y<sub>12</sub> receptors control muscularis macrophage functions in health and disease could be a fruitful area for future work since these data suggest that P2Y<sub>12</sub> receptors are involved in both pro- and anti-inflammatory pathways.

The results of our experiments support the conclusion that P2Y<sub>12</sub> receptors control macrophage pathways that exert regulatory influences over enteric motor neurocircuits. Muscularis macrophages engage in bidirectional communication with enteric neurons and glia that function to shape ENS synaptic pathways during development and sustain both ENS and macrophage health in mature animals [60]. Muscularis macrophages secrete factors such as bone morphogenic protein 2 (BMP2) that act on receptors expressed by

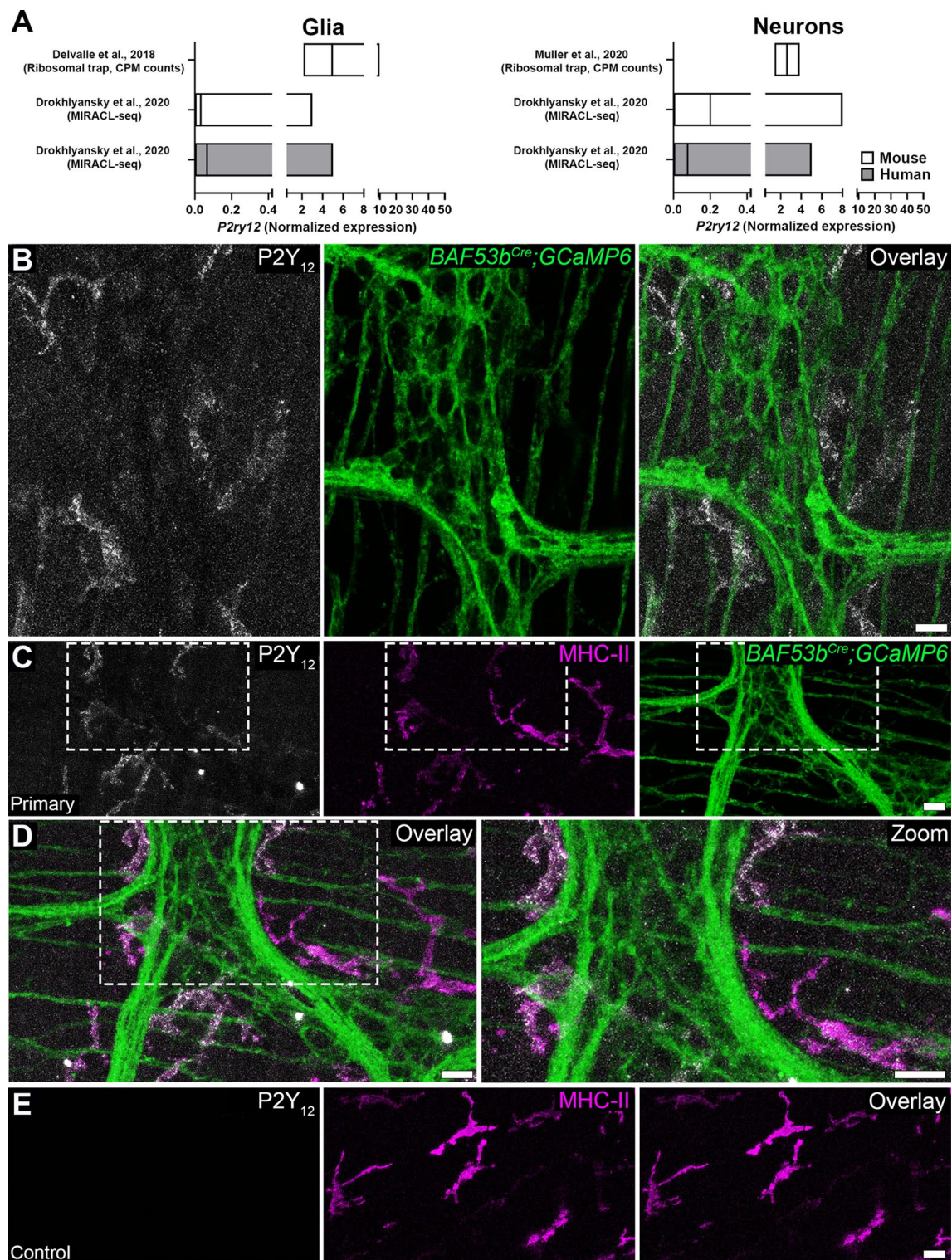


**Fig. 5** Cellular distribution of P2Y<sub>12</sub> receptors in the colonic myenteric plexus. **A** *P2ry12* cell-specific transcript expression in combined glial and neuronal subpopulations (left and right, respectively) from mouse and human colon prepared from published data sets. Graphs are prepared from the published datasets by [26, 35, 36] and data show normalized expression levels (mean ± range). **B** Representative confocal images showing P2Y<sub>12</sub> immunoreactivity (grey) and

GCaMP6f fluorescence (green) in neurons within the myenteric plexus of *BAF53b<sup>Cre</sup>;GCaMP6* mice. **C–D** Representative confocal images showing P2Y<sub>12</sub> immunoreactivity (grey), major histocompatibility complex type II (MHC-II) immunoreactivity (magenta), and GCaMP6f fluorescence (green) in the myenteric plexus of *BAF53b<sup>Cre</sup>;GCaMP6*. **E** Isotype control for P2Y<sub>12</sub> antibody. Scale bars in B–E = 20 μm. *n* = 3 animals

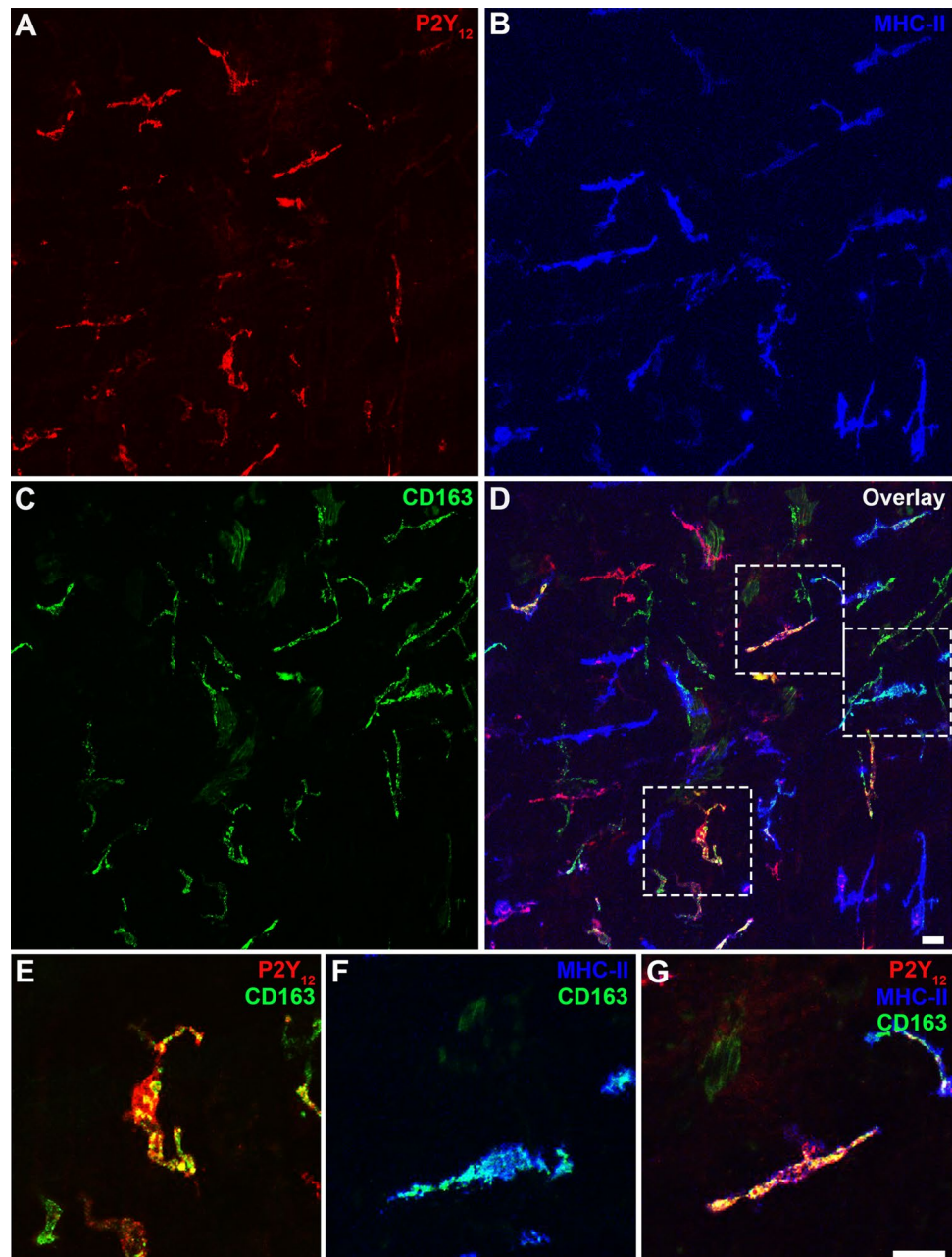
**Table 2** Quantification of cells co-expressing P2Y<sub>12</sub>, MHC-II, and CD163

|       | Antibody<br>MHC-II | CD163 | % of<br>P2Y <sub>12</sub> + Cells | Number of<br>P2Y <sub>12</sub> + Cells |
|-------|--------------------|-------|-----------------------------------|----------------------------------------|
| Total | -                  | -     | 1.0                               | 1                                      |
|       | -                  | +     | 17.2                              | 17                                     |
|       | +                  | -     | 24.2                              | 24                                     |
|       | +                  | +     | 57.6                              | 57                                     |
|       |                    |       | 100                               | 99                                     |

*MHC-II* major histocompatibility complex type II. 137 Cells Quantified

enteric neurons with subsequent effects on gut motor activity [51, 52]. BMP2 exerts suppressive effects on enteric neurons and macrophage ablation leads to hypercontractility and abnormal motility patterns. Interestingly, these effects are mainly mediated by CD163 + macrophages and are not recapitulated by Iba-1 + macrophages that repopulate the gut following ablation of resident cells [52]. Our results show that P2Y<sub>12</sub> receptors are enriched in this population and that blocking P2Y<sub>12</sub> increases spontaneous ENS activity and switches ganglionic responses to ADP from glia to neurons. This effect was most pronounced when ADP was applied in the bath solution but was also present when ADP was focally applied to individual ganglia. The differential

**Fig. 6** P2Y<sub>12</sub> receptors distribution in muscularis macrophage subtypes near the mouse colon myenteric plexus. Representative confocal images showing the immunoreactivity of P2Y<sub>12</sub> (A, red), major histocompatibility complex type II (MHC-II) (B, blue), CD163 (C, green), and overlay (D) in macrophages near the myenteric plexus of a wild-type (WT) mouse. Note P2Y<sub>12</sub> immunoreactivity is varied in MHC-II + and CD163 + macrophage subtypes (boxed regions correlate to E–G). Representative macrophage subtypes that are P2Y<sub>12</sub> + and CD163 + (E); MHC-II + and CD163 + (F); and P2Y<sub>12</sub> +, MHC-II + and CD163 + (G). Scale bars in D and G = 20 µm and applies to A–D and E–G, respectively. *n* = 3 animals



effects of P2Y<sub>12</sub> inhibition on focal and bath ADP application were likely due to different cellular activation patterns. Focal application directly activates neurons and glia within the myenteric plexus with minimal actions on cells outside the ganglion. In contrast, bath application stimulates cells outside the ganglia first, including P2Y<sub>12</sub>-expressing macrophages identified here.

The normal uniform response dominated by glia was mostly lost following P2Y<sub>12</sub> inhibition when responses were characterized by stochastic neuronal firing. While the data show that both neurons and glia exhibited increased spontaneous activity, glia responses under these conditions were small amplitude events and we cannot rule out the possibility that these apparent responses were not due to elevated activity in neuronal processes intertwined with glia. The switch to neuron dominated ADP responses was quite striking in many cases and suggests that P2Y<sub>12</sub> inhibition causes neuronal hyper- and glia hypo-activity. The mechanisms underlying neuronal hyperactivity likely involve a loss of inhibitory signals from macrophages such as BMP2 but the reason for the suppressive effects on glia is unclear. It is possible that increased activity of inhibitory nitrergic neurons under these conditions increases tonic inhibition of enteric glia. However, such an increase in inhibition would be expected to also decrease activity among the adjacent enteric neurons and this is not the case. Alternate explanations could be that macrophages secrete unknown factors that potentiate glial purinergic responses or that neurons normally potentiate glial activity and this mechanism is lost when macrophage cues are blocked. Glia help to refine signaling among enteric neurons and coordinate synaptic pathways so the loss of glial responses could contribute to the observed stochastic neuronal responses to ADP following P2Y<sub>12</sub> inhibition. These observations certainly warrant further work in this area to understand the nature of macrophage-neuron-glia communication and the cues involved that regulate ENS activity.

The results of this study provoke several questions, not the least of which is the reason for why such a system whereby macrophage P2Y<sub>12</sub> pathways suppress ENS activity has evolved. One possibility is that homeostatic macrophages slow gut motor activity to aid in digestion while a switch in macrophage phenotype during disease increases ENS activity and speeds expulsion of potential hazards. However, the source and nature of the driving signal for homeostatic macrophage P2Y<sub>12</sub> receptors is unclear. Our results would indicate a significant amount of constitutive activity of macrophage P2Y<sub>12</sub> receptors since blocking P2Y<sub>12</sub> increased spontaneous activity in the absence of other stimuli. ADP is the main known agonist for P2Y<sub>12</sub> and is produced by ATP hydrolysis through the actions of extracellular nucleoside triphosphate diphosphohydrolyase-2 (NTPDase2). Interestingly, NTPDase2 is nearly exclusively expressed by enteric glia in the mouse

colon [16]. This arrangement may indicate a signaling loop whereby glia generate signals for macrophage P2Y<sub>12</sub> receptors that stimulate the release of factors that suppress neuron activity. In support, deleting NTPDase2 decreases enteric neuron numbers and enhances inhibitory neuromuscular transmission in the gut [16]. Such mechanisms would be important to understand in more detail especially given that purines are major contributors to inflammatory signaling and changes in purinergic transmission between glia, neurons, and macrophages would have profound impacts on gut motor function.

There is a growing appreciation that ongoing neuro-immune communication regulates key aspects of gut physiology and disease [61]. The results of this study build on this concept by showing that macrophage P2Y<sub>12</sub> receptors are involved in regulating enteric neuron and glial responses to ADP mediated through P2Y<sub>1</sub> receptors. Our results agree with prior studies suggesting phenotypic and functional similarities between muscularis macrophages and microglia and with prior data showing suppressive effects of macrophage signals on ENS-driven motor activity. There are still many unanswered questions regarding this pathway including the source of the stimulus for P2Y<sub>12</sub> receptors, the nature of cues responsible for effects on enteric neurons and glia, and potential contributions to disease processes. However, this initial study adds an important link in mechanisms regulating neuro-immune crosstalk in the myenteric plexus. These mechanisms are relevant to changes in purinergic transmission that occur during gut inflammation in common diseases such as inflammatory bowel disease and irritable bowel syndrome. Several current antiplatelet drugs target P2Y<sub>12</sub> receptors such as Ticagrelor, Prasugrel, and Clopidogrel [62]. It is possible that understanding functions of macrophage P2Y<sub>12</sub> in more detail will identify ways to leverage these and other existing strategies to improve gut function in various disease states.

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**Author contributions** Conceptualization, B.D.G.; methodology, B.J.H., J.L.M., A.Z., L.S. and B.D.G.; formal analysis, B.D.G.; investigation, B.J.H., J.L.M., A.Z., L.S. and B.D.G.; resources, B.D.G.; data curation, J.L.M. and B.D.G.; writing—original draft preparation, B.J.H. and J.L.M.; writing—review and editing, J.L.M. and B.D.G.; visualization, B.J.H., J.L.M., L.S. and B.D.G.; supervision, B.D.G.; project administration, B.D.G.; funding acquisition, B.D.G. All authors have read and agreed to the published version of the manuscript.

**Data availability** Primary data described in this study will be made available upon reasonable request from the corresponding author.

## Declarations

**Competing interests** The authors declare no competing interests.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Disclosures** The Authors have no financial, professional, or personal conflicts that are relevant to the manuscript. Main resources generated from this study including mouse lines, analytic methods, study materials, and results will be made available upon request.

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