Macrophages and glia are the dominant P2X7-expressing cell types in the gut nervous system – no evidence for a role of neuronal P2X7 receptors in colitis

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1 **Summary**

2 Blockade or deletion of the pro-inflammatory P2X7 receptor channel has been shown
3 to reduce tissue damage and symptoms in models of inflammatory bowel disease (IBD)
4 and P2X7 receptors on enteric neurons were suggested to mediate neuronal death and
5 associated motility changes. Here we used P2X7-specific antibodies and nanobodies as
6 well as a BAC transgenic P2X7-EGFP reporter mouse model and *P2rx7^{-/-}* controls to
7 perform a detailed analysis of cell type-specific P2X7 expression and possible
8 overexpression effects in the enteric nervous system. In contrast to previous studies, we
9 did not detect P2X7 in neurons but found dominant expression in glia and macrophages
10 which closely interact with the neurons. P2X7 overexpression *per se* did not induce
11 significant pathological effects. Our data indicate that macrophages and/or glia account
12 for P2X7-mediated neuronal damage in IBD and provide a refined basis for the
13 exploration of P2X7-based therapeutic strategies.

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Introduction

Inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, are chronic relapsing-remitting inflammatory diseases with rising incidence in industrialized countries. They are thought to result from an abnormal host-microbial response of the intestinal immune system in a genetically susceptible host (Baumgart and Carding, 2007; Orholm et al., 1991). The resulting imbalance of homeostatic immunomodulatory mechanisms is characterized by increased macrophage and T-cell activation and up-regulation of proinflammatory cytokines (Sanchez-Munoz et al., 2008; Xavier and Podolsky, 2007). In addition, IBDs are associated with enteric neurodegeneration and abnormal gut motility and the resulting breakdown of enteric nervous system (ENS) control is a major contributing factor in the development of functional bowel disorders (Gulbransen et al., 2012; Niesler et al., 2021; Spear and Mawe, 2019).

Extracellular purines play important roles in immune cell regulation and enteric signaling (Antonioli et al., 2015; Burnstock, 2016; Diezmos et al., 2016; Gulbransen and Sharkey, 2009; Vuerich et al., 2020). High concentrations of ATP are sensed as a danger signal by the P2X7 receptor and it is generally accepted that activation of this non-specific cation channel acts as a secondary stimulus to induce NLRP3 inflammasome assembly and subsequent maturation and release of proinflammatory IL-1 β from macrophages. In addition, it has been shown that ATP, via the P2X7 receptor, induces apoptosis in several cell types (Di Virgilio et al., 2017; Di Virgilio et al., 2018; Proietti et al., 2014; Rissiek et al., 2015). While pathophysiological roles of P2X7 receptors have been well established in *P2rx7*^{-/-} mouse models (Kaczmarek-Hajek et al., 2012; Rumney and Gorecki, 2020), possible physiological roles are comparably poorly investigated.

P2X7 expression was shown to be increased in IBD patients and animal models, and its deletion or blockade reduced tissue damage in different rodent colitis models (Antonioli et al., 2019; Evangelinellis et al., 2021; Vuerich *et al.*, 2020). In a short phase IIa clinical study with a limited number of patients, oral application of an P2X7 antagonist ameliorated the pain component in the Crohn's disease activity index but did not change biomarkers of inflammation (Eser et al., 2015).

A variety of cell types in the colon has been shown to express P2X7 receptors and were involved in colitis pathology. These include immune cells like mast cells (Kurashima et al., 2012; Matsukawa et al., 2016; Ohbori et al., 2017) and different types of T cells (Figliuolo et al., 2017; Schenk et al., 2011), epithelial cells (Cesaro et al., 2010) (Hofman et al., 2015) (Diezmos et al., 2018), and enteric neurons of both the submucosal and myenteric plexus (da Silva et al., 2017; Palombit et al., 2019) (Antonioli et al., 2014; Evangelinellis *et al.*, 2021; Gulbransen *et al.*, 2012). P2X7-mediated neuronal cell death was implicated in motor dysfunction associated with colitis (da Silva *et al.*, 2017; Palombit *et al.*, 2019) (Antonioli *et al.*, 2014; Brown et al., 2016; Gulbransen *et al.*, 2012). Accordingly, elevated levels of extracellular ATP chronically activate a neuronal P2X7/Pannexin/Asc/caspase complex that leads to preferential loss of nitrergic neurons (Gulbransen *et al.*, 2012). While numerous mechanisms and cell types have been proposed to mediate protective effects of P2X7 blockade, localization of P2X7 receptors in specific cell types *in situ* has been challenging due to a lack of metabolically stable and selective pharmacological tools. In addition, specificity of the used antibodies has not been demonstrated in most studies. So far, the role of P2X7 receptors in neurons remains unclear but its cell type-specific expression, in particular in neurons versus immune cells, has important implications for potential treatments. In this study, we therefore set out to perform a

65 detailed analysis of P2X7-expressing cell-types in the colon using a BAC transgenic
66 P2X7-EGFP reporter mouse model (Kaczmarek-Hajek et al., 2018) in combination
67 with a P2X7-selective nanobody and *P2rx7^{-/-}* mice as negative control.

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Results

Expression of P2X7 in different layers of the colon

To determine P2X7 localization across the different layers of the colon, we first analyzed cross sections of the distal colon in the P2X7-EGFP transgenic mouse model (Kaczmarek-Hajek *et al.*, 2018) (Fig. 1A,B). In this mouse, an EGFP-tagged P2X7 receptor is overexpressed under the control of BAC-derived P2X7 promoters. The respective BAC contains the full-length *P2rx7* gene flanked by about 100 kb and 10 kb up- and downstream sequences, respectively, and careful analysis of P2X7-EGFP localization in the central nervous system (CNS) has previously confirmed an P2X7-EGFP expression pattern that is identical to the endogenous P2X7 receptor and revealed predominant expression in microglia, oligodendrocytes and Bergmann glia (Kaczmarek-Hajek *et al.*, 2018). Co-staining of colon slices with antibodies against GFP and P2X7 revealed good co-localization of both signals in single cells throughout the lamina propria (arrow heads in Fig. 1 B) and in two distinct layers adjacent to and in the muscular layer (arrows in Fig. 1 B). The cells in the lamina propria show more intense staining and based on their size, morphology, and localization, represent most likely macrophages, in agreement with the known high P2X7 expression in this cell type (Di Virgilio *et al.*, 2018). A weaker EGFP staining and more intense signal of the P2X7 antibody was seen in the apical side of the mucosa. Since the anti-P2X7 antibody should detect both P2X7-EGFP and endogenous P2X7, this could indicate a lower expression of the P2X7-EGFP protein in enterocytes which would be in agreement with the dynamic regulation of P2X7 expression in epithelial cells (Hofman *et al.*, 2015). Alternatively, unspecific staining or higher background signal of the P2X7 antibody in these cells or in this preparation could account for the difference. To further confirm epithelial P2X7-EGFP expression, we also performed co-staining of P2X7-EGFP with

an anti-P2X7 nanobody in planar mucosa preparations (Fig. 1C). This revealed an almost complete signal overlap, confirming a good correlation between P2X7-EGFP and endogenous P2X7 localization in epithelial cells. As presence of P2X7 receptors in enteric neurons has been frequently reported (Antonioli *et al.*, 2014; da Silva *et al.*, 2017; Gulbransen *et al.*, 2012; Palombit *et al.*, 2019) we next performed co-staining for P2X7 and the neuronal markers HuC/D (RNA-binding proteins HuC and HuD) to further determine the identity of the P2X7-expressing cells in the tunica muscularis (Fig. 1D). While both P2X7 and HuC/D signals were present in the same layers, they were clearly separated, indicating localization in different cell types or different subcellular structures.

To compare P2X7 expression in immune cells of the colon from P2X7-EGFP transgenic and WT mice, we performed flow cytometry and stained immune cells isolated from colon with an anti-P2X7 monoclonal antibody. Among T cells, CD4⁺, CD8⁺, and $\gamma\delta$ T cells from P2X7-EGFP transgenic and WT mice exhibited a similar P2X7 expression pattern, though cells from P2X7-EGFP transgenic mice showed in general higher P2X7 level due to transgene overexpression (Fig. 1E). Similarly, colon-derived innate immune cells, differentiated by the expression of CD11b and CD11c, showed a comparable P2X7 expression pattern in P2X7-EGFP transgenic and WT mice. Here, P2X7 expression was prominent on CD11b+CD11c⁻ and CD11b+CD11c⁺ cells co-expressing CD64⁺ (Fig. 1F).

P2X7 expression in macrophages of the myenteric plexus

To further investigate P2X7 expression in the muscular layer and identify the respective P2X7-expressing cell types, we next stained whole-mount preparations of the longitudinal muscle and myenteric plexus (LMMP) from P2X7-EGFP, wt, and P2rx7⁻

^{-/-} mice with the P2X7-specific nanobody. This revealed specific staining of enteric ganglia that appeared as mesh-like structures and large isolated extraganglionic cells with ramified morphology (Fig. 2A, compare also Figs. 3A and 5A). Staining of the latter was particularly strong in the P2X7-EGFP mice, which was confirmed by co-staining with an antibody against GFP (Fig. 2B). In contrast, the EGFP signal in the mesh-like structures was less consistent and had a patchy appearance, suggesting that EGFP was only present in some clusters of the cells that are forming this structure (compare two representative images in Fig. 2B). Co-staining with antibodies against F4/80 (EGF-like module-containing mucin-like hormone receptor-like 1), Iba1, and CD68 confirmed that the extraganglionic cells represent macrophages, in agreement with the known expression of P2X7 receptors in these cell types (Fig. 2C). Two different types of macrophages could be differentiated: macrophages with longitudinal orientation and bipolar morphology likely represent muscular macrophages whereas those with ramified morphology were localized in the vicinity of ganglia. Interestingly, only the ramified macrophages were positive for Iba1 (see also Suppl. Fig. 1). Since macrophage numbers appeared to be increased in P2X7-EGFP mice in comparison to wt mice (Fig. 2 D, compare also Fig. 3A), we performed a quantitative analysis by counting the F4/80-positive macrophages in randomly chosen images of both genotypes. This revealed about two times more macrophages in the myenteric plexus of P2X7-overexpressing mice (97.03 ± 12.91 and 202.7 ± 32.03 macrophages per image (0.125 mm^2 , $n=5$ images/mouse) in WT ($N=3$) and transgenic mice ($N=4$), respectively). Finally, co-stainings with antibodies against Anoctamin-1 (ANO-1) and CD117 excluded the presence of P2X7 receptors in myenteric interstitial cells of Cajal that function as pacemaker cells (Suppl. Fig. 2). The good correlation between stainings

of ANO-1 and CD117, which represents also a mast cell marker, is in agreement with a low number of mast cells in the myenteric plexus.

No evidence for P2X7 expression in neurons of the myenteric and submucosal plexuses

We next performed co-staining of HuC/D and P2X7 in the myenteric plexus (Fig. 3A and Suppl. Fig. 3) and the submucosal plexus (Fig. 3C) to examine a possible neuronal localisation of P2X7. These stainings revealed a clearly different localization of HuC/D and P2X7 in both wt and P2X7-EGFP mice. Similar results were obtained if co-stainings were performed using the anti-P2X7 nanobody or the anti-GFP antibody for P2X7 staining (Suppl. Fig. 3). As positive control for a neuronal expression pattern, we used antibodies against P2X2 and P2X4 receptors, which were previously shown to be expressed in enteric and other neurons (Galligan and North, 2004; Linan-Rico et al., 2015; Nieto-Pescador et al., 2013; Ren et al., 2003). Accordingly, these stainings revealed good co-localization of HuC/D with both receptors in neuronal cell bodies (Fig. 3B). While a uniform staining in all neurons was seen for the P2X4R, P2X2R staining appeared more variable between cells. In contrast to P2X2Rs, a punctate P2X4R staining was also detected in extraganglionic cells. Here P2X4 co-localized with CD68, in agreement with its known expression in lysosomes of macrophages (Suppl. Fig. 4), (Duveau et al., 2020).

HuC/D is localized in the cell body but under pathophysiological conditions, has been reported to show a prevalent nuclear localization (Desmet et al., 2014). Thus, to exclude P2X7 expression in specific neuronal substructures that are not stained with HuC/D, we also performed co-localization studies of P2X7 with β 3-tubulin, which stains neuronal processes, the neuronal marker proteins PGP9.5 (protein gene product 9.5),

calbindin and calretinin (Fig. 4A) as well as with the synaptic marker proteins synaptophysin, and vesicular glutamate transporter subtype 2 (vGlut2) (Fig. 4B). Again, none of these stainings revealed a co-localization with P2X7, thus arguing against a physiological expression of P2X7 protein in enteric neurons. Interestingly, the strong staining of the P2X7-EGFP-expressing macrophages enabled the visualization of close interactions of macrophages with the HuC/D-positive neurons (Fig. 4A), as previously described (Gabanyi et al., 2016; Muller et al., 2014; Phillips and Powley, 2012). In addition, a close association of macrophages with blood vessels in the submucosal plexus could be observed (Fig. 3C, right panel).

Identification of P2X7 expression in enteric glia cells

Based on the above data, we concluded that the intraganglionic P2X7-expressing cells that are forming the mesh-like structure in which the neurons are embedded represent most likely enteric glia cells. Enteric glia cells are generally differentiated by their localization and morphology whereas expression of specific marker proteins appears highly dynamic (Boesmans et al., 2015; Gulbransen and Sharkey, 2012). Using antibodies against the widely used glia marker GFAP (glial fibrillary acidic protein) and S100 β (S100 calcium-binding protein B), we detected a clear and specific co-localization of P2X-EGFP and S100 β in wt and transgenic mice (Fig. 5). However, while a uniform intraganglionic and interganglionic staining of S100 β is seen, the anti-GFP antibody shows a patchy pattern, in agreement with the incomplete EGFP P2X7 co-staining in these structures (compare also Fig. 2B above).

No induction of neuronal P2X7 expression in the DSS colitis model

The above data show a dominant expression of P2X7 in macrophages and glia cells but not in enteric neurons. This is in contrast to previous studies, where neuronal P2X7 receptors have been implicated in colitis-associated enteric neuron death (Gulbransen *et al.*, 2012). We therefore wondered, whether neuronal P2X7 expression could be induced by Bz-ATP application in an *ex vivo* model in which we incubated *myenteric plexus* preparations from wt, P2X7-EGFP and *P2rx7^{-/-}* mice for 60 min with 300 μ M Bz-ATP. In agreement with previous observations (Gulbransen *et al.*, 2012), Bz-ATP-treatment resulted in a reduction of neuron numbers. This appeared to be increased in transgenic mice, although the difference was not significant (Fig. 6A). Most importantly, no upregulation of P2X7 expression in neurons was observed, neither in wt, nor in transgenic mice. To examine a possible neuronal P2X7 upregulation under more physiological conditions and investigate if P2X7 overexpression is associated with a higher disease susceptibility, we next subjected wt and transgenic mice to the DSS colitis model. Figures 6B and C show only a slightly increased weight loss of DSS-treated P2X7-EGFP mice compared to DSS-treated wt mice at day 10, but no effects on the fecal score during the entire experiment and colon length at day of sacrifice. Microscopic analysis of HuC/D-stained tissue from DSS-treated mice shows massive tissue damage but no neuronal P2X7 localization (Fig. 6D).

Discussion

Despite its importance as a drug target, the localization, regulation, and functions of P2X7 receptors in different cell types remains controversial. This is partly due to the complex pharmacology of purinergic receptors and the unclear specificity of the available antibodies in different tissues. In the study presented above, we analysed the cell type-specific P2X7 localization in the colon with a focus on the myenteric plexus.

We demonstrate that myenteric macrophages and enteric glia represent the dominant P2X7-expressing cell types in the myenteric plexus under both physiological and pathological conditions and visualize the close interaction of the macrophages with enteric neurons. Based on our data we propose that P2X7 receptors in macrophages and/or glia cells indirectly contribute to neuronal damage in colitis pathology.

P2X7 in enteric neurons and glia

The enteric nervous system and enteric glia cells have emerged as important regulators of gut barrier function and homeostasis in gastrointestinal diseases including IBD (Sharkey et al., 2018). P2X7 receptors have been described in enteric neurons (da Silva et al., 2017; Palombit et al., 2019)(Antonioli et al., 2014; Brown et al., 2016; Gulbransen et al., 2012)(Evangelinellis et al., 2021) and a role of neuronal P2X7 receptors in neuronal cell death and colitis-associated motility disorders was proposed. However, using antibodies against a variety of neuronal marker proteins in distinct neuronal substructures in combination with P2X7-specific anti- and nanobodies and *P2rx7^{-/-}* mouse controls as well as a transgenic P2X7-EGFP reporter mouse model, we could not detect neuronal P2X7 receptors. In contrast, a clear staining was found with P2X2 and P2X4-specific antibodies that were used as positive controls. Based on these data we conclude that P2X7 protein is either absent in neurons or below the detection limit and that P2X7 receptors do not play a physiological role in enteric neurons.

Presence and function in glia cells

Enteric glia are closely associated with enteric neurons. They modulate motility, secretion, function of the epithelial barrier, neuroinflammation, and have also been involved in nociceptor sensitization and immune cell modulation (Grubisic et al., 2020). Using both an antibody and a nanobody against P2X7, we could detect identical patterns of P2X7 expression that were absent in *P2rx7^{-/-}* mice and correlated well with

the expression of the enteric glia cell marker S100 β . Since the S100 β antibody and both the P2X7-specific antibody and nanobody carry rabbit Fc domains, no co-staining could be performed for these proteins. Using the P2X7-EGFP reporter mouse, however, the co-localization of P2X7-EGFP and S100 β could be confirmed although P2X7-EGFP expression appeared more irregular than that of the endogenous P2X7. The reason for this is unclear but might reflect the highly dynamic protein expression and subtype differentiation that has been reported for enteric glia (Boesmans *et al.*, 2015; Gulbransen and Sharkey, 2012). In support of our finding, glial responses to ATP and expression of P2X7 in enteric glia was previously reported in rats (da Silva *et al.*, 2017; Mendes *et al.*, 2019; Van Crombruggen *et al.*, 2007; Vanderwinden *et al.*, 2003) and a glial cell line (Bhave *et al.*, 2017). However, purinergic neuron to glia signaling and ATP-mediated Ca²⁺ signals in enteric glia cells were generally believed to be mediated by P2Y1 receptors (Boesmans *et al.*, 2015; Gulbransen and Sharkey, 2012) (Boesmans *et al.*, 2013; Gomes *et al.*, 2009). Thus, while the importance of glia in neuro-immune interaction in colitis associated visceral pain has recently been shown (Grubisic *et al.*, 2020), the role of glial P2X7 receptors remains completely unclear. Interestingly, enteric glia in the myenteric plexus share marker proteins and functional properties with satellite glia in the DRG and astrocytes in the CNS and common roles in inflammatory processes and abdominal pain generation were described (for a review see (Morales-Soto and Gulbransen, 2019)). Both satellite glia and Bergmann glia, a specific type of S100 β -positive astrocytes, express also P2X7, suggesting common roles of P2X7 receptors in these cells. Further studies are needed to clarify the functions of glial P2X7 receptors and their involvement in colitis pathology and visceral pain.

Presence and function in macrophages

Based on their localization and specific functions, intestinal resident macrophages are classified into two major groups: the highly phagocytic lamina propria macrophages that represent the first line of defence against pathogens and act primarily as innate immune effector cells and the muscularis macrophages, which represent the dominant immune cell population in the muscular layer and closely and bidirectionally interact with the enteric nervous system and also with smooth muscle cells (Viola and Boeckxstaens, 2020). Although of different developmental origin, macrophages share many marker proteins with microglia, the resident immune cells of the CNS (Ginhoux and Prinz, 2015). Muscularis macrophages resemble microglia also morphologically and functionally (Verheijden et al., 2015). P2X7 receptors are highly expressed in microglia and macrophages and staining of overexpressed P2X7-EGFP with P2X7-specific nanobodies and antibodies or antibodies against GFP resulted in a clear and detailed visualization of macrophages and revealed their close interaction with neuronal cell bodies and processes, similar to microglia in the CNS. The P2X7-EGFP mice might therefore provide a good model to further study these interactions. As the maturation and release of cytokines, such as IL-1 β , is the best investigated function of P2X7 receptors in macrophages, this represents the most obvious mechanism how P2X7 could contribute to inflammation but also nociceptor sensitization (Grubisic *et al.*, 2020). P2X7-mediated production of reactive oxygen species (Kopp et al., 2019) could further contribute to ATP-induced neuronal damage or death as observed in (Gulbransen *et al.*, 2012). Since intestinal macrophages, similar to microglia in the CNS, are considered gatekeepers of tissue homeostasis, they are also discussed as therapeutic targets in IBD (Na et al., 2019). The physiological mechanism or relevance

of the increased macrophage number in P2X7-EGFP mice remains to be determined but its reminiscent of a trend towards higher microglia numbers that was found in a preliminary analysis of the retina of P2X7-EGFP mice (Kaczmarek-Hajek *et al.*, 2018). An intriguing possibility would be that P2X7 overexpression has an effect on macrophage migration and/or proliferation.

Involvement of P2X7 in other cell types

P2X7 receptors in other cell types besides macrophages and glia have also been involved in colitis pathology. Thus, P2X7 receptors are highly expressed in mast cells and P2X7-mediated mast cell activation was shown to initiate and exacerbate colitis via cytokine induction and neutrophil infiltration (Kurashima *et al.*, 2012). In support of this finding, inhibition of ATP-induced mast cell activation ameliorated colitis (Matsukawa *et al.*, 2016; Ohbori *et al.*, 2017). P2X7 receptors have also been involved in T cell activation and its expression is dynamically regulated during T cell differentiation. T cell subsets, including follicular helper T cells, regulatory T cells and Th17 T cells, are sensitive to P2X7-mediated cell death (Proietti *et al.*, 2014; Rissiek *et al.*, 2015) and it was reported that P2X7-mediated death of regulatory T cells decreases immune tolerance and thereby promotes inflammation (Figliuolo *et al.*, 2017; Schenk *et al.*, 2011). In epithelial cells, activation of P2X7 receptors was shown to induce IL-1 β release (Cesaro *et al.*, 2010) and thereby, suggested to promote inflammation. Furthermore, it was shown to inhibit epithelial cell proliferation and P2X7 receptor inhibition or deletion promoted re-epitheliation and recovery of inflammatory lesions but also enhanced tumor susceptibility (Hofman *et al.*, 2015). Based on a study on human colonic mucosal strips, it was suggested that epithelial P2X7 receptors represent therapeutic targets for IBD (Diezmos *et al.*, 2018).

In conclusion, P2X7 receptors in numerous cell types appear to contribute to colitis pathology as well as associated motility disorders and visceral sensitivity. Here we demonstrate that in the intestinal nervous system, P2X7 receptors are dominantly expressed in macrophages and S100 β -positive glia. This is resembling the situation in the CNS, where P2X7 receptors are highly expressed in microglia and S100 β -positive astrocytes. We propose that P2X7 fulfills similar functions in both systems and that ATP-induced neuronal damage in the enteric system is an indirect effect of macrophage or glial P2X7 activation. Since numerous studies support beneficial effects of P2X7 blockade in central and peripheral inflammatory diseases it represents a promising drug target. and this study provides a basis to further elucidate its cell type-specific functions and develop novel therapies.

Author contributions

Conceptualization: AN, TJ, JZ, SZ

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Investigation: TJ, JZ, TRJ, BR

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Declaration of Interest

The authors declare no conflict of interest

Figure Legends

Figure 1: P2X7 localization in different layers of the mouse colon. (A) Schematic drawing (created with BioRender.com) of the histological structure of the colon explaining the cell layers and perspectives of the images shown in B-D. (B) Co-staining of colonic cross section and a planar mucosa preparation from P2X7-EGFP mice with anti-P2X7 and anti-GFP antibodies. Arrow heads and arrows in (B) indicate examples of single cells and layers with clear co-localization. (D) Co-staining of colonic cross section with anti-P2X7 antibodies and an antibody against the neuronal marker HuCD. All scale bars: 50 μ m, cell nuclei were counterstained with DAPI (blue). (E) Flow cytometric analyses of colonic immune cells from wt (black) and transgenic (red) mice. Within the CD3⁺ T cell population, CD4⁺ and CD8⁺ $\alpha\beta$ T cells and $\gamma\delta$ T cells were analyzed for cell surface P2X7 expression using the mAb RH23A44. The right-shifted histogram indicates higher P2X7 expression in transgenic mice. (F) Comparison of P2X7 expression in innate immune cells. Among CD3-Ly6G-innate immune cells, CD11b⁺CD11c⁻, CD11b⁺CD11c⁺, and CD11b⁻CD11c⁺ cells were analyzed for cell surface P2X7 expression using mAb RH23A44. Cells were co-stained with anti-CD64.

Figure 2: P2X7 staining in whole mount LMMP preparations. (A) Specificity of the P2X7-specific nanobody. Preparations from P2X7-EGFP, wt, and *P2rx7*^{-/-} mice were stained as indicated and confocal images were taken with identical settings. Arrows indicate extraganglionar P2X7 positive cells. (B) Two representative confocal images of preparations from P2X7-EGFP transgenic mice co-stained with anti-GFP and anti-P2X7 antibody or nanobody, as indicated. (C) Tissues from P2X7-EGFP mice were co-stained with anti-P2X7 or anti-GFP antibodies and antibodies against different macrophage marker proteins. (D) Representative images of tissue from P2X7-EGFP

and wt mice stained with an antibody against the macrophage marker F4/80 and statistical analysis of macrophage numbers in both genotypes. n=5 images of 0.125 mm² from the distal colon were analyzed per mouse. Data are represented as mean $\pm$ SEM from N = 3 - 4 mice per group (large symbols). Numbers for individual mice are represented in small symbols with different color intensities for each mouse. 97.03 $\pm$ 12.91 and 202.7 $\pm$ 32.03 macrophages per image were counted in WT and transgenic mice, respectively. Statistical significance was tested by Welch's t-test (p=0,0438). Normal distribution (Shapiro-Wilk, Kolmogorov-Smirnov, QQ plot) was confirmed for each group.

Figure 3: Analysis of neuronal P2X7 localization in LMMP and submucosal plexus preparations. (A) Specificity of staining with the P2X7-specific antibody in combination with antibodies against the neuronal marker HuC/D. LMMP preparations from P2X7-EGFP, wt, and *P2rx7*^{-/-} mice are shown . (B) Co-staining of HuC/D and P2X2 receptors or P2X4 receptors (both used as positive controls for neuronal P2X expression) in LMMP preparations from P2X7-EGFP mice. (C) Co-staining of P2X7 (using the P2X7 nanobody) and HuC/D in submucosal plexus preparations of *P2rx7*^{-/-} and P2X7-EGFP mice, as indicated (upper panel). The images from P2X7-EGFP mice demonstrate staining of ganglia (right panel) and blood vessels (lower left image). V indicates the lumen of the blood vessel. The lower right image confirms P2X7 co-staining with the macrophage marker F4/80.

Figure. 4: Analysis of neuronal and synaptic P2X7 localization in whole mount LMMP preparations. Tissues from P2X7-EGFP and wt mice were co-stained with P2X7-specific nanobody or anti-GFP antibodies as indicated and antibodies against

different neuronal (A) or synaptic (B) marker proteins. β 3-Tub, β 3-tubulin; PGP9.5, protein gene product 9.5; Calr, Calretinin; Calb, Calbindin; vGluT2, vesicular glutamate transporter 2; Syn, synaptophysin.

Figure 5: Analysis of P2X7 localization in enteric glia of the myenteric plexus. (A)

Specificity of staining with the P2X7-specific antibody in combination with antibodies against the glial marker GFAP. LMMP preparations from P2X7-EGFP, wt, and *P2rx7*^{-/-} mice are shown. (C) Co-staining of P2X7-EGFP (using the anti-GFP antibody) and the glial marker S100 β in tissue from P2X7-EGFP mice. Two representative images are shown to demonstrate the patchy distribution of P2X7-EGFP expression in enteric glia.

Figure 6: Comparison of wt and P2X7-EGFP overexpressing mice in an *ex vivo* model and in the *in vivo* DSS colitis model. (A)

Representative images of LMMP preparations after treatment with 300 μ M Bz-ATP (line 15) and statistical analysis of neuron numbers per 0.501 mm². Data from knockout mice, and transgenic P2X7-EGFP lines 15 and 17, and their respective wildtype litter mates were analyzed by Brown-Forsythe and Welch ANOVA with a post-hoc Tamhane T2 test to correct for inhomogeneity of variance. Data are represented as mean $\pm$ SEM from N = 2 - 4 mice per group (large symbols). Numbers from n = 9 - 10 images per mouse are represented in small symbols with different color intensities for each mouse.

(B) Delta of weight variation (left) and fecal score (right) over time in P2X7-EGFP (TG, line 17) mice and their wild-type control littermates (WT) treated or not with DSS 1%. (C) Pictures (left) and statistical analysis of colon length (right) at day 10 in P2X7-EGFP (TG) mice and their wild-type control littermates (WT) treated or not with DSS

427 1%. ns: not significant; *: $P < 0.05$; **: $P < 0.01$. Fecal score: 0, normal solid faeces;
 428 1, soft faeces; 2, liquid faeces; 3, visible blood traces on faeces. Data are represented as
 429 mean $\pm$ SD. All mice were in C57/BL6 background. Female mice of 16 weeks of age
 430 were used in (B) and (C). (D) Triple staining of tissue from DSS-treated wt and P2X7-
 431 EGFP transgenic mice with antibodies against GFP, the neuronal marker HuC/D and
 432 the glial marker S100 β .

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Material and Methods

Animals

BAC transgenic P2X7-EGFP mice (Tg(RP24-114E20P2X7451P-StrepHis-EGFP)Ani15 and 17) were recently described (Kaczmarek-Hajek *et al.*, 2018) and in FVB/N or C57BL6 background. Identical P2X7 expression patterns were observed in both lines and strains. *P2rx7^{-/-}* mice (*P2rx7^{tmid(EUCOMM)Wtsi}*) were bred as described (Kaczmarek-Hajek *et al.*, 2018) and in C57BL/6 background. 12-18 weeks old animals of both sexes were used. Mice were housed in standard conditions (22°C, 12 h light–dark cycle, water/food ad libitum). All animal experiments were performed in accordance with the principles of the European Communities Council Directive (2010/63/EU). Procedures were reviewed and approved by the State of Upper Bavaria (55.2-1-54-2532-59-2016). All efforts were made to minimize suffering and number of animals.

Flow cytometric analysis

For purification of leukocytes from the large intestine, the colon was separated from the small intestine, opened by longitudinal section and feces was washed out thoroughly in Hanks Balanced Salt Solution (HBSS). The cleaned colon was cut into 1 cm pieces and further washed in 20 ml HBSS at 37°C for 30 min. Colon pieces were then digested for 30 min at 37°C in a digestion solution containing collagenase (1 mg/ml) and DNase (0.1 mg/ml). The generated cell suspension was filtered through a 100 µm cell strainer and centrifuged for 5 min at 300 g. Leukocytes were separated from debris by resuspending the pellet in 5 ml 33% Percoll solution (GE Healthcare). Erythrocytes were lysed using an ACK lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.2). Isolated cells were stained with an antibody mix in FACS buffer

(containing 1 mM EDTA (Sigma) and 0.1 % bovine serum albumin (Sigma)) for 30 min at 4 °C (antibodies see key resource table) to identify major immune cell populations, including T cells and innate immune cells. Cells were washed once with FACS buffer and were subsequently analyzed on a Becton Dickinson FACSymphony A3 cell analyzer.

Whole mount myenteric plexus and submucosal plexus preparation

For whole mount myenteric plexus and submucosal plexus preparation, mice were sacrificed by cervical dislocation and 1 cm segments were taken 1 cm aboral from the proximal colon and transferred to ice-cold Krebs solution (containing in mM: 117 NaCl, 4.7 KCl, 1.2 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, 11 glucose, aerated with carbogen to pH 7.4) in Sylgard (Dow Corning)-filled dissecting dishes. After flushing with Krebs buffer, segments were opened along the mesenteric border, pinned out, and fixed for 4 hr at 4°C (4% PFA and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4). Tissue was rinsed (3 × 10 min) with phosphate buffer and dissected in PBS (pH 7.4). Using forceps and a stereomicroscope, the mucosa and submucosa, were carefully removed and the submucosal plexus was then transferred to a microscopic slide. For myenteric plexus preparations, the circular musculature was also removed. Preparations were blocked (0.5%, Triton X-100, 0.1% NaN₃, 4% goat serum (Sigma-Aldrich) in PBS, 1 hr, RT), incubated with primary antibodies in the above solution (12 hr at RT), rinsed 3 × 10 min with PBS, and incubated with secondary antibodies (2 hr at RT). After washing (3x in PBS) preparations were mounted in PermaFluor (Thermo Fisher Scientific) on slides and images were acquired with a Zeiss 880 Airyscan confocal microscope and processed with ImageJ.

Preparation and staining of colon cryo-sections

Animals were anesthetized with isoflurane (IsoFlo®) and euthanized by cervical dislocation. The distal colon was prepared, flushed 3 times with PBS, embedded in Tissue Tek® and after freezing at -80°C cut in 10 µm cross-sections using a Cryostat Microm HM560 (Leica Biosystems).

For immunofluorescence staining, colon sections were dried for 1 h at RT, fixed for 20 min (2% PFA in 150 mM sodium phosphate buffer pH 7,4), and washed 2 x 10 min with PBS and 1 x 10 min with water. After drying at RT, slices were blocked for 1 h (10% goat serum, 0,5% Triton X-100, in PBS, pH 7,4) and then incubated overnight at 4°C with 100 µl of the primary antibody dilution in blocking solution using a humid chamber.

After washing (3x 10 min in PBS), 100 µl of the secondary antibody dilution in blocking buffer were applied for 1 h at RT and slices were then washed (2 x 10 min with PBS, 1 x 10 min with water). Sections were subsequently treated for 1 min with 0.1% DAPI, washed 3 x 5 min with PBS, and embedded in PermaFluor™ Mounting Medium. Preparations were analyzed using a Zeiss 880 Airyscan confocal microscope..

Counting analysis

For counting analysis, 1cm² whole-mount LMMP preparations taken 1 cm aboral from the beginning of distal colon were used. 5 and 10 z-stack images (0.5 mm²) were randomly selected for counting of macrophages or neurons, respectively. Only F4/80-positive macrophages or HuCD-positive neurons that were completely within the image were counted using the image J point tool.

Bz-ATP treatment

For *ex vivo* Bz-ATP treatment of LMMP preparations a modified protocol of (Gulbransen *et al.*, 2012) was used. The distal colon was prepared and fixed with minuten pins in a Sylgard dish, and opened along the mesenteric border as described above. Mucosa, submucosa, and plexus submucosus were prepared off the muscle layer. The circular muscle layer was maintained to avoid shearing stress of the MP. The tissue was then incubated for 1 h in a cell culture incubator with 20ml of 300 μ M BzATP in Krebs buffer (containing in mM: 117 NaCl, 4.7 KCl, 1.2 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, 11 glucose, aerated with carbogen to pH 7.4). The tissue was then washed 3 x 10 min with Krebs buffer and incubated for another 2 h with 20 ml Krebs buffer before fixation for 4 h in 4% PFA (4% PFA and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4) at 4°C. Subsequently, the circular muscle layer was removed and immunofluorescence staining was performed as described above.

DSS colitis model

BAC transgenic P2X7-EGFP mice (Tg(RP24-114E20P2X7451P-StrepHis-EGFP)Ani17) (Kaczmarek-Hajek *et al.*, 2018) and their wild-type (wt) control littermates were maintained and treated in accordance with the Swiss Federal Veterinary Office guidelines at IRB animal facility. Experiments were approved by "Dipartimento della Sanità e Socialità" with authorization numbers TI-25/2018 and TI-24/2019. For experimental procedures 16 weeks old female mice were used. Mice were housed in standard conditions (20-22°C, 12 h light–dark cycle, water/food ad libitum) and monitored and weighted daily. The following values were used to establish the daily fecal score: 0, normal solid faeces; 1, soft faeces; 2, liquid faeces; 3, visible blood

traces on faeces. All efforts were made to minimize suffering and limit the number of animals used in experiments.

Dextran sodium sulphate (DSS, TdB Labs) was added at a concentration of 1% in drinking water for 6 days, then DSS 1% was replaced with normal drinking water for other 4 days. After 10 days from the beginning of the experiment, mice were sacrificed by inhalation of CO₂ for retrieval of organs.

Statistical analysis

Graph Pad Prism software was used for statistical analysis and data were presented as means $\pm$ standard derivation (SD). Student's t- test, one way or two way ANOVA (Turkey's multiple comparisons test) was used to determine statistical differences between groups. Significance was accepted at * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$.

546 **Key Resource Table: Antibodies used for immunohistochemistry and flow**
 547 **cytometry**

Antibody	Supplier	Cat.# and RRID	Dilution
GFP (rb pAb)	Abcam	ab6556 AB_305564	1:2000
GFP (chk pAb)	Thermo Fisher	CA10262 AB_2534023	1:200
P2X7 C-term (rb pAb)	Synaptic Systems	177003 AB_887755	1:500
P2X7 ECD 7E2-rbIgG	Nolte lab	Nanobody rbIgG fusion construct	0.1 µg/mL
P2X2 (rb pAb)	Alomone	APR-003 AB_2040054	1:200
P2X4 (rb pAb)	Alomone	APR-002 AB_2040058	1:200
Iba1 (rb pAb)	WAKO	019-19741 AB_839504	1:500
CD68 (rat FA-11)	Biorad	MCA1957 AB_322219	1:200
F4/80 (rat BM8)	Thermo Fisher	14-4801-81 AB_467557	1:100
Hu C/D (ms 16A11)	Thermo Fisher	A-21271 AB_221448	1:200
β3-Tubulin (gp pAb)	Synaptic Systems	302304 AB_10805138	1:200
PGP9.5 (rb pAb)	Acurate Chemical & Scientific Corp.	UCRA95101 AB_2313685	1:30.000
Calretinin (ms 37C9)	Synaptic Systems	214111 AB_2619904	1:200
Calbindin D28k (ms CB-955)	Sigma-Aldrich	C9848 AB_476894	1:200
vGlut2 (rb pAb)	Synaptic Systems	135 403 AB_887883	1:500
Synaptophysin (ms 7.2)	Synaptic Systems	101011 AB_887824	1:500
GFAP (ms pAb)	Millipore	MAB360 AB_11212597	1:200
S100β (rb pAb)	Synaptic Systems	287003 AB_2620024	1:200
Ano-1 (rb pAb)	Alomone	ACL-011 AB_10920576	1:50
CD117 (rat ACK2)	eBioscience	14-1172-82 AB_467436	1:1.000
A594 gt anti-rb	Thermo Fisher	A11037 AB_2534095	1:400
A594 gt anti-ms	Thermo Fisher	A11032 AB_2534091	1:400

A594 gt anti-rat	Thermo Fisher	A11007 AB_10561522	1:400
A546 gt anti-ms	Thermo Fisher	A11003 AB_2534071	1:400
A488 gt anti-rb	Thermo Fisher	A11008 AB_143165	1:400
A488 gt anti-chk	Thermo Fisher	A11039 AB_2534096	1:400
anti-P2X7-A647	Nolte lab	-	1:100
anti-Ly6G-A700	Biolegend	127622	1:100
anti-CD3eBv650	Biolegend	100229	1:100
anti-CD8a-Bv711	Biolegend	100747	1:100
anti-CD4-Bv605	Biolegend	100547	1:100
anti-gdTCR-Bv421	Biolegend	118119	1:100
anti-CD11b-APC- Cy7	Biolegend	101225	1:100
anti-CD11c-PE- Dazzle	Biolegend	117347	1:100
anti-CD64-PE-Cy7	Biolegend	139313	1:100

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Fig. 1

Fig. 2

Fig. 3

Fig. 4

A

B

Fig. 5

Fig. 6

A

B

C

D