TRPV4 stimulates colonic afferents through mucosal release of ATP and glutamate

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RESEARCH ARTICLE

TRPV4 stimulates colonic afferents through mucosal release of ATP and glutamate

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Background and Purpose: Abdominal pain is a leading cause of morbidity for people living with gastrointestinal disease. Whereas the transient receptor potential vanilloid 4 (TRPV4) ion channel has been implicated in the pathogenesis of abdominal pain, the relative paucity of TRPV4 expression in colon-projecting sensory neurons suggests that non-neuronal cells may contribute to TRPV4-mediated nociceptor stimulation.

Experimental Approach: Changes in murine colonic afferent activity were examined using ex vivo electrophysiology in tissues with the gut mucosa present or removed. ATP and glutamate release were measured by bioluminescence assays from human colon organoid cultures and mouse colon. Dorsal root ganglion sensory neuron activity was evaluated by Ca^{2+} imaging when cultured alone or co-cultured with colonic mucosa.

Key Results: Bath application of TRPV4 agonist GSK1016790A elicited a robust increase in murine colonic afferent activity, which was abolished by removing the gut mucosa. GSK1016790A promoted ATP and glutamate release from human colon organoid cultures and mouse colon. Inhibition of ATP degradation in mouse colon enhanced the afferent response to GSK1016790A. Pretreatment with purinoceptor or glutamate receptor antagonists attenuated and abolished the response to GSK1016790A when given alone or in combination, respectively. Sensory neurons co-cultured with colonic mucosal cells produced a marked increase in intracellular Ca^{2+} to GSK1016790A compared with neurons cultured alone.

Conclusion and Implications: Our data indicate that mucosal release of ATP and glutamate is responsible for the stimulation of colonic afferents following TRPV4

Abbreviations: CRD, colorectal distension; DRG, dorsal root ganglion; IBS-D, irritable bowel syndrome with diarrhoea; KYNA, kynurenic acid; LSN, lumbar splanchnic nerve; MTEP, 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline; PAR2, protease-activated receptor 2; POM-1, polyoxotungstate-1; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; scRNA-seq, single-cell RNA sequencing; TRPV4, transient receptor potential vanilloid 4.

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activation. These findings highlight an opportunity to target the gut mucosa for the development of new visceral analgesics.

KEYWORDS
ATP, colonic afferents, glutamate, intestinal mucosa, TRPV4, visceral hypersensitivity

1 | INTRODUCTION

The **transient receptor potential vanilloid 4 (TRPV4) channel** is a non-selective cation channel that displays greater conductance for divalent over monovalent ions, resulting in the influx of extracellular Ca^{2+} upon activation. TRPV4 is a polymodal sensor of heat and hypo-osmolality and can be activated by endogenous lipids, including **arachidonic acid** and its epoxyeicosatrienoic acid (EET) metabolite **5,6-EET** (Güler et al., 2002; Liedtke et al., 2000; Watanabe et al., 2003). Whereas TRPV4 appears to play roles in homeostatic functions, such as detecting extracellular fluid osmolality and shear stress-induced vasodilation, it also has been implicated in disease (Köhler et al., 2006; Lechner et al., 2011). For example, levels of 5,6-EET are elevated in colonic biopsies from patients with irritable bowel syndrome with diarrhoea (IBS-D) and correlate with the magnitude and frequency of abdominal pain, thus linking TRPV4 activation to disease pathophysiology in IBS-D (Cenac et al., 2015). Consistent with these findings, stimulation of TRPV4 with 5,6-EET and other agonists such as **4α-phorbol 12,13-didecanoate (4α-PDD)** or **GSK1016790A** is pro-nociceptive, increasing intracellular [Ca^{2+}] in sensory neurons, promoting nerve discharge in afferent fibres innervating the bowel and enhancing pseudoeffective pain behaviours such as the visceromotor response evoked by colorectal distension (CRD) (Brierley et al., 2008; Cenac et al., 2008; McGuire et al., 2018; Poole et al., 2013; Sipe et al., 2008). In addition, TRPV4 serves as a downstream effector of visceral hypersensitivity evoked by other algogenic and inflammatory mediators elevated in the bowel of IBS-D patients such as tryptase, **serotonin** (5-HT) and **histamine** (Barbara et al., 2004, 2007; Dunlop et al., 2005). These effects are driven by the potentiation of TRPV4-mediated ion currents, due to increased membrane expression and phosphorylation of tyrosine and serine/threonine residues in TRPV4 following cognate receptor activation, that is, **protease-activated receptor 2 (PAR2)** or **histamine H_1 receptors** (Cenac et al., 2010; Fan et al., 2009; Poole et al., 2013). In particular, TRPV4 is critical for the activation of colonic afferents and development of visceral hypersensitivity evoked by PAR2 due to the generation of 5,6-EET following PAR2 stimulation (Cenac et al., 2008; Grant et al., 2007; Poole et al., 2013; Sipe et al., 2008).

Our recent single-cell RNA sequencing (scRNA-seq) analysis of colonic sensory neurons revealed that TRPV4 mRNA is present in a relatively small population of colonic neurons (Hockley et al., 2019). This contrasts with the marked excitatory effect of TRPV4 on colonic afferent activity discussed above and thus suggests that non-neuronal cell types may provide a substantial contribution to TRPV4-mediated colonic afferent responses by releasing secondary signalling mediators. Consistent with this hypothesis, **ATP** release following TRPV4 activation has previously been demonstrated from the oesophageal epithelium and in astrocytes where TRPV4 also evokes **glutamate** release (Mihara et al., 2011; Shibasaki et al., 2014). These observations prompted us to evaluate the contribution of ATP and glutamate release from the gut in TRPV4-mediated colonic afferent activation.

What is already known

- Activation of TRPV4 causes visceral hypersensitivity via the stimulation of colonic afferents.

What does this study add

- TRPV4-mediated colonic afferent activation is dependent on mucosal release of ATP and glutamate.

What is the clinical significance

- Mucosal TRPV4-mediated colonic afferent activation provides a gut restricted target for treating abdominal pain.

2 | METHODS

2.1 | Materials

Stock concentrations were prepared for all drugs as recommended and diluted to working concentrations on the day of testing. GSK1016790A, ATP, **D-aminophosphonovaleric acid (D-AP5)**, **kynurenic acid (KYNA)** and **capsaicin** were purchased from Sigma-Aldrich. **HC067047**, **CGS 15943**, **pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS)**, polyoxotungstate-1 (POM-1), **bradykinin**, **ASP7663**, **L-701,324** and **2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX)** were purchased from Tocris. **3-[(2-Methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) hydrochloride** was purchased from Abcam, and RO4 (AF-353) was a gift from Dr Wendy Winchester.

2.2 | Ethical approval

All animal experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 and following local ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

2.3 | Animals

Experiments were performed using tissue from male C57BL/6J mice (8–14 weeks) purchased from Charles River (Cambridge, UK, RRID: IMSR_JAX:000664). This species and strain were selected to allow us to compare our findings with previous studies exploring investigating TRPV4-associated roles in the gut and visceral hypersensitivity (Cenac et al., 2015; Sipe et al., 2008). Mice were conventionally housed in groups of up to eight per cage with nesting material, enrichment (shelter, tubes and chewing blocks), and access to food and water ad libitum. Rooms were temperature-controlled (21°C) with a 12-h light/dark cycle. Animals were euthanized by a rising concentration of CO_2 followed by exsanguination.

2.4 | Ex vivo electrophysiological recording from the lumbar splanchnic nerve (LSN)

2.4.1 | Nerve recording

Electrophysiological recordings were conducted as previously described (Barker et al., 2023). Briefly, following euthanasia, the colorectum with associated LSNs was isolated. Tissue was cannulated as a tubular preparation in a recording bath with a Sylgard base (Dow Corning, UK), luminally perfused (200 µl·min^{-1}) by a syringe pump (Harvard Apparatus, MA) and serosally superfused (7.5 ml·min^{-1}, 31–34°C) with carbogenated (95% O_2–5% CO_2) Krebs buffer (in mM: NaCl 124, KCl 4.8, NaH_2PO_4 1.3, $CaCl_2$ 2.5, $MgSO_4{\cdot}7H_2O$ 1.2, D(+)-glucose 11.1 and $NaHCO_3$ 25). Krebs buffer was supplemented with **atropine** (10 µM, Sigma-Aldrich) and **nifedipine** (10 µM, Sigma-Aldrich) to inhibit smooth muscle activity (Bhebhe et al., 2023; Cibert-Goton et al., 2021). For mucosa-free preparations, the tissue was opened along the mesenteric border, pinned to the Sylgard base with the mucosal side up and the mucosal layer gently dissected away (Figure S1a–d). Complete removal of the mucosa was confirmed using haematoxylin and eosin staining (Figure S1e,f). Confirmation of spontaneous contractility and comparison of baseline activity before and after mucosal removal were used to ensure that tissue viability was retained (Figure S1g). Multi-unit LSN activity was recorded from teased nerve bundles using borosilicate glass suction electrodes. Signals were amplified (gain 5 kHz), bandpass filtered (100–1300 Hz; Neurolog, Digitimer Ltd, UK) and digitally filtered (Humbug, Quest Scientific, Canada) to remove 50-Hz noise. Data were digitized at 20 kHz (Micro1401; Cambridge Electronic Design, UK) prior to display using Spike2 software (Cambridge Electronic Design, UK).

2.4.2 | Electrophysiology protocols

Data were collected following a 30-min stabilization period to establish baseline firing, with a signal-to-noise ratio sufficient to allow accurate spike counting. Agonists and antagonists were diluted from stock solutions into final volumes of 20- or 50-ml Krebs respectively and applied by serosal superfusion via the in-line heater. A washout period of 30 min was given between repeat drug applications on the same tissue preparation. Antagonist studies were conducted blinded, except for the coloured drug PPADS (red/orange) where blinding was not possible.

2.4.3 | Data analysis

Action potentials were determined by counting spiking waveforms passing through a threshold set at twice the background noise (typically 30–50 µV), and ongoing nerve discharge was expressed as a mean frequency (time base 60 s). The change in nerve activity after treatment was determined by subtracting a baseline pretreatment firing rate (mean firing for the 6-min period prior to treatment) from the mean firing rate. Data from individual experiments were averaged to give a mean and standard error of the mean (SEM) value of nerve discharge over time. The area under the curve (AUC) was calculated as an indicator of multi-unit nerve activity for 900 s following initial GSK1016790A, bradykinin or ATP application.

2.5 | Primary culture of mouse dorsal root ganglion (DRG) neurons

DRG neurons were cultured as previously described (Higham et al., 2024). Briefly, isolated DRGs (T12–L5, spinal segments innervating the distal colon) were incubated in 1-mg·ml^{-1} type 1A collagenase (15 min) followed by 1-mg·ml^{-1} trypsin (30 min), both with 6-mg·ml^{-1} bovine serum albumin (BSA) in Leibovitz's L-15 Medium and GlutaMAX™ Supplement (supplemented with 2.6% [v/v] $NaHCO_3$). DRGs were resuspended in 2-ml Leibovitz's L-15 Medium and GlutaMAX™ Supplement containing 10% (v/v) fetal bovine serum (FBS), 2.6% (v/v) $NaHCO_3$, 1.5% (v/v) D(+)-glucose, and 300-U·ml^{-1} penicillin and 0.3-mg·ml^{-1} streptomycin (P/S). DRGs were mechanically dissociated and centrifuged (100 *g*), and the supernatant was collected for five triturations. Following centrifugation and resuspension, the supernatant (50 µl) was plated onto poly-D-lysine-coated 35-mm glass bottom culture dishes (MatTek, MA, USA), further coated with laminin (Thermo Fisher). Dishes were incubated for 3 h to allow cell adhesion before flooding with 2-ml supplemented L-15 medium and cultured for 24 h. All incubations were carried out at 37°C with 5% CO_2.

2.6 | Primary culture and co-culture of mouse colonic mucosal cells

The protocol for mucosal cell culture was adapted from Psichas et al. (2017). Luminal contents were flushed from the colon with cold phosphate-buffered saline (PBS), and the outer muscle layer was removed. Tissue was minced and digested with 0.35-mg·ml^{-1} type 1A collagenase (5× 10 min). Crypt-containing fractions were centrifuged (100 *g*) for 3 min and resuspended with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 2% (v/v) $NaHCO_3$, 1.5% (v/v) glucose, P/S and Y-27632 (10 μM) before filtering through a 100-μm cell strainer. Crypts were plated at a density of 1000 crypts per cm^2 onto poly-D-lysine-coated 35-mm glass bottom culture dishes (MatTek, MA, USA), further coated with laminin (Thermo Fisher). Dishes were incubated for 3 h before flooding with 2-ml supplemented DMEM. For co-culture experiments, DRGs were isolated and cultured as described, then plated on top of mixed colonic mucosal cell cultures isolated 24 h previously and flooded before use 24 h later. All incubations were carried out at 37°C with 5% CO_2.

2.7 | RNA extraction and polymerase chain reaction (PCR)

Total RNA was isolated from primary cultured colonic mucosal cells using the RNeasy® Mini Kit and QIAshredder (Qiagen) according to the manufacturer's instructions and DNase (Promega) treated to maximize RNA quality. A 500-ng RNA sample was subjected to reverse transcription to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). A negative reverse transcription reaction was performed without reverse transcriptase (RT) to check for genomic DNA contamination during the PCR. PCR was performed with specific primer sets (Merck) directed against TRPV4 and β-actin (Table 1) with cycling conditions of 1 cycle of 95°C for 30 s and 30 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 1 min.

2.8 | Colon histology and staining

The colonic mucosa and underlying muscle were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) overnight at 4°C, followed by cryopreservation in 30% (w/v) sucrose overnight (Sigma-Aldrich) at 4°C. Tissue was embedded in an M-1 Embedding Matrix (Thermo Fisher Scientific) and snap frozen in liquid nitrogen. Embedded tissue was sectioned into 10-μm slices using a Leica CM3000 cryostat and mounted on SuperFrost slides (Thermo Fisher Scientific). Sections were stained with haematoxylin (0.2% w/v; Sigma-Aldrich) and eosin (0.5% w/v; Acros Organics) to confirm mucosa removal. Slides were mounted with 70% (v/v) glycerol and imaged using an Olympus Bx51 microscope.

TABLE 1 Primers designed for the amplification of TRPV4 and the housekeeping gene β-actin, based upon the reference genome for mouse (*Mus musculus*, GRCm39).

Primer	Sequence
TRPV4-F	5′-TCCTATCTGTGTGCCATGGTC-3′
TRPV4-R	5′-AGAGAATTCACTCCAGGGCATT-3′
β-Actin-F	5′-CGATATCGCTGCGCTGGTC-3′
β-Actin-R	5′-TTCTCCATGTCGTCCCAGTTG-3′

Abbreviation: TRPV4, transient receptor potential vanilloid 4.

2.9 | Immunocytochemistry

Unless otherwise stated, all incubations were carried out at room temperature. Antibodies used are summarized in Table 2. MatTek plated cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.05% Triton X-100 for 5 min and blocked in 5% donkey serum in 0.2% Triton X-100 for 30 min. Cells were incubated in primary antibody for 24 h at 4°C. Secondary antibodies were incubated for 1 h followed by 4′,6-diamidino-2-phenylindole (DAPI; 1:1000, Abcam) for 5 min. Immunostained cells were imaged on an Olympus Bx51 microscope, and images were captured on a Qicam CCD camera (QImaging) with a 100-ms exposure and false coloured (green: βIII-tubulin [Abcam, Cat# ab18207, RRID:AB_444319]; blue: DAPI; and red: E-cadherin [R&D Systems, Cat# AF748, RRID:AB_355568] and vimentin [Novus Biologicals, Cat# NB300-223, RRID:AB_10003206]). Staining was not observed when primary antibodies were omitted (data not shown). The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology*.

2.10 | Ca^{2+} imaging

2.10.1 | Data acquisition

Extracellular solution (ECS, in mM: NaCl 140, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, D(+)-glucose 4 and HEPES 10) was prepared and adjusted to pH 7.4 using NaOH and an osmolality of 290–310 mOsm using sucrose. Cells were incubated for 30 min with 100 μl of 10-μM Ca^{2+} indicator Fluo-4-AM (37°C; shielded from light). For inhibitor studies requiring pre-incubation, 200 μl of antagonist was added for 10 min prior to imaging. Dishes were mounted on the stage of an inverted microscope (Nikon Eclipse TE-2000S), and cells were visualized at 10× magnification with bright-field illumination. Fluorescent images were captured with a CCD camera (Retiga Electro, Photometrics) at 2.5 fps with 100-ms exposure and a 470-nm filter cube (Cairn Research, Faversham, UK). Emission at 520 nm was recorded with μManager32. All protocols began with a 10-s baseline of ECS before drug superfusion to establish baseline fluorescence. Finally, cells were stimulated with 50-mM KCl for 10 s to determine cell viability, identify neuronal cells and allow normalization of fluorescence. A fresh dish was used for each protocol, and all solutions were diluted in ECS.

TABLE 2 Antibodies used for immunohistochemistry of primary DRG and colonic mucosal cell co-cultures.

Primary antibodies					
Antigen	**Host**	**Dilution**	**Source**	**Cat#**	**RRID**
βIII-Tubulin	Rabbit	1:500	Abcam	ab18207	RRID:AB_444319
E-cadherin	Goat	1:100	R&D Systems	AF748	RRID:AB_355568
Vimentin	Chicken	1:400	Novus Biologicals	NB300-223	RRID:AB_10003206
Secondary antibodies					
Antibody label		**Dilution**	**Source**	**Cat#**	**RRID**
Donkey anti-rabbit IgG Alexa Fluor 488		1:1000	Thermo Fisher	A-21206	RRID:AB_2535792
Donkey anti-goat IgG Alexa Fluor 568		1:1000	Thermo Fisher	A-11057	RRID:AB_2534104
Donkey anti-chicken IgG Alexa Fluor 594		1:1000	Thermo Fisher	A-78951	RRID:AB_2921073

Abbreviations: DRG, dorsal root ganglion; IgG, immunoglobulin; RRID, Research Resource Identifier.

2.10.2 | Data analysis

Individual cells were circled on a bright-field image, and outlines were overlaid onto fluorescent images using ImageJ (National Institutes of Health [NIH], MA, USA). Pixel intensity was measured and analysed with custom-written scripts in RStudio (RStudio, MA, USA). Background fluorescence was subtracted from each cell, and fluorescence intensity (F) was baseline corrected and normalized to the maximum fluorescence elicited during 50-mM KCl stimulation (F_{max}). Maximum KCl fluorescence was denoted as 1 F/F_{max}. Further analysis was confined to cells with a fluorescence increase ≥5 standard deviations away from the mean baseline before 50-mM KCl application. Neurons were deemed responsive to a drug challenge if a fluorescence increase of 0.1 F/F_{max} was seen in response to drug perfusion. The proportion of responsive neurons and magnitude of the fluorescence response were measured for each experiment, with peak responses calculated from averaging fluorescence values from individual neurons at each time point.

2.11 | ATP and glutamate secretion assays

2.11.1 | Mouse colon tissue preparation

The ascending colon to rectum was removed, luminal contents were flushed and the tissue opened longitudinally. Incubations were performed at 37°C in a 1.5-ml Eppendorf tube containing Krebs solution with NTPDase 1–3 inhibitor POM-1 (100 μM). Samples were stabilized in 1-ml Krebs solution for 10 min before Krebs was replaced with GSK1016790A (0.1–10 μM) or dimethyl sulfoxide (DMSO) (0.01%). For inhibition studies, samples were incubated with HC067047 (10 μM) or DMSO (0.01%) for 10 min prior to GSK1016790A (10 μM). After 10 min, supernatants were removed and cellular debris were removed by centrifugation (200 *g*, 5 min, 4°C).

2.11.2 | Human colon organoid preparation

Human tissue no longer required for pathological assessment was collected following informed patient consent from individuals undergoing surgical bowel resections as part of their standard clinical treatment for colorectal cancer or endometriosis at the Toulouse University Hospital (CODECOH National Agreement DC2015-2443, COLIC Collection). Colon crypts were isolated and cultured as previously described (d'Aldebert et al., 2020). Organoid cultures were expanded and seeded on transwell inserts (24-well plates, 0.4-μm pore size, Corning COSTAR). Organoids were collected and dissociated into single cells by incubation in pre-warmed TrypLE Express Enzyme for 10 min at 37°C in agitation (1000 rpm). After addition of 5-ml Advanced DMEM/F12 with 2-mM GlutaMAX, 10-mM HEPES and 10% FBS and centrifugation (400 rpm, 5 min, 4°C), dissociated cells were resuspended in organoid culture medium (50:50 volume L-WRN CM and Advanced DMEM/F12, GlutaMAX/HEPES containing FBS (37.5:7 v/v DMEM/FBS)). Cells were seeded at a density of 4×10^5 on individual transwells pre-coated with Matrigel (1:40 v/v in PBS). A 600-μl culture medium supplemented with **SB431542** (10 μM) and Y-27632 (10 μM) was added to the basolateral side, and cells were incubated at 37°C in 5% CO_2. Medium was replaced every 2–3 days until cells reached confluence. On the day of the experiment, cells were washed with PBS, medium was replaced with PBS in the basolateral compartment, and PBS containing GSK1016790A (1 μM) or DMSO (0.01%) was added to the apical compartment. Medium from the basolateral compartment was collected before and 10 min after apical exposure to GSK1016790A or DMSO.

2.11.3 | Secretion assays

ATP secretion was determined using the CellTiter-Glo® 2.0 (Promega) ATP kit. Glutamate secretion was determined using the Glutamate-Glo® Assay (Promega) with samples diluted in Krebs 1:40 prior to measurement. All samples were run as technical triplicates, and

luminescence was measured with a plate reader (FluoStar Omega; BMG Labtech). Concentrations were calculated from luminescence signals using a standard curve generated from ATP or glutamate standards and linear regression using Prism 9 (GraphPad Software, USA).

2.12 | Statistics

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022). Statistical analysis and compilation of figures were conducted using GraphPad Prism Version 9.0.0 for Windows (GraphPad, Inc.). Data were examined to ensure that the assumptions for parametric analysis were fulfilled and the appropriate non-parametric analyses were used if required. Normality testing was performed using the Shapiro–Wilk test. For two groups, Student's *t*-test or Mann–Whitney *U*-test was applied as appropriate. For pairwise comparison, a paired Student's *t*-test was performed. Nerve response traces were analysing using two-way repeated-measures analysis of variance (ANOVA). DRG and co-culture peak F/F_{max} values and % neuron responses were analysed using two-way ANOVA. For more than two groups, a one-way ANOVA was used. Holm–Šídák's multiple comparisons post hoc test was performed if F achieved $P < 0.05$. Data are displayed as mean ± SEM. The level of probability deemed to constitute statistical significance was established as $^*P < 0.05$.

2.13 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in https://www.guidetopharmacology.org/ and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos, et al., 2021; Alexander, Mathie, et al., 2021).

3 | RESULTS

3.1 | TRPV4-induced colonic afferent activation is inhibited by purinoceptor and *N*-methyl-D-aspartate (NMDA) receptor antagonists

Bath application of the selective TRPV4 agonist GSK1016790A at a minimum of 100 nM was required to evoke an increase in colonic afferent firing (Figure S2a,b, Thorneloe et al., 2008; Willette et al., 2008). The response to GSK1016790A at 10 μM consisted of a robust, prolonged increase in nerve discharge that did not return to baseline within 30 min of treatment or elicited a further increase in firing to a second repeat application of GSK1016790A (Figures 1a and S2c). Pretreatment with the TRPV4 antagonist HC067047 (10 μM) abolished the colonic afferent response to GSK1016790A (10 μM) but not to bradykinin (1 μM), highlighting the selectivity of HC067047 (Figures S2a and S3).

The contribution of ATP and glutamate to the activation of colonic afferents by GSK1016790A was evaluated using a cocktail of purinoceptor antagonists previously shown to block the afferent response to ATP (Figure S4) and **NMDA receptor** antagonists D-AP5, KYNA and L-701,324. The AUC of the afferent response to GSK1016790A (10 μM) was significantly reduced by the purinoceptor antagonist cocktail and by NMDA receptor antagonists D-AP5 (200 μM), KYNA (30 μM) and L-701,324 (100 nM, Figure 1e). Furthermore, combined incubation with both the purinoceptor antagonist cocktail and D-AP5 virtually abolished afferent firing in response to GSK1016790A (Figure 1e). The inhibition of afferent firing to GSK1016790A by all pretreatments persisted over time (Figures 1c–e and S5). By contrast, pretreatment with the purinoceptor antagonist cocktail, D-AP5 alone or in combination, had no significant effect on the AUC and colonic afferent response to bradykinin (1 μM, Figure 1f–i). These findings indicate that ATP and glutamate are required for the activation of colonic afferents by GSK1016790A and that the respective antagonist pretreatments are specific for the afferent response evoked by GSK1016790A rather than a generalized reduction in afferent sensitivity.

3.2 | TRPV4-evoked colonic afferent activation requires the gut mucosa

Having established that ATP and glutamate are involved in TRPV4-mediated stimulation of colonic afferents by GSK1016790A, we next sought to understand its site of action. Investigation of *Trpv4* expression alongside other algogenic receptors ***Bdkrb2***, **Trpa1** and ***Trpv1*** within our published scRNA-seq database of transcript expression in mouse colon-projecting neurons (Hockley et al., 2019, https://hockley.shinyapps.io/ColonicRNAseq/) revealed that expression of *Trpv4* is restricted to a small population of neurons (39 of 314 cells) in contrast to the wider expression of *Bdkrb2*, *Trpa1* and *Trpv1*, which are found in 206, 170 and 271 of the 314 cells studied, respectively (Figure 2a,b). The majority of cells expressing *Trpv4*, *Bdkrb2* and *Trpa1* also co-expressed *Trpv1* (84.6%, 94.1% and 94.7%, respectively), consistent with expression in nociceptors (albeit a small subpopulation for *Trpv4*) and a role in nociception (Figure 2c–f). Given that GSK1016790A evokes a robust colonic afferent response greater than that evoked by bradykinin (Figure 1), in contrast to the restricted expression of *Trpv4* relative to *Bdkrb2* (or *Trpa1* and *Trpv1*) in colonic sensory neurons, we hypothesized that non-neuronal cell types contributed to the activation of colonic afferents by GSK1016790A. To interrogate this possibility further, we examined the afferent response to GSK1016790A (10 μM) following removal of the colonic mucosa, which abolished the AUC and afferent response to GSK1016790A (Figure 2g,h). This was in marked contrast to the AUC and colonic afferent response to bradykinin (1 μM) or the TRPA1 agonist ASP7663 (100 μM), which were unchanged following removal of the colonic mucosa (Figure 2i–l). These findings demonstrate that the mucosal expression of TRPV4 is required for the robust activation of colonic afferents by GSK1016790A.

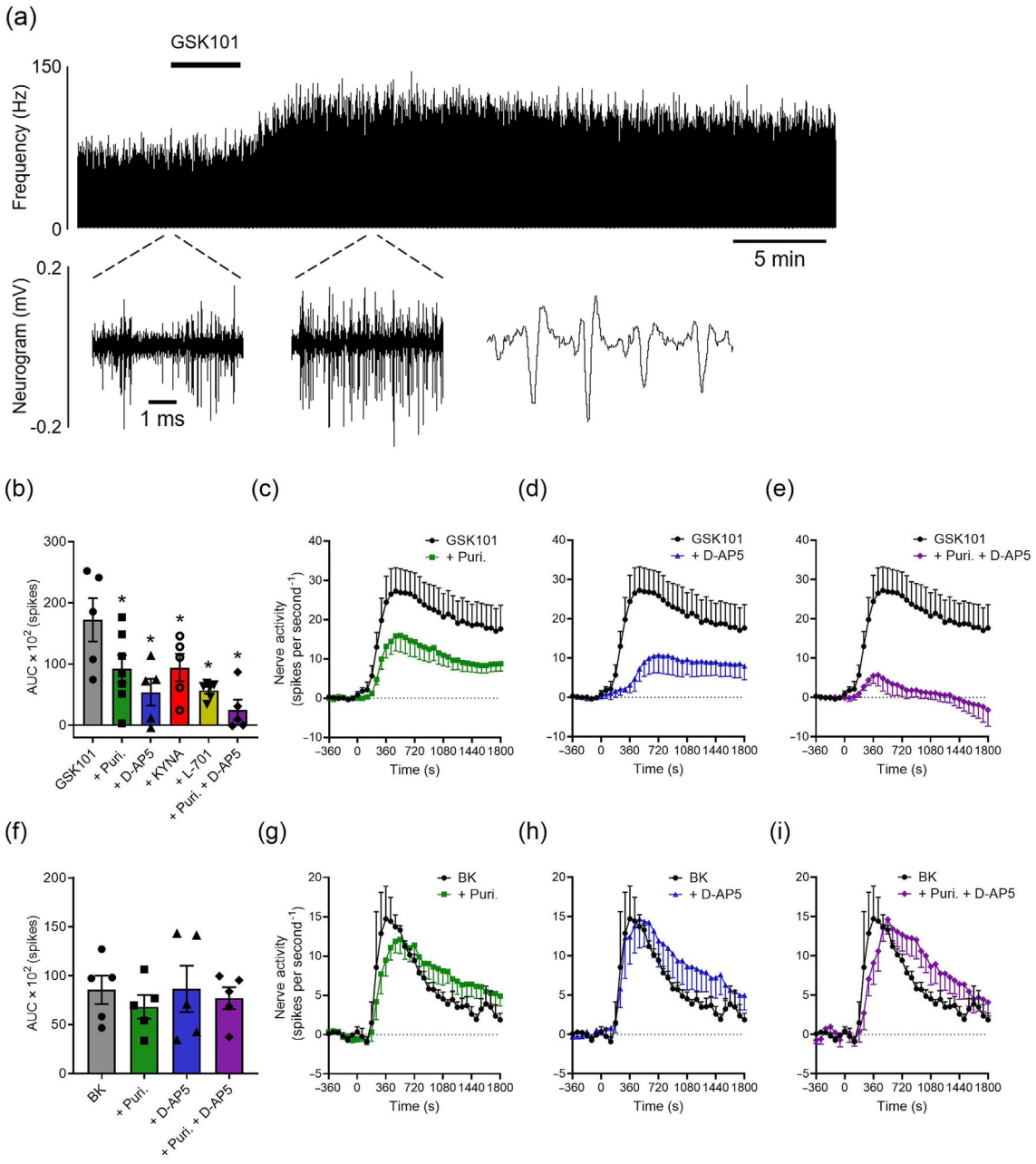

FIGURE 1 ATP and glutamate drive transient receptor potential vanilloid 4 (TRPV4)-induced lumbar splanchnic nerve (LSN) firing. (a) Example rate histogram demonstrating an increase in LSN activity following application of GSK1016790A (GSK101, 10 μM). Bin width = 1 s. (b) Area under the curve (AUC) of nerve activity from $t = 0$–900 s following application of GSK101 (10 μM) alone or following pretreatment with the purinoceptor antagonist cocktail (Puri.: CGS 15943 [3 μM], RO4 [10 μM] and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid [PPADS] [100 μM]), D-aminophosphonovaleric acid (D-AP5) (200 μM), kynurenic acid (KYNA, 30 μM), L-701,324 (L-701, 100 nM), or the purinoceptor antagonist cocktail and D-AP5 combined. One-way analysis of variance (ANOVA) with Holm–Šídák's multiple comparisons test compared to the GSK101-only group (n = 5–7). (c) Change in nerve activity following application of GSK101 (10 μM) alone or following pretreatment with the purinoceptor antagonist cocktail. (d) Change in nerve activity following application of GSK101 (10 μM) alone or following pretreatment with D-AP5. (e) Change in nerve activity following application of GSK101 (10 μM) alone or following pretreatment with the purinoceptor antagonist cocktail and D-AP5. GSK101-only dataset repeated in (c)–(e) for ease of interpretation. (c–e) Two-way repeated-measures ANOVA ($N = 5$–7). (f) AUC of nerve activity from $t = 0$–900 s following application of bradykinin (1 μM) alone or following pretreatment with the purinoceptor antagonist cocktail (Puri.: CGS 15943 [3 μM], RO4 [10 μM] and PPADS [100 μM]), D-AP5 (200 μM), or the purinoceptor antagonist cocktail and D-AP5 combined. One-way ANOVA (n = 5). (g) Change in nerve activity following application of bradykinin alone or following pretreatment with the purinoceptor antagonist cocktail. (h) Change in nerve activity following application of bradykinin alone or following pretreatment with D-AP5. (i) Change in nerve activity following application of bradykinin alone or following pretreatment with the purinoceptor antagonist cocktail and D-AP5. Bradykinin-only dataset repeated in (g)–(i) for ease of interpretation. (g–i) Two-way repeated-measures ANOVA (n = 5). GSK101 and bradykinin applied from $t = 0$–240 s. $^{*}P < 0.05$.

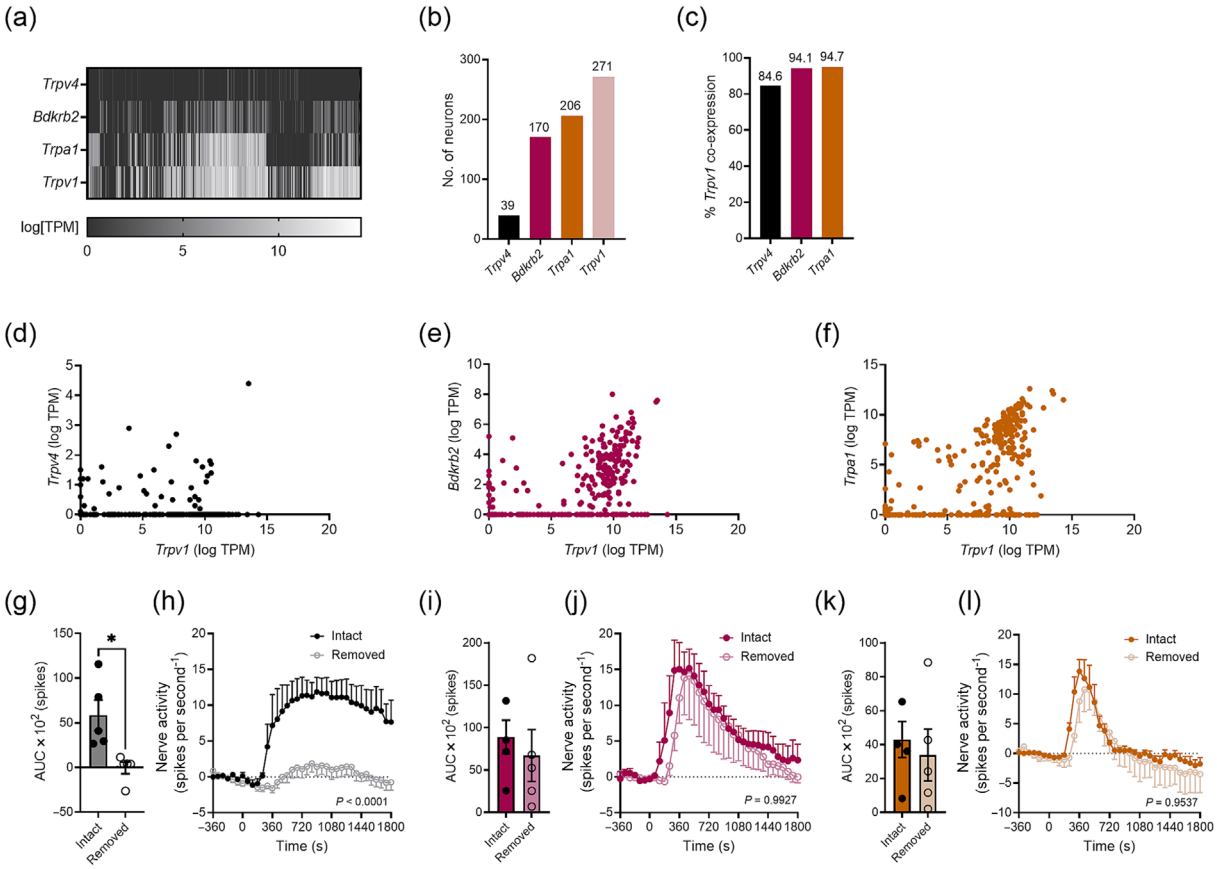

FIGURE 2 Transient receptor potential vanilloid 4 (TRPV4) acts on the colonic mucosa to induce robust colonic afferent activity. (a) Heatmap of gene expression in murine colonic sensory neurons as log[TPM] (transcripts per million). (b) Number of neurons expressing *Trpv4*, *Bdkrb2* and *Trpa1* transcripts in 314 cells tested. (c) % of *Trpv4*-, *Bdkrb2*- and *Trpa1*-positive colonic sensory neurons co-expressing *Trpv1*. (d) Scatter plot of *Trpv1* co-expression with *Trpv4*. (e) Scatter plot of *Trpv1* co-expression with *Bdkrb2*. (f) Scatter plot of *Trpv1* co-expression with *Trpa1*. Each point denotes a single neuron. Data from (a)–(f) redrawn from Hockley et al. (2019). (g) Area under the curve (AUC) of nerve activity from $t = 0$–900 s after the addition of GSK1016790A (10 μM) in preparations with the mucosa intact and removed. Unpaired *t*-test (n = 5). (h) Change in nerve activity following application of GSK1016790A (10 μM) added from $t = 0$–240 s in preparations with the mucosa intact and removed. Two-way repeated-measures analysis of variance (ANOVA) (n = 5). (i) AUC of nerve activity from $t = 0$–900 s after the addition of bradykinin (1 μM) in preparations with the mucosa intact and removed. Unpaired *t*-test (n = 5). (j) Change in nerve activity following application of bradykinin (1 μM) added from $t = 0$–240 s in preparations with the mucosa intact and removed. Two-way repeated-measures ANOVA (n = 5). (k) AUC of nerve activity from $t = 0$–900 s after the addition of ASP7663 (100 μM) in preparations with the mucosa intact and removed. Unpaired *t*-test ($n = 5$). (l) Change in nerve activity following application of ASP7663 (100 μM) added from $t = 0$–240 s in preparations with the mucosa intact and removed. Two-way repeated-measures ANOVA (n = 5). *$P < 0.05$.

3.3 | TRPV4 releases ATP to drive colonic afferent activation

To confirm that activation of TRPV4 by GSK1016790A evokes ATP release from the gut mucosa, we measured ATP levels in the basolateral compartment of human colon organoids stimulated apically with GSK1016790A, observing a significant increase in ATP release following addition of 1 μM of GSK1016790A with no sex-specific effect (Figures 3a and S6a). Consistent with this finding, we observed significant concentration-dependent increases in ATP (Figure S6b) following incubation of male mouse colon with GSK1016790A, which were abolished at 10 μM by pretreatment with HC067047 (10 μM, Figure 3b). Furthermore, pretreatment with the NTPDase 1–3 inhibitor POM-1 (100 μM) produced a marked increase in the AUC and colonic afferent response to GSK1016790A (10 μM, Figure 3c–e) but had no effect on the AUC and colonic afferent response to bradykinin (1 μM, Figure 3f–h), providing further evidence for a specific ATP-mediated increase in afferent activity following stimulation of TRPV4.

3.4 | TRPV4-driven colonic afferent activation is mediated by release of glutamate

Because the receptor subunits for alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (**AMPA**) and **kainate** receptors as well as excitatory **glutamate-activated metabotropic receptors** are expressed alongside NMDA receptor subunits in colonic sensory afferents (Figure 4a), we next sought to confirm the contribution of glutamate

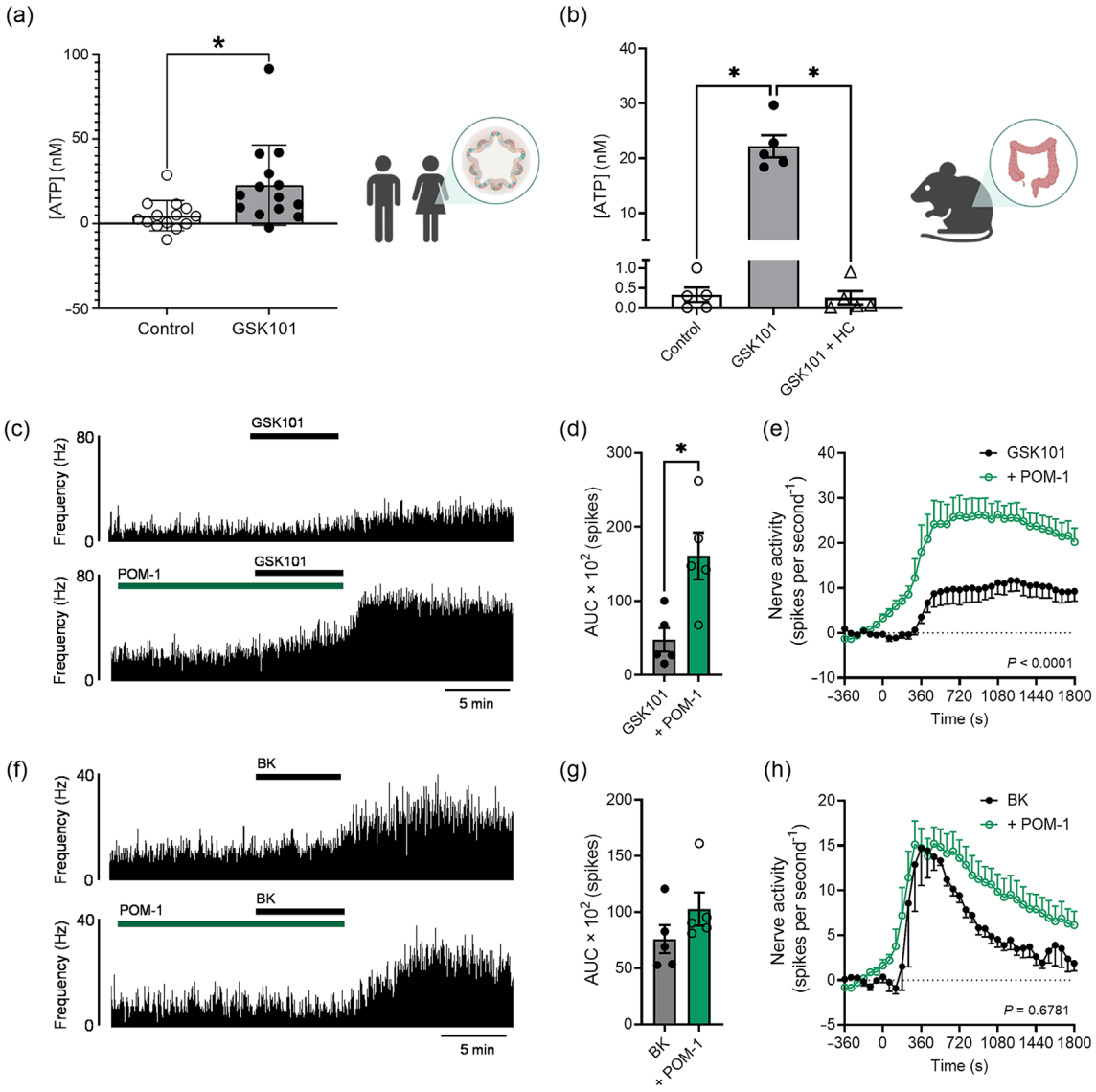

FIGURE 3 Transient receptor potential vanilloid 4 (TRPV4)-mediated colonic afferent activation requires ATP. (a) Differences in ATP concentration measured in the basolateral compartment of human colon organoids before and 10 min after apical exposure to dimethyl sulfoxide (DMSO) (0.01%, control) or GSK1016790A (1 μM). Paired *t*-test (n = 14). (b) ATP concentration measured from mouse colonic tissue supernatant after stimulation for 10 min with DMSO (0.01%, control), GSK1016790A (10 μM) or pretreatment for 10 min with HC067047 (HC, 10 μM) followed by GSK1016790A (10 μM) for 10 min. One-way analysis of variance (ANOVA) with Holm–Šídák's multiple comparisons test (n = 5). (c) Rate histogram of lumbar splanchnic nerve (LSN) activity following application of GSK1016790A (10 μM) alone or following pretreatment with polyoxotungstate-1 (POM-1) (100 μM) in two separate nerve preparations. Bin width = 1 s (d) Area under the curve (AUC) of nerve activity from *t* = 0–900 s after application of GSK1016790A (10 μM) alone or following pretreatment with POM-1 (100 μM). Unpaired *t*-test (n = 5). (e) Change in nerve activity after GSK1016790A (10 μM) added from *t* = 0–240 s alone or following pretreatment with POM-1 (100 μM). Two-way repeated-measures ANOVA (*n* = 5). (f) Rate histogram of LSN activity following application of bradykinin (1 μM) alone or following pretreatment with POM-1 (100 μM) in two separate nerve preparations. Bin width = 1 s. (g) AUC of nerve activity from *t* = 0–900 s after application of bradykinin (1 μM) alone or following pretreatment with POM-1 (100 μM). Unpaired *t*-test (n = 5). (h) Change in nerve activity after bradykinin (1 μM) added at *t* = 0 s alone or following pretreatment with POM-1 (100 μM). Two-way repeated-measures ANOVA (n = 5). Illustrations produced using BioRender. *$P < 0.05$.

and other excitatory glutamate receptors to the colonic afferent response to GSK1016790A. We detected a significant increase in glutamate release from the basolateral compartment of human colon organoids stimulated apically with GSK1016790A (1 μM, Figure 4b), with no sex-specific effect being observed (Figure S7a), establishing that GSK1016790A provokes glutamate release from the human intestinal mucosa. In addition, we also observed a significant increase in glutamate release from the mouse colon to 10-μM GSK1016790A (Figure S7b), which was abolished by pretreatment with HC067047 (10 μM, Figure 4c), confirming that responses were TRPV4-mediated. Consistent with the release of glutamate and glutamate receptor expression in colonic neurons, the AUC of the afferent response to

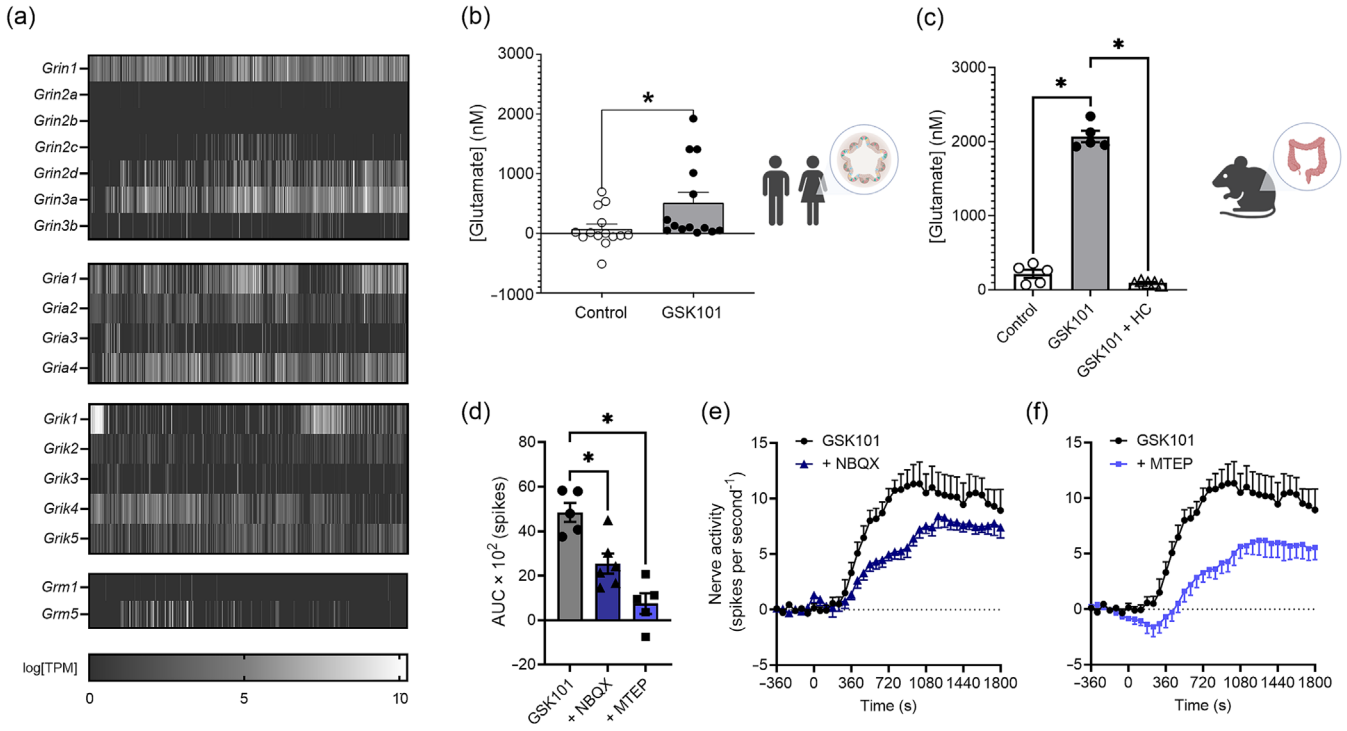

FIGURE 4 Transient receptor potential vanilloid 4 (TRPV4)-mediated colonic afferent activation requires glutamate. (a) Heatmap of gene expression in murine colonic sensory neurons as log[TPM] (transcripts per million) for subunits of *N*-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methylisooxazole-4-propionic acid (AMPA), and kainate receptors and excitatory metabotropic glutamate receptors. Data redrawn from Hockley et al. (2019). (b) Differences in glutamate concentration measured in the basolateral compartment of human colon organoids before and 10 min after apical exposure to dimethyl sulfoxide (DMSO) (0.01%, control) or GSK1016790A (1 μM). Paired *t*-test (n = 14). (c) Glutamate concentration measured from mouse colonic tissue supernatant after stimulation for 10 min with DMSO (0.01%, control), GSK1016790A (10 μM) or pretreatment for 10 min with HC067047 (HC, 10 μM) followed by GSK1016790A (10 μM) for 10 min. One-way analysis of variance (ANOVA) with Holm–Šídák's multiple comparisons test (n = 5). (d) Area under the curve (AUC) of nerve activity from *t* = 0–900 s following GSK1016790A alone or after pretreatment with 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline (NBQX) (10 μM) or 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl] pyridine (MTEP) hydrochloride (100 μM). One-way ANOVA with Holm–Šídák's multiple comparisons test compared to GSK1016790A alone (n = 5–6). (e) Change in nerve activity after GSK1016790A (10 μM) added from *t* = 0–240 s alone or following pretreatment with NBQX (10 μM). (f) Change in nerve activity after GSK1016790A (10 μM) added from *t* = 0–240 s alone or following pretreatment with MTEP hydrochloride (100 μM). GSK1016790A-only dataset repeated in (e) and (f) for ease of comparison. (e, f) Two-way repeated-measures ANOVA (n = 5–6). Illustrations produced using BioRender. **P* < 0.05.

GSK1016790A (10 μM) was markedly reduced by pretreatment with the combined AMPA/kainate blocker NBQX (10 μM) or the **mGlu$_5$** antagonist MTEP hydrochloride (100 μM, Figure 4d), effects that persisted over time (Figure 4e,f). These data demonstrate a role for AMPA/kainate and mGlu$_5$ receptors alongside NMDA receptors in TRPV4-mediated colonic afferent activation.

3.5 | TRPV4 is expressed and functional in the gut mucosa

To confirm the contribution made by the mucosa to TRPV4-driven afferent activation, we examined the effect of GSK1016790A on intracellular Ca^{2+} concentrations ($[Ca^{2+}]$) in sensory neurons isolated from mouse DRG cultured alone or co-cultured with primary cells from the mouse colonic mucosa. Cells obtained from the mouse colonic mucosa were predominantly comprised of epithelial cells and fibroblasts and they expressed transcripts corresponding to TRPV4 (Figure 5a–c). The application of GSK1016790A (1000 nM) to DRG neurons co-cultured with mucosal cells produced an increase in $[Ca^{2+}]_i$ of significantly greater magnitude compared to DRG neurons cultured alone (Figure 5d–f). Furthermore, GSK1016790A elicited an increase in $[Ca^{2+}]_i$ in a significantly greater proportion of co-cultured DRG neurons (Figure 5g), thus highlighting a difference in the mechanism of Ca^{2+} mobilization and requirement for mucosal cells to fully realize the more marked response in DRG neurons to GSK1016790A. Comparison of the size of co-cultured DRG neurons demonstrated that smaller sized cells produced a response when stimulated by application of 1000 nM GSK1016790A indicative of the activation of a nociceptive population (Figure 5h). The increase in $[Ca^{2+}]_i$ within co-cultured DRG neurons to GSK1016790A (1000 nM) was significantly inhibited by pretreatment with the TRPV4 antagonist HC067047 (10 μM), a purinoceptor antagonist cocktail and the NMDA receptor antagonist D-AP5 and, to an even greater extent, by combining the

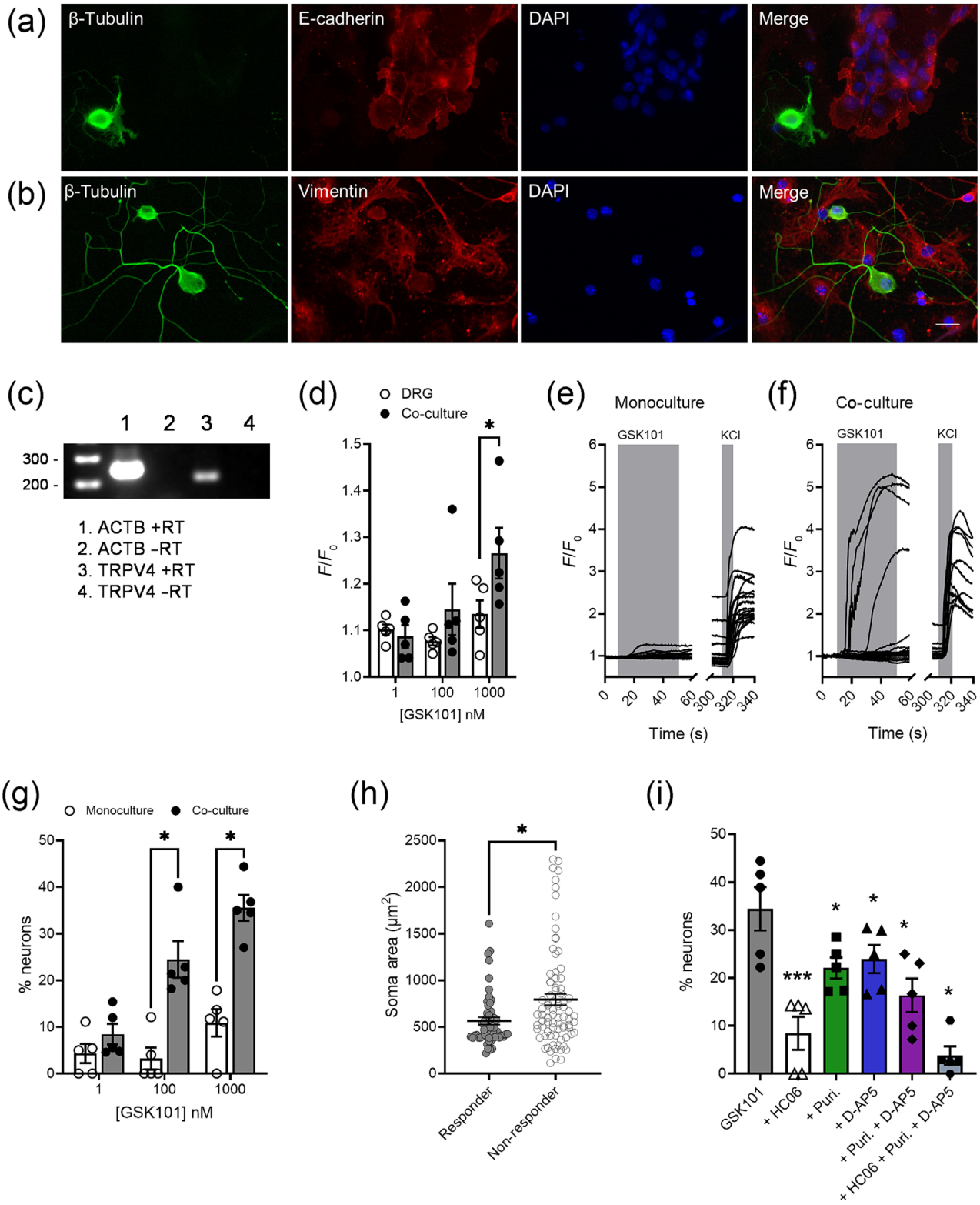

FIGURE 5 Legend on next page.

FIGURE 5 Mouse colonic mucosal cells enhance dorsal root ganglion (DRG) neuron responses to GSK1016790A. (a) DRG neuron and colonic mucosal cell co-cultures stained with anti-β-tubulin antibody (green, neuronal marker), anti-e-cadherin antibody (red, epithelial marker) and 4′,6-diamidino-2-phenylindole (DAPI) (blue, nuclear marker). (b) DRG neuron and colonic mucosal cell co-cultures stained with anti-β-tubulin antibody (green), anti-vimentin antibody (red, fibroblast marker) and DAPI (blue). Scale bar = 20 μm. (c) Reverse transcription polymerase chain reaction (RT-PCR) analysis of transient receptor potential vanilloid 4 (TRPV4) and β-actin (ACTB) mRNA expression in primary cultured colonic mucosal cells. No signals were detected when reverse transcriptase (RT) was omitted. (d) Peak F/F_0 values of GSK1016790A (1–1000 nM) responses from DRG neuron monocultures and co-cultured dishes. Two-way analysis of variance (ANOVA) with Holm–Šídák's multiple comparisons test (N = 5; n = 5, no. of dishes; no. of animals). (e) Representative response profiles from individual neurons in DRG neuron monocultures in response to GSK1016790A (1000 nM) applied from t = 10–40 s (shaded grey area) and KCl (50 mM) applied from t = 310–320 s (shaded grey area). (f) Representative response profiles from individual neurons in co-cultures in response to GSK1016790A (1000 nM) applied from t = 10–40 s (shaded grey area) and KCl (50 mM) applied from t = 310–320 s (shaded grey area). (g) Proportion of GSK1016790A (1–1000 nM) sensitive neurons in DRG neuron monocultures and co-cultured dishes. Two-way ANOVA with Holm–Šídák's multiple comparisons test (N = 5; n = 5, no. of dishes; no. of animals). (h) Neuronal soma area for GSK1016790A (1000 nM) sensitive and insensitive DRG neurons from co-cultured dishes. Mann–Whitney U-test (n = 54 [responder] and 81 [non-responder], no. of cells). (i) % of co-cultured DRG neurons responding to GSK1016790A (1000 nM) alone or following pretreatment with HC067047 (HC06, 10 μM), the purinoceptor antagonist, D-aminophosphonovaleric acid (D-AP5) (200 μM), the purinoceptor antagonist cocktail and D-AP5 combined, or HC06, the purinoceptor antagonist cocktail and D-AP5 combined. One-way ANOVA with Holm–Šídák's multiple comparisons test compared to GSK1016790A alone (N = 5; n = 5, no. of dishes; no. of animals). *P < 0.05.

purinoceptor antagonist cocktail with D-AP5 or with D-AP5 and HC067047 (Figure 5i). Overall, these results indicate that the DRG neuron response to GSK1016790A was mediated through the release of ATP and glutamate following activation of TRPV4.

4 | DISCUSSION

An improved understanding of how nociceptors are stimulated during gastrointestinal diseases is key to the development of new visceral analgesic drugs. A large body of data has identified TRPV4 as an important effector of visceral pain and hypersensitivity, making it an attractive therapeutic target for the treatment of gastrointestinal pain (Vergnolle, 2014). Recent single-cell transcriptomic data indicate that only a small proportion of colon-projecting neurons express TRPV4, suggesting that the pro-nociceptive effects of TRPV4 are also driven by non-neuronal cells (Hockley et al., 2019). Consequently, the goal of this study was to explore the contribution of the gut mucosa to TRPV4-mediated colonic afferent activation. Our findings revealed an essential role for the mucosa, which can release ATP and glutamate following agonist activation of TRPV4 to stimulate colonic afferents downstream (Figure 6).

Within the study, we observed a robust increase in colonic afferent firing in response to application of the TRPV4 agonist GSK1016790A, which was sustained over time, indicative of channel sensitization, likely through the activation of signalling cascades by secondary signalling mediators culminating in the production of TRPV4-sensitizing agents such as **prostaglandin E_2** and histamine (Alessandri-Haber et al., 2003; Cenac et al., 2010). This effect was abolished by pretreatment with the TRPV4 antagonist HC067047, thus confirming the selectivity of GSK1016790A for TRPV4. The stimulatory effect of GSK1016790A was lost following removal of the mucosa, which demonstrates a mucosal site of action. Importantly, the colonic afferent response to both bradykinin and the TRPA1 agonist ASP7663 was unchanged between mucosa intact and removed

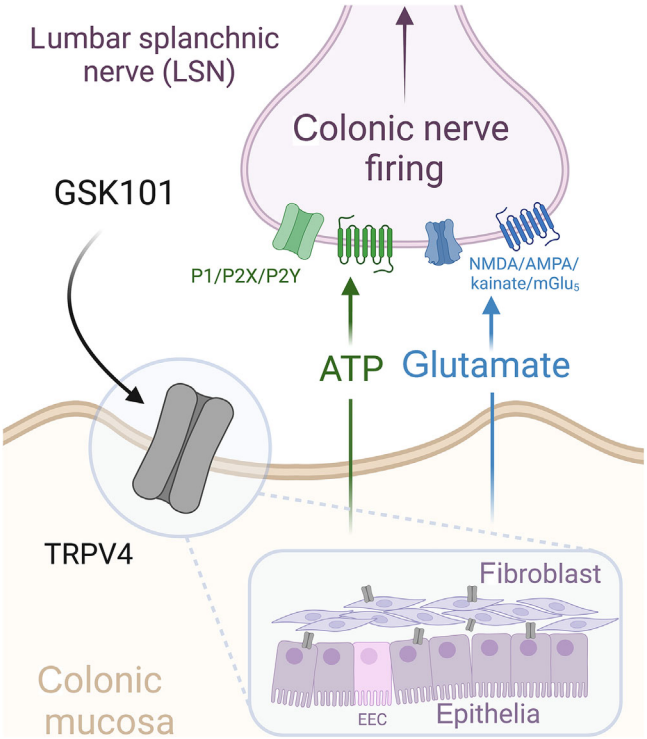

FIGURE 6 Proposed mechanism of colonic sensory nerve firing by transient receptor potential vanilloid 4 (TRPV4). GSK1016790A activates TRPV4 expressed upon cells of the colonic mucosa (most likely epithelial cells including enteroendocrine cells [EECs] and fibroblasts). This drives the release of ATP and glutamate, which act upon purinergic (P1, P2X and P2Y) and glutamatergic (*N*-methyl-D-aspartate [NMDA], amino-3-hydroxy-5-methylisooxazole-4-propionic acid [AMPA], kainate and $mGlu_5$) receptors at the nerve terminal to drive lumbar splanchnic nerve (LSN) activation. Illustration produced using BioRender.

preparations, demonstrating that removal of the mucosa had not damaged the colonic innervation of the external smooth muscle layers and myenteric plexus by nociceptors. Furthermore, the co-expression

of TRPV4 with TRPA1 and the bradykinin B_2 receptor in colonic sensory neurons also indicated that removal of the mucosa had not affected the small population of TRPV4-expressing colonic neurons (Hockley et al., 2019). While the exact location of TRPV4-expressing spinal afferents in the gut remains unclear, anterograde tracing studies by Spencer et al. (2014, 2020) highlight the presence of spinal afferent endings in the mucosa, submucosa and myenteric ganglia—locations that cannot be excluded for TRPV4-expressing afferents.

The essential requirement for an intact gut mucosa to realize the afferent response to GSK1016790A suggests that the activation of TRPV4 promotes the release of mucosal mediators, which in turn stimulate colonic afferents. We hypothesized that this was driven by the release of ATP and glutamate, which we confirmed using several approaches. We demonstrated inhibition of the colonic afferent response to GSK1016790A in the presence of a pan-purinoceptor antagonist cocktail and individual pretreatments with NMDA, AMPA/kainate or $mGlu_5$ receptor antagonists, with combined pretreatment with the pan-purinoceptor antagonist cocktail and NMDA receptor antagonist D-AP5 abolishing the response to GSK1016790A. The involvement of a broad spectrum of purinergic and glutamatergic ion channels and G protein-coupled receptors (GPCRs) and their respective downstream signalling pathways are likely drivers of the sustained colonic afferent response to TRPV4 activation. In addition, we observed an increase in ATP and glutamate release from the mouse colon following treatment with GSK1016790A, which was repeated in primary human organoid cultures. The use of transwell organoid culture allowed confirmation of increased ATP and glutamate release from the basolateral compartment in response to apical GSK1016790A. Finally, we showed that marked activation of DRG neurons to GSK1016790A was only seen when co-cultured with primary colonic mucosa cells, an effect attenuated by individual and combined pretreatment with a pan-purinoceptor antagonist cocktail and D-AP5. A small proportion of DRG neurons responded to GSK1016790A when cultured alone, consistent with the restricted TRPV4 transcript expression in scRNA-seq databases (Hockley et al., 2019; Jung et al., 2023; Usoskin et al., 2015).

Our findings that TRPV4 stimulation promotes colonic afferent activation through mucosal ATP release are entirely consistent with previous work showing TRPV4-mediated release of ATP from epithelial cells in the oesophagus, stomach, lung and bladder as well as intestinal cell lines (Aizawa et al., 2012; Bonvini et al., 2016; Mihara et al., 2011, 2016, 2018). Importantly, we demonstrate here that not only cell lines but also fully differentiated primary organoid cultures generated from surgically resected human bowel, release ATP in response to TRPV4 agonist stimulation. This release was measured in the basolateral compartment, highlighting how ATP can be released in the vicinity of primary afferent endings. Studies in the lung and bladder also demonstrated a role for ATP in TRPV4-mediated sensory afferent firing (Aizawa et al., 2012; Bonvini et al., 2016), which we now confirm for the colon. In keeping with these and other studies looking at the colonic epithelium and cell lines (D'Aldebert et al., 2011; Liu et al., 2019), we found expression of TRPV4 in primary colonic mucosal cell cultures containing epithelial and fibroblast cells. While congruent with the published literature, we also showed that a cocktail of purinoceptor antagonists targeting subtypes of P1 (**adenosine receptors**) and P2 receptors (**P2X receptors** and **P2Y receptors**) was required to abolish the colonic afferent response to exogenous ATP, thereby highlighting the diversity of purinergic signalling in colonic afferents (Hockley et al., 2016; Shinoda et al., 2009).

Importantly, we revealed for the first time that TRPV4 activation promotes human intestinal epithelial cell glutamate release in keeping with (i) the expression of glutamate transporters (**excitatory amino acid transporter 3**, EAAT3) on colonic epithelial cells (Holmseth et al., 2012; Hu et al., 2018), (ii) the utilization of glutamate as a fuel source and (iii) the expression of **glutaminase** in colonic enterocytes (Cherbuy et al., 1995), thereby allowing the synthesis of glutamate from **glutamine**. The released glutamate in turn stimulated colonic afferents through a combination of NMDA, AMPA/kainate and $mGlu_5$ receptor activation, consistent with the marked expression of these receptor subtypes in colon-projecting sensory neurons (Hockley et al., 2019), supported by published work implicating NMDA and $mGlu_5$ receptor activation in the development of visceral hypersensitivity (Lindström et al., 2008; McRoberts et al., 2001). Furthermore, given the significant contribution TRPV4 makes to colorectal afferent mechanosensitivity, our findings may provide a mechanistic explanation for the inhibition of the visceromotor response and pelvic afferent response to CRD following pretreatment with glutamate receptor antagonists, namely, TRPV4-mediated mucosal glutamate release (Brierley et al., 2008; Cenac et al., 2008, 2010). Similarly, our findings suggest that TRPV4-mediated mucosal ATP release may contribute to the colonic afferent response to CRD, in line with its subsequent inhibition by purinoceptor antagonists and studies showing that CRD promotes mucosal ATP release (Shinoda et al., 2009; Wynn et al., 2003). As such, further experiments are now warranted to confirm the role of TRPV4 in mechanically evoked ATP and glutamate release from the bowel and the contribution of specific epithelial and stromal cell types to TRPV4-evoked ATP and glutamate release. In addition to nociceptors, GSK1016790A may activate afferents involved in physiological reflex pathways that do not necessarily result in the conscious perception of pain.

Whereas GSK1016790A may activate afferents involved in physiological reflex pathways, our findings that the majority of TRPV4-mediated colonic afferent activation is mucosally driven highlight the potential utility of mucosa-restricted treatments for visceral pain in gastrointestinal disease. Indeed, many of the mediators implicated in the sensitization of TRPV4 signalling in gastrointestinal disease also express receptors in gut epithelial cells and fibroblasts. The most pertinent of these is PAR2, which, like TRPV4, was not found to be widely expressed in colon-projecting sensory neurons but is highly expressed in colonic epithelial cells (Green et al., 2000; Kong et al., 1997), its activation causing visceral hypersensitivity (Cenac et al., 2007; Vergnolle, 2004), and neurogenic inflammation (Nguyen et al., 2003), thus emphasizing that mucosa may be a site of action for PAR2-mediated visceral hypersensitivity (Rolland-Fourcade et al., 2017), particularly through the modulation of TRPV4

signalling. Furthermore, significant increases in TRPV4 expression are found in mucosal biopsies from patients with irritable bowel syndrome and inflammatory bowel disease (Cheng et al., 2022; D'Aldebert et al., 2011; Fichna et al., 2015; Rizopoulos et al., 2018), suggesting that mucosal TRPV4 activation is likely to produce a greater effect on colonic afferent sensitization in disease states. Given that mucosal TRPV4 expression has been implicated in the development of colitis in people with inflammatory bowel disease (D'Aldebert et al., 2011), targeting mucosal TRPV4 activity may be effective for the treatment of both pain and inflammation in gastrointestinal diseases.

In summary, our study demonstrates that TRPV4-mediated colonic afferent activation is driven by the mucosal release of ATP and glutamate, which act on a broad array of glutamate and purinoreceptors to stimulate colonic afferents.

AUTHOR CONTRIBUTIONS

M. Y. Meng: Conceptualization; data curation; formal analysis; methodology; visualization; writing—original draft. **L. W. Paine:** Data curation; formal analysis; methodology. **D. Sagnat:** Data curation; formal analysis; methodology; validation. **I. Bello:** Data curation; formal analysis; methodology; validation. **S. Oldroyd:** Formal analysis; methodology; validation. **F. Javid:** Methodology; validation. **M. T. Harper:** Methodology; resources; validation. **J. R. F. Hockley:** Conceptualization; methodology. **E. St. John Smith:** Conceptualization; methodology; resources. **R. M. Owens:** Methodology; validation. **L. Alric:** Resources. **E. Buscail:** Resources. **F. Welsh:** Conceptualization; project administration; resources; supervision. **N. Vergnolle:** Investigation; methodology; resources; supervision. **D. C. Bulmer:** Conceptualization; funding acquisition; investigation; project administration; resources; supervision; writing—original draft.

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CONFLICT OF INTEREST STATEMENT

Michelle Y. Meng and Luke W. Paine are supported by an AstraZeneca Studentship. Fraser Welsh is employed by AstraZeneca. David C. Bulmer and Ewan St. John Smith receive research funding from AstraZeneca.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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