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ARTICLE

Serotonin-induced vascular permeability is mediated by transient receptor potential vanilloid 4 in the airways and upper gastrointestinal tract of mice

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Abstract

Endothelial and epithelial cells form physical barriers that modulate the exchange of fluid and molecules. The integrity of these barriers can be influenced by signaling through G protein-coupled receptors (GPCRs) and ion channels. Serotonin (5-HT) is an important vasoactive mediator of tissue edema and inflammation. However, the mechanisms that drive 5-HT-induced plasma extravasation are poorly defined. The Transient Receptor Potential Vanilloid 4 (TRPV4) ion channel is an established enhancer of signaling by GPCRs that promote inflammation and endothelial barrier disruption. Here, we investigated the role of TRPV4 in 5-HT-induced plasma extravasation using pharmacological and genetic approaches. Activation of either TRPV4 or 5-HT receptors promoted significant plasma extravasation in the airway and upper gastrointestinal tract of mice. 5-HT-mediated extravasation was significantly reduced by pharmacological inhibition of the 5-HT_{2A} receptor subtype, or with antagonism or deletion of TRPV4, consistent with functional interaction between 5-HT receptors and TRPV4. Inhibition of receptors for the neuropeptides substance P (SP) or calcitonin gene-related peptide (CGRP) diminished 5-HT-induced plasma extravasation. Supporting studies assessing treatment of HUVEC with 5-HT, CGRP, or SP was associated with ERK phosphorylation. Exposure to the TRPV4 activator GSK1016790A, but not 5-HT, increased intracellular Ca^{2+} in these cells. However, 5-HT pre-treatment enhanced GSK1016790A-mediated Ca^{2+} signaling, consistent with sensitization of TRPV4. The functional interaction was further characterized in HEK293 cells expressing 5-HT_{2A} to reveal that TRPV4 enhances the duration of 5-HT-evoked Ca^{2+} signaling through a PLA_2 and PKC-dependent mechanism. In summary, this study demonstrates that TRPV4 contributes to 5-HT_{2A}-induced plasma extravasation in the airways and upper GI tract, with evidence supporting a mechanism of action involving SP and CGRP release.

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Introduction

The vasculature performs several important functions that are essential for maintaining fluid homeostasis. Endothelial cells make up the physical barrier in blood vessels that enables the control of fluid and molecule exchange from the circulation to the surrounding tissues. Physiological regulation of these barriers controls the extravasation of plasma proteins through inter-endothelial gaps, where cytoskeletal reorganization and disassembly of VE-cadherin junctions are essential regulators of endothelial permeability [1]. Disruption of these processes, as occurs in disease, is associated with unregulated movement and accumulation of fluids, leading to tissue edema.

Several inflammatory mediators, including proteases (e.g., thrombin), histamine, substance P (SP), and serotonin (5-HT) can activate specific receptors on vascular endothelial cells to promote changes in endothelial permeability. These changes can be mediated by an increase in intracellular calcium ($[Ca^{2+}]_i$) and activation of signaling pathways that regulate the contractile apparatus of cells, leading to cytoskeletal remodeling and disassembly of VE-Cadherin junctions [2]. This, in turn, causes endothelial cell contraction and cell junction disruption, resulting in increased endothelial permeability and tissue edema [2–6]. For example, increased endothelial $[Ca^{2+}]_i$ in pulmonary blood vessels leads to their constriction and to subsequent edema formation [3, 7, 8].

It has been demonstrated that systemic administration of 5-HT produces detrimental effects on the integrity of the endothelial barrier, leading to plasma extravasation into the surrounding tissue [9]. The biological actions of 5-HT are mediated through specific serotonin receptors ($5\text{-}HT_{1-7}$) [10], all of which are G protein-coupled receptors (GPCRs), with the notable exception of the $5\text{-}HT_3$ ligand-gated ion channel [11]. In addition to their well-defined roles in neurotransmission and clinical association with the pathogenesis of neurological diseases and neuropsychiatric disorders, 5-HT receptors are also key regulators of the homeostatic control of vasoconstriction and vascular permeability [12–14].

As previously described, elevated $[Ca^{2+}]_i$ in endothelial cells is required to cause barrier dysfunction. This elevation in $[Ca^{2+}]_i$ is triggered by mechanical stimuli or by activation of GPCRs and occurs in two phases, initiated by the release of Ca^{2+} from ER-stores and followed by entry of extracellular Ca^{2+} through cation channels [15]. Additionally, GPCR activation can also promote Ca^{2+} entry by activating cation channels, including transient receptor potential channels (TRP), which are the main non-selective cation channels in endothelial cells [1, 16]. The principal TRP channels that mediate endothelial cell permeability are TRPM2, TRPC1, 4 and 6 and vanilloid family members TRPV1 and 4 [17].

Transient Receptor Potential Vanilloid 4 (TRPV4) is an established enhancer of vascular permeability and edema that is expressed by a variety of cells including endothelia, peripheral sensory neurons, and immune cells [18–21]. TRPV4 is also a recognized promoter of neurogenic inflammation through enhanced release of neuropeptides, including SP and calcitonin gene-related peptide (CGRP), from peptidergic peripheral nerve endings [22, 23]. The sensitivity to ligand or mechanical activation, as well as the magnitude and duration of TRPV4 activity can be augmented by functional interactions (termed 'coupling') with GPCRs. These interactions are also known to be reciprocal, where functional coupling of a GPCR to an ion channel such as TRPV4 can lead to augmentation of GPCR signaling outputs. Furthermore, coupling between GPCRs and TRPV4 is proposed to contribute to disease-associated processes, including neurogenic inflammation and pain [24].

A well-characterized example of reciprocal coupling is illustrated through functional interactions between protease-activated receptors 1 and 2 (PAR1 and PAR2) and TRPV4 [20, 25, 26]. PAR activation can "sensitize" or reduce the activation threshold through channel phosphorylation and enhance TRPV4 signaling through the production of endogenous TRPV4 activators (e.g., arachidonic acid and 5′,6′-EET) [25–27]. Conversely, TRPV4 activity augments PAR1- and PAR2-dependent signaling, and this bidirectional PAR-TRPV4 relationship drives a significant component of PAR-evoked edema [20, 26, 27].

A variety of cell types co-express 5-HT receptors and TRPV4, highlighting their broader potential to functionally interact. Indeed, studies have demonstrated an important role for TRPV4 as an enhancer of 5-HT signaling associated with arterial smooth muscle proliferation [28, 29], pulmonary artery smooth muscle contraction [8, 19], itch [30], and visceral pain [31]. Immunohistochemistry and in situ hybridization studies have demonstrated that nociceptive dorsal root ganglion neurons mainly express 5-HT receptor subtypes 2A and 3 [32, 33]. Activation of $5\text{-}HT_{2A}$ expressed by these neurons promotes 5-HT-induced nociception and the release of SP and CGRP from peripheral nerve terminals, leading to a sustained increase in vascular permeability [34–38]. Neurogenic inflammation is initiated by the release of these neuropeptides [36, 39] and further studies have supported the involvement of neurogenic inflammation in 5-HT-evoked plasma extravasation by demonstrating significant inhibition of plasma protein extravasation with antimigraine drugs [40, 41].

Although there is strong evidence to support the importance of TRPV4 as an amplifier of 5-HT receptor signaling, the relative contribution of TRPV4 to 5-HT-induced edema

has not been defined in detail. We hypothesized that 5-HT-induced plasma extravasation is augmented by TRPV4 activity and is mediated, in part, through release of SP and CGRP from nerve fibers associated with microvasculature. In the present study, we found that the systemic administration of 5-HT-induced plasma extravasation in the airway and upper GI tract, particularly by the activation of $5HT_{2A}$, where the pharmacological inhibition or genetic deletion of TRPV4 attenuates 5-HT-induced plasma extravasation in the airways and upper GI tract, indicating a reciprocal coupling between 5-HT_{2A} and TRPV4 in vivo. In addition, in vitro studies indicated that 5-HT_{2A} interact with TRPV4 through the activation of PLA_2 and PKC. Moreover, we establish that inhibitors of NK_1R (SR140333) or the CGRP receptor (Olcegepant; BIBN4096) block 5-HT- and TRPV4-induced plasma extravasation in mice. These observations provide further mechanistic understanding of the important contribution that GPCR-TRP channel interactions have in fundamental biological processes, including the control of vascular permeability.

Materials and methods

Drugs and reagents

Evans Blue dye and GSK1016790A were purchased from Sigma-Aldrich (St. Louis, MO); 5-HT, HC067047, SR140333, GF 109203X (GFX), and BIBN 4096 (Olcegepant) were purchased from Tocris Bioscience (Bristol, UK); YM 26734 was from Cayman Chemical; WAY-100635 Maleate, GR 55562 dihydrochloride, GR113808 and SB 269970 hydrochloride were purchased from Abcam Australia (Melbourne, VIC Australia). Ketanserin and RS-127445 were purchased from Selleck Chemicals (Houston, TX. USA); Evans Blue was dissolved in sterile 0.9% saline. All drugs administered to mice were prepared on the day of experimentation in sterile 1% dimethyl sulfoxide (DMSO) in 0.9% saline.

Animals

All animal experiments adhered to the ARRIVE guidelines [42] and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. This study was approved by the Animal Ethics Committees of RMIT and Monash Institute of Pharmaceutical Sciences. Wild-type C57Bl/6J and $TRPV4^{-/-}$ (kindly provided by Dr. W Liedtke, Duke University) (6–12 weeks, male) were obtained from the Animal Resources Center (Canning Vale, WA), or from Monash Animal Research Platform, Monash University. All animals were maintained in a temperature-controlled (24 °C) environment with a 12 h light/dark cycle and with access to food and water ad libitum.

Measurement of plasma extravasation

Mice were anaesthetized with a combination of Ketamine (100 mg/kg i.p.) and Xylazine (10 mg/kg i.p.) and kept on a warming pad. The skin at the throat was removed to expose the jugular veins. Substances were i.v. administered by passing a needle through the *pectoralis major* muscle to prevent bleeding on withdrawal. Evans Blue dye (20 mg/kg) or 0.9% saline were administered into the jugular vein, 1 min before injection of agonist (5-HT or GSK1016790A, dosing as indicated in relevant sections) or vehicle (1% DMSO in 0.9% saline). Mice were killed (5 min post-agonist administration) by exsanguination and perfused with saline solution. Tissue samples were collected, weighed, and placed in formamide (≥18 h at 37 °C) to facilitate dye extraction. Absorbance of the extracts was determined against standard concentrations of Evans Blue at 620 nm using a FlexStation III plate reader (Molecular Devices, Sunnyvale, CA). Antagonists of 5-HT_{1A} (WAY-100635, 80 µg/kg) [43], 5-HT_{1B} (GR 55562, 300 µg/kg) [44], 5-HT_{2A} (ketanserin, 2 mg/kg) [45], 5-HT_{2B} (RS-127445, 300 µg/kg) [46], 5-HT_4 (GR 113808, 1 mg/kg) [47], 5-HT_7 (SB269973, 300 µg/kg) [46], TRPV4 (HC0670471, 10 mg/kg), NK_1R (SR140333, 1 mg/kg), or CGRP receptor (Olcegepant, 1 mg/kg) were i.p. injected 60 min prior to anesthetics. Results were expressed as the amount of Evans Blue dye per wet weight tissue (ng of EB/mg of tissue).

Human umbilical vein endothelial cell (HUVEC) culture

HUVEC were grown in endothelial growth medium (EGM, Lonza, Mount Waverley, VIC, Australia) containing 2% fetal bovine serum and a SingleQuots Supplement Pack (Lonza) as described [20].

Transient transfection

Constructs of pcDNA3.1$^+$ human 5-HT receptors subtype 1A, 1B, 2A, 2B, 4, and 7 (hHT_{1A-7}) plasmids were purchased from the cDNA Resource Center (Bloomsburg, PA, USA). Human Embryonic Kidney 293 cell line with tetracycline-inducible (T-Rex™ 293) TRPV4 overexpression (HEK-TRPV4) was grown at 37 °C in 5% CO_2 in DMEM containing 10% FBS (5 µg/mL blasticidin S). Cells were transiently transfected with hHT_{1A-7} plasmids (75 ng DNA/well, HEK-5-HT_{1A-7}) using the standard protocol for the FuGENE reagent system (Promega

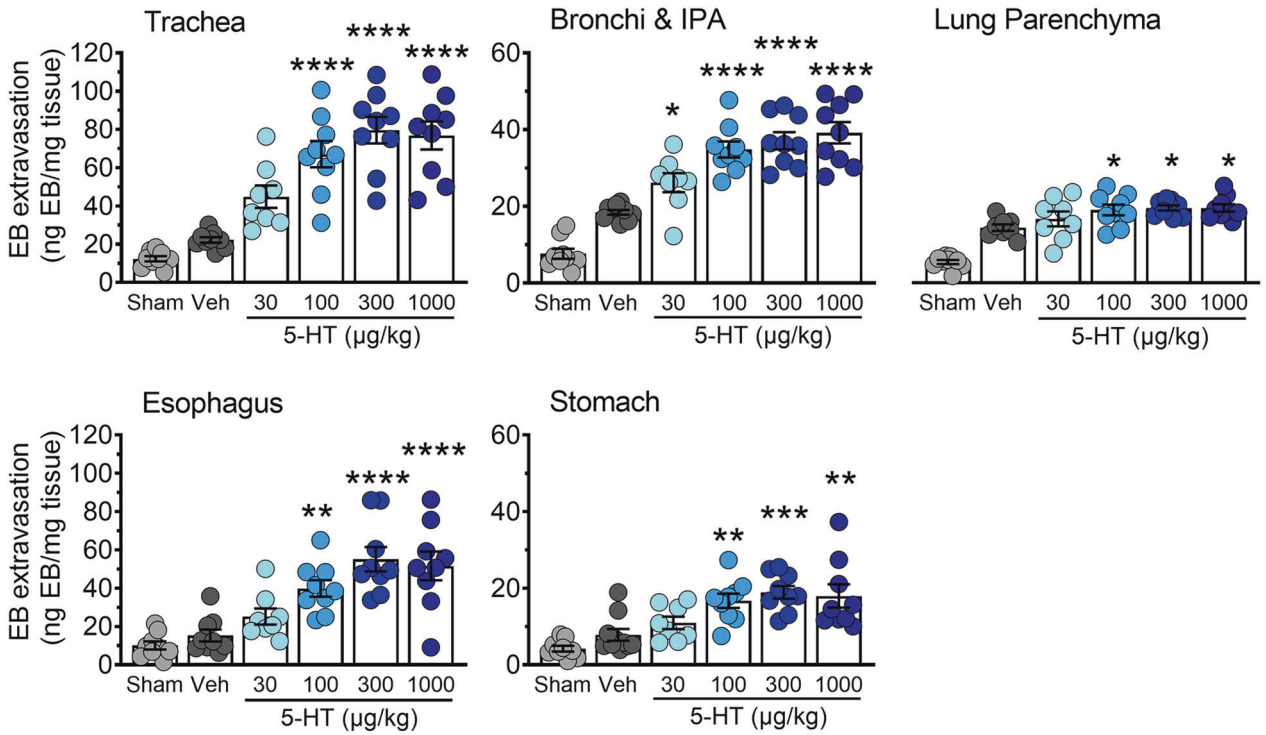

Fig. 1 5-HT causes vascular hyperpermeability in the airways and upper GI tract. Vascular hyperpermeability was assessed by the presence of Evans Blue in tissues of the airways, esophagus, and stomach following the intravenous injection of increasing concentrations of 5-HT (30–1000 µg/kg). Data are expressed as mean ± S.E.M., $n = 6$–9 mice per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; significantly different compared to vehicle treatment (1% DMSO in 0.9% saline); one-way ANOVA and Dunnett's multiple comparisons test.

Corporation Madison, WI USA). Expression of TRPV4 was induced overnight with 0.1 µg/mL tetracycline.

Ca^{2+} signaling assays

HUVEC or HEK cells were seeded onto poly-D-lysine coated 96-well plates (15,000 cells/well) and cultured for 48 h. Cells were loaded with Fura2-AM ester (1 µM) in Hank's Balanced Salt Solution (HBSS) supplemented with probenecid (2 mM) and pluronic acid (0.5 µM) for 45 min at 37 °C. Fluorescence was measured at 340/380 nm excitation and 530 nm emission wavelengths using a FlexStation III plate reader. Baseline measurements were recorded for 20 s prior to agonist addition. Responses to agonists were recorded for 200 s post-addition. For the PKC and PLA_2 inhibition assay, cells were incubated 30 min prior to 5-HT addition, as previously described with GF 109203X (GFX, 100 nM) [26] or YM26734 (30 µM) [48].

ERK phosphorylation assays

HUVEC were seeded onto non-coated 96-well plates (15,000 cells/well) and cultured for 48 h. Cells were serum starved for 6 h and treated as described in the results section. Phospho-ERK 1/2 (pERK1/2) was measured using the AlphaScreen SureFire p-ERK 1/2 (Thr202/Tyr204) Assay Kit (PerkinElmer, USA), according to the manufacturer's specifications. Fluorescence was measured using the EnVision multilabel plate reader (PerkinElmer). Data were normalized to the positive control (PDBu, 1 µM).

Statistical analysis

Data were analyzed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). All treatments were analyzed using one-way ANOVA with Dunnett's post-test. All data are presented as mean ± S.E.M., with a p value < 0.05 considered to be significantly different to the null hypothesis at the 95% confidence level.

Results

5-HT induces plasma extravasation in the airways and upper gastrointestinal tract

Evans Blue dye is commonly used as an indicator of altered vascular permeability to macromolecules due to its high

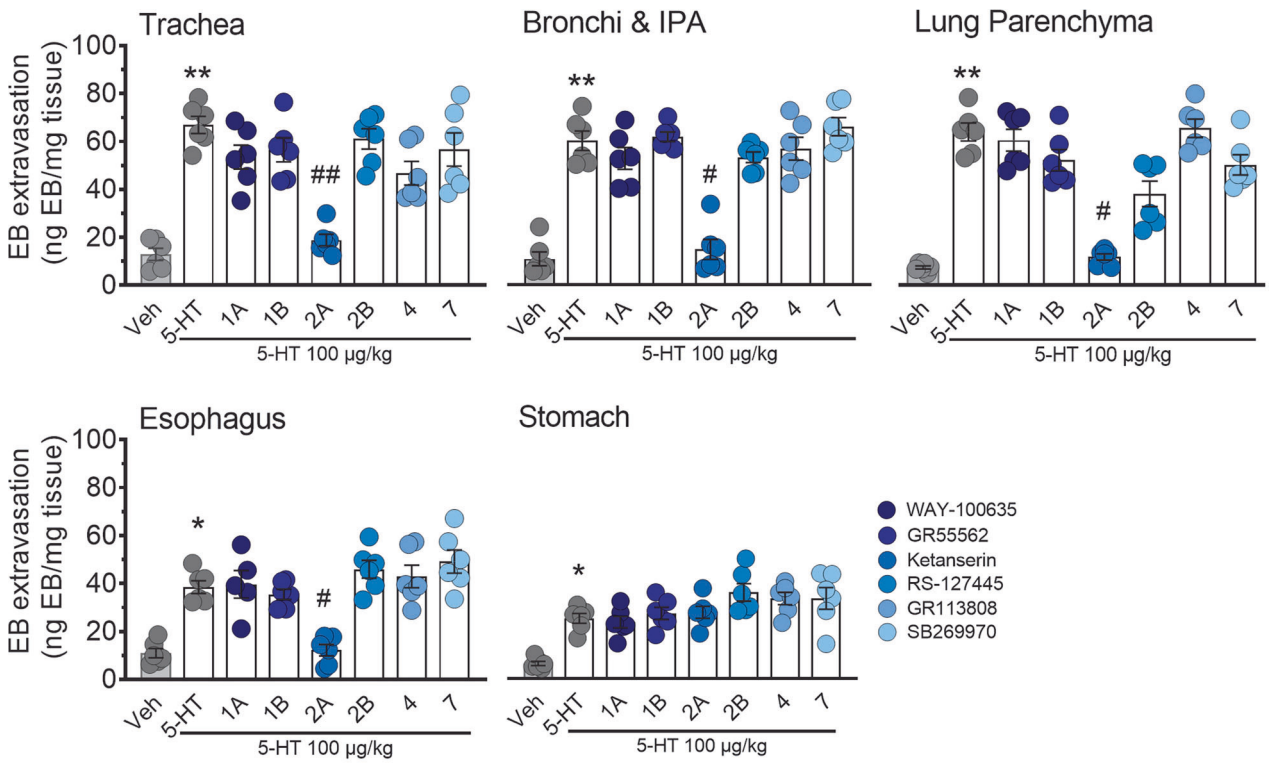

Fig. 2 5-HT-induced vascular hyperpermeability is inhibited in the airways and upper GI tract by the 5-HT$_{2A}$ selective antagonist ketanserin. Effect of WAY-100635 (5-HT$_{1A}$ antagonist, 80 µg/kg), GR 55562 (5-HT$_{1B}$ antagonist, 300 µg/kg), RS-127445 (5-HT$_{2B}$ antagonist, 300 µg/kg), GR 113808 (5-HT$_4$ antagonist, 1 mg/kg), or SB269973 (5-HT$_7$ antagonist, 300 µg/kg) in the airways and upper GI tract. Data are expressed as mean ± SEM for $n = 5$–6 experiments. *$p < 0.05$; **$p < 0.01$; One-way ANOVA and Dunnett's multiple comparisons test. * Indicates statistical significance compared to vehicle treatment, # indicates statistical significance compared to 5-HT 100 µg/kg treatment.

affinity for albumin. Under normal conditions, the vascular endothelium is impermeable to albumin, restricting albumin-bound Evans Blue to blood vessels. When inflammation occurs, albumin-bound Evans Blue is able to diffuse into surrounding tissues under conditions due to regulated, increased permeability of the vascular endothelium. Known as plasma extravasation, this process is important for promoting leukocyte infiltration, to initiate wound healing processes and subsequent swelling can also physically protect affected tissue [49].

To determine the effect of 5-HT on plasma extravasation, we examined the tissue distribution of Evans Blue following the administration of either vehicle (1% DMSO in 0.9% saline) or 5-HT (30–1000 µg/kg). For assessment of the natural absorbance of each tissue, an additional control group received an injection of saline solution without Evans Blue, followed by vehicle treatment. The vehicle treatment group did not exhibit significant basal leakiness of Evans Blue in the airways (trachea, bronchi and lung parenchyma) and upper gastrointestinal (GI) tract (esophagus and stomach) (Fig. 1). In contrast, the systemic administration of 5-HT elicited a dose-dependent increase in the amount of Evans Blue in tissues of the airways and upper GI tract, indicative of plasma extravasation (Fig. 1). A submaximal dose of 5-HT (100 µg/kg) was used in all subsequent experiments.

The pharmacological inhibition of 5-HT$_{2A}$ attenuates plasma extravasation in the airways and esophagus

To study the specific subtype of 5-HT receptor that is involved in 5-HT-induced plasma extravasation, mice were pre-treated with selective antagonists for 5-HT subtypes 1A (WAY-100635), 1B (GR 55562), 2A (ketanserin), 2B (RS-127445), 4 (GR 113808), or 7 (SB269973). The inhibition of 5-HT$_{2A}$ by ketanserin significantly attenuated plasma extravasation compared with vehicle pre-treated mice in the airways and esophagus (Fig. 2). However, ketanserin did not attenuate 5-HT-induced plasma extravasation in the stomach (Fig. 2). The inhibition of the 5-HT receptor subtypes 1A, 1B, 2B, 4, and 7 had no significant effect on 5-HT-induced plasma extravasation in the airways and upper GI tract compared with vehicle pre-treated mice, indicating that only 5-HT$_{2A}$ plays an important role on plasma extravasation.

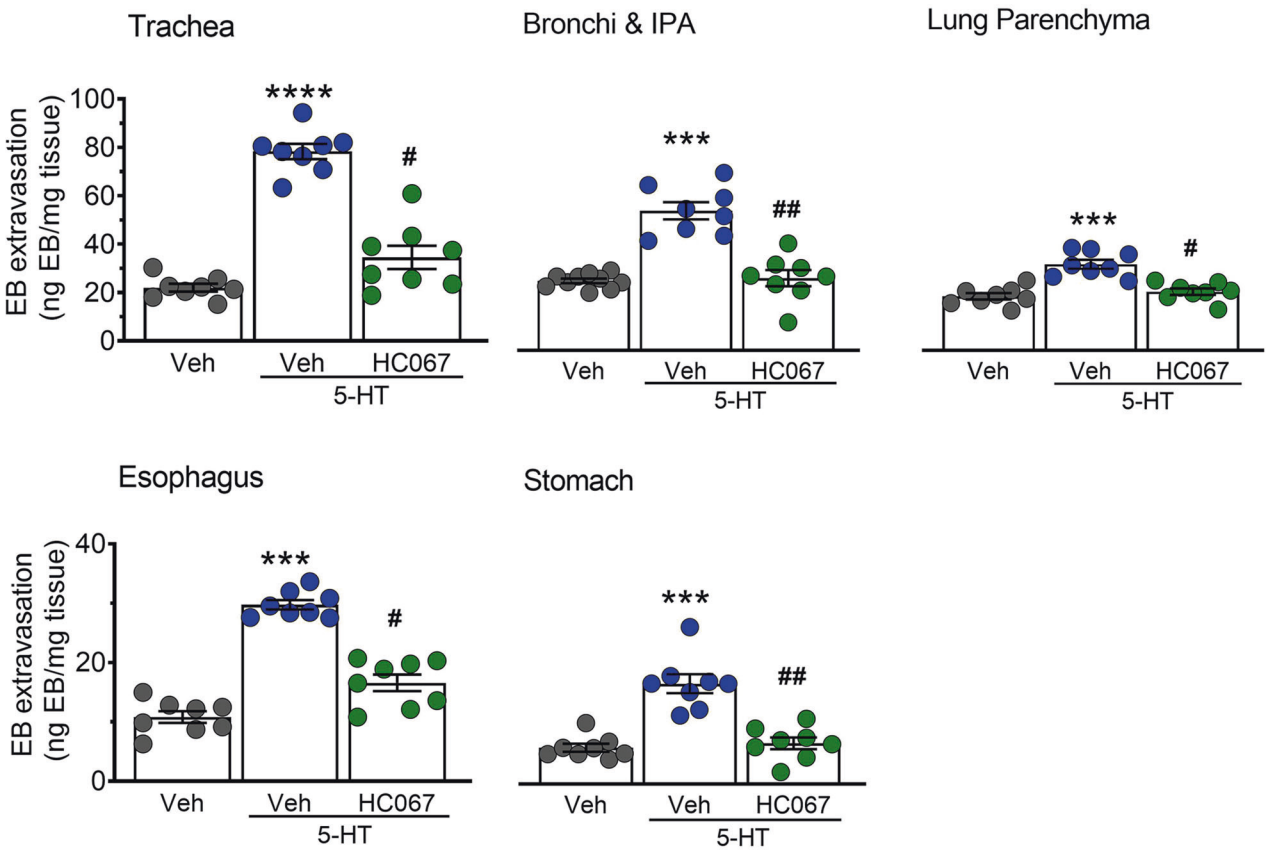

Fig. 3 Selective inhibition of TRPV4 suppresses 5-HT-induced edema. Effects of pre-treatment with the TRPV4 inhibitor HC067047 (10 mg/kg, HC067) on 5HT-induced plasma extravasation. HC067 significantly reduced Evans Blue leakage induced by 5-HT (100 µg/kg, i.v.). Data are expressed as mean ± S.E.M., $n = 8$ mice per group. $^{***}p < 0.001$; $^{****}p < 0.0001$; significantly different compared to vehicle treatment. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; significantly different compared to 5-HT treatment; one-way ANOVA and Dunnett's multiple comparisons test.

TRPV4 mediates 5-HT-induced plasma extravasation in the airways and upper GI tract

We have previously demonstrated that TRPV4 contributes to PAR1- and PAR2-dependent intracellular signaling and to PAR2-induced plasma extravasation [20, 26]. To determine whether TRPV4 plays an equivalent role in 5-HT-induced plasma extravasation, we administered the selective TRPV4 blocker HC067047 (HC067; 10 mg/kg, i.p.) prior to delivery of 5-HT. Inhibition of TRPV4 significantly decreased 5-HT-induced Evans Blue extravasation in the airways and upper GI tract, consistent with a TRPV4-dependent mechanism of action (Fig. 3).

5-HT-induced plasma extravasation requires TRPV4 expression

To confirm that 5-HT-induced plasma extravasation requires TRPV4 expression, we performed equivalent studies in TRPV4$^{-/-}$ mice or matched TRPV4$^{+/+}$ littermates. Previously, we reported that the selective TRPV4 activator GSK1016790A (GSK101) induced a dose-dependent increase in plasma extravasation in wild-type mice [20]. Consistent with our prior report, the administration of GSK101 (100 µg/kg) to wild-type mice induced a significant increase in plasma extravasation in the airways and upper GI tract (Fig. 4). Both GSK101- and 5-HT-induced plasma extravasation were abolished in TRPV4$^{-/-}$ mice (Fig. 4) when compared to TRPV4$^{+/+}$ mice. These data demonstrate the TRPV4-dependence of the 5-HT-evoked extravasation described.

TRPV4 enhanced 5-HT$_{2A}$ calcium signaling in HEK cells

The direct effect of 5-HT receptor signaling on TRPV4 activity was examined in an isolated cell system using HEK cells expressing the serotonin receptors (1A, 1B, 2A, 2B, or 4) alone or with co-expression of TRPV4. Assessment of 5-HT-mediated Ca^{2+} signaling over time (100 µM) demonstrated that HEK cells expressing 5-HT$_{1A,\ 1B,\ 2B}$ did not exhibit increased $[Ca^{2+}]_i$ in response to 5-HT (Fig. 5A).

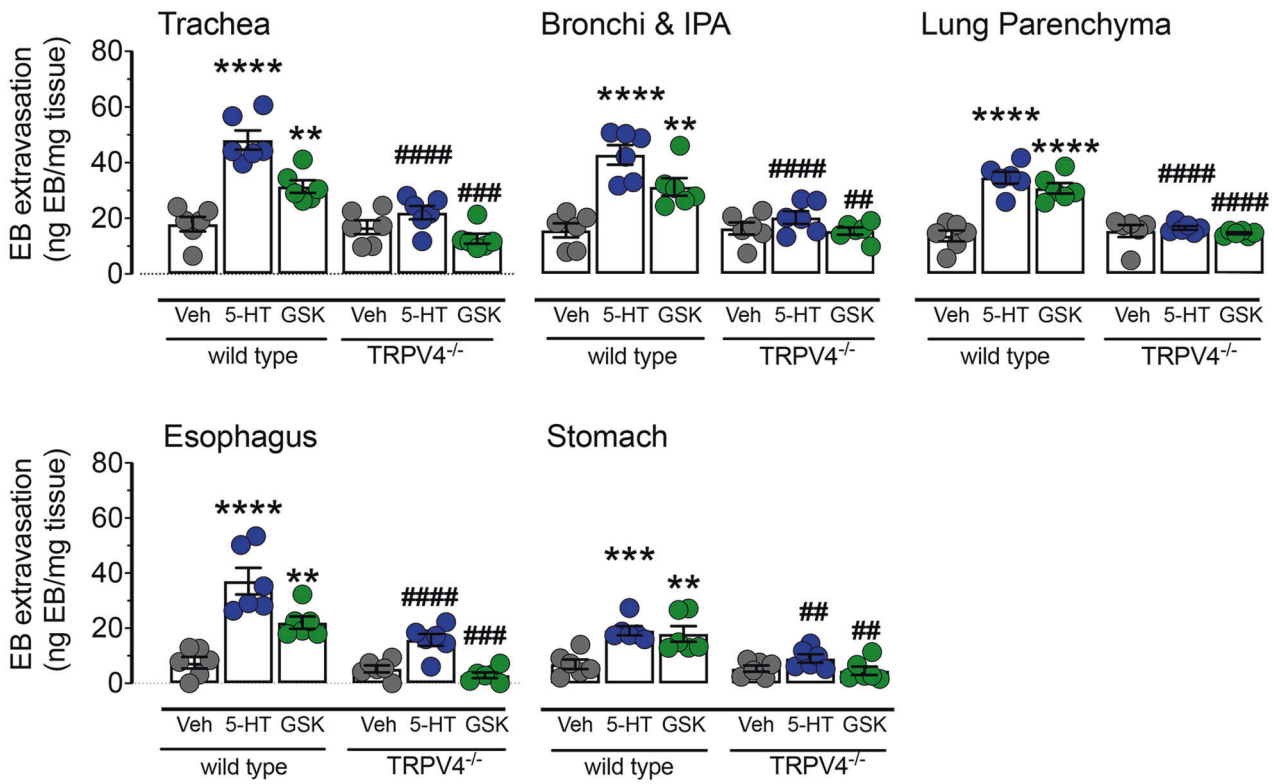

Fig. 4 5-HT and TRPV4-induced edema is absent in TRPV4$^{-/-}$ mice. The TRPV4 agonist GSK1016790A (100 µg/kg, i.v.; GSK) or 5-HT (100 µg/kg, i.v.) caused significant leakage of Evans Blue in the airway and upper GI tract of wild-type mice. Both 5-HT- and TRPV4-induced edema was significantly reduced in TRPV4$^{-/-}$ mice compared to wild-type littermate controls. Data are presented as mean ± S.E.M., $n = 6$ mice per group. * Significantly different compared to vehicle treated wild-type; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. # Significantly different compared to 5-HT- or GSK1016790A-treated wild-type; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$; one-way ANOVA and Dunnett's multiple comparisons test.

Expression of TRPV4 did not influence this response. In contrast, stimulation of HEK-5-HT$_{2A}$ cells resulted in a rapid, transient elevation in $[Ca^{2+}]_i$ that returned to baseline within 40 s, consistent with Gq-coupled signaling. Furthermore, the duration of the Ca^{2+} response was markedly sustained in cells functionally expressing TRPV4 (Fig. 5B). Stimulation of HEK-5HT$_4$ also revealed a Ca^{2+} transient that was only moderately enhanced in cells co-expressing TRPV4 (Fig. 5C).

Based on the robust nature of 5HT$_{2A}$-TRPV4 coupling, we focused on 5-HT$_{2A}$ and used known mediators of GPCR-TRPV4 coupling [24] to define the signaling mechanisms involved. The 5-HT$_{2A}$ dependence of the Ca^{2+} response was initially confirmed using ketanserin (Fig. 5B). Changes in $[Ca^{2+}]_i$ were then quantified over time by assessing the amplitude of the acute phase after 5-HT stimulation (0–20 s) and the magnitude of the sustained plateau phase (20–80 s post-stimulation). 5-HT$_{2A}$ transactivates phospholipase A2 (PLA_2) to generate arachidonic acid (AA), an endogenous activator of TRPV4 [50–53]. G_q-coupled activation of Protein Kinase C (PKC) can lead to rapid phosphorylation of intracellular regulatory domains of non-selective cation channels to modulate their ionic permeability [24]. To determine if PLA_2 and PKC serve as intermediates of 5-HT$_{2A}$-TRPV4 coupling, cells were treated with the PLA_2 inhibitor YM 26734 (30 µM) or the PKC inhibitor GF 109203X (GFX; 100 nM). Neither inhibitor affected the initial peak of the 5-HT response (Fig. 5D, E). Both inhibitors significantly suppressed the sustained phase (Fig. 5D, F). In addition, removal of extracellular Ca^{2+} abolished the transient and sustained phase of the 5-HT-evoked $[Ca^{2+}]_i$ response (Fig. 5D). These results suggest that coupling to TRPV4 enhances 5-HT$_{2A}$ receptor signaling predominantly through influx of extracellular Ca^{2+}.

Neuropeptide receptors contribute to TRPV4- and 5-HT-induced edema

Neuropeptides including CGRP and SP are released from sensory terminals that innervate blood vessels. These neuropeptides can influence endothelial barrier function and promote tissue edema through direct actions on microvascular endothelial cells [22, 54]. We assessed the contribution of CGRP and SP receptors to 5-HT- and TRPV4-induced plasma extravasation using selective antagonists of either the CGRP receptor (Olcegepant) or NK_1R

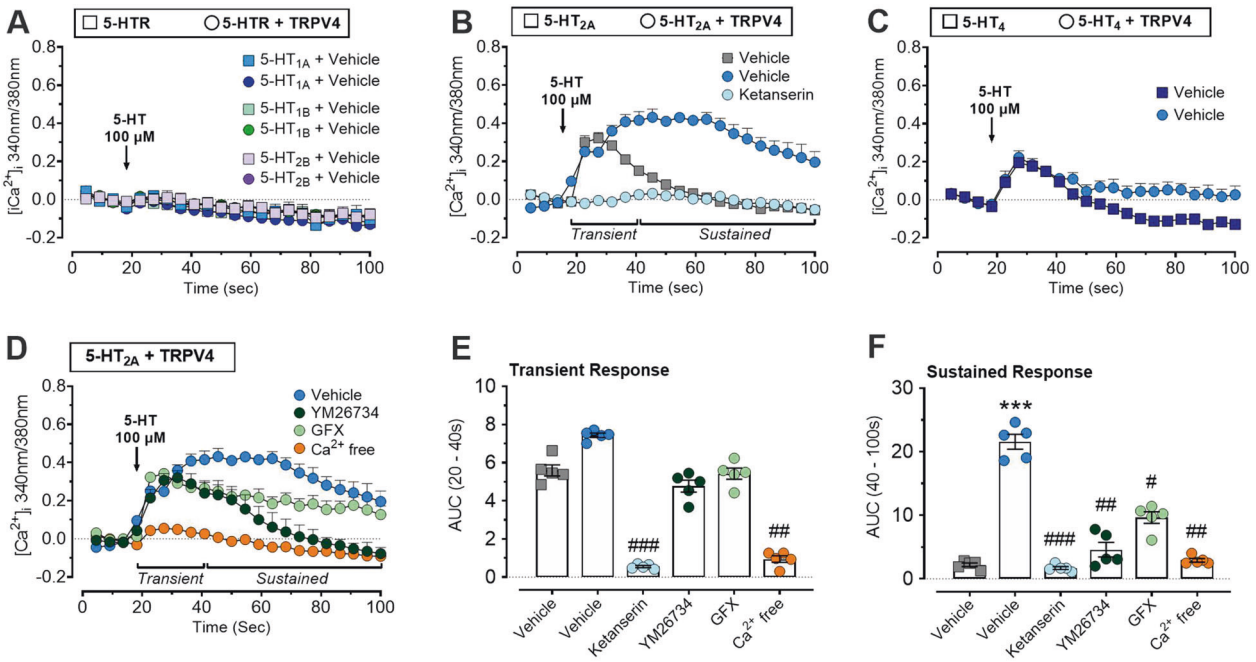

Fig. 5 5-HT induced a sustained increase in $[Ca^{2+}]_i$ in HEK cells co-expressing 5-HT_{2A} receptor and TRPV4. A Time traces showing responses to 5-HT (100 µM) by HEK cells expressing 5-HT_{1A}, 5-HT_{1B} or 5-HT_{2A} alone (circles) or with coexpression of TRPV4 (squares). **B** Time traces showing responses to 5-HT (100 µM) by HEK cells expressing 5-HT_{2A} or coexpressing 5-HT_{2A}/TRPV4. 5-HT-induced $[Ca^{2+}]_i$ was abolished by ketanserin (10 µM). **C** Time traces showing responses to 5-HT (100 µM) by HEK cells expressing 5-HT_4 alone or with coexpression of TRPV4. **D** Effect of the depletion of extracellular Ca^{2+}, PLA_2 inhibitor YM 26734 (30 µM), or PKC inhibitor GF 109203X (GFX; 100 nM) in HEK cells co-expressing 5-HT_{2A}/TRPV4. **E**, **F** Area under the curve analysis from 60 to 100 s post 5-HT (100 µM) addition. Data are expressed as mean ± SEM for n = 5–6 independent replicates. *p < 0.05; **p < 0.01; One-way ANOVA and Dunnett's multiple comparisons test. * Indicates statistical significance compared to HEK cells expressing 5-HTR subtype, # indicates statistical significance compared to HEK co-expressing 5-HTR and TRPV4.

(SR140333). Both antagonists significantly decreased tissue edema in the airways, esophagus and stomach in animals treated with GSK101 (Fig. 6) and 5-HT (Fig. 7), consistent with a neurogenic mechanism of action.

5-HT signaling in vascular endothelial cells is independent of TRPV4

The direct effects of 5-HT-TRPV4 coupling on vascular endothelial cells were examined using HUVEC, which are known to functionally express both targets [20, 55]. Focusing initially on Ca^{2+} mobilization, exposure to 100 nM or 1 µM 5-HT did not increase in $[Ca^{2+}]_i$. This is consistent with signaling through a G_q-independent mechanism (Fig. 8A). In contrast, GSK101 evoked a concentration-dependent elevation of $[Ca^{2+}]_i$ and this was attenuated with prior treatment with HC067 (Fig. 8A), thus confirming functional expression of TRPV4. Pre-treatment with 5-HT (100 nM; 30 min) enhanced GSK101-mediated $[Ca^{2+}]_i$ signaling in HUVECs (Fig. 8A, B). Specifically, 5-HT pre-treatment promoted a modest shift in pEC_{50} from −8.69 M to −9 M and increased E_{max} from 49.52 to 64.58 (Fig. 8A). This demonstrates a significant 5-HT-evoked amplification of TRPV4 signaling. Functional expression of 5-HT receptors was further confirmed by measuring levels of phosphorylated ERK (pERK), which allows for assessment of signaling through convergent pathways downstream of GPCRs. ERK activation was maximal at 2 min post-5-HT addition (100 nM or 1 µM) and decreased gradually over the 30 min assessment period. In contrast, GSK101 did not stimulate pERK in these cells (Fig. 8C). We confirmed that exposure to either SP (100 nM or 1 µM) or CGRP (100 nM or 1 µM) promotes a rapid and robust increase in pERK levels in HUVEC (Fig. 8D). Together, these data indicate that 5-HT receptors can sensitize and augment TRPV4 activity. These observations suggest that enhanced vascular permeability in response to 5-HT is potentially mediated through an indirect mechanism involving the TRPV4-dependent release of the neuropeptides SP and CGRP, possibly from external cellular sources such as primary afferent terminals.

Discussion

TRPV4 activation is important for the pathogenesis of pulmonary edema associated with heart failure or

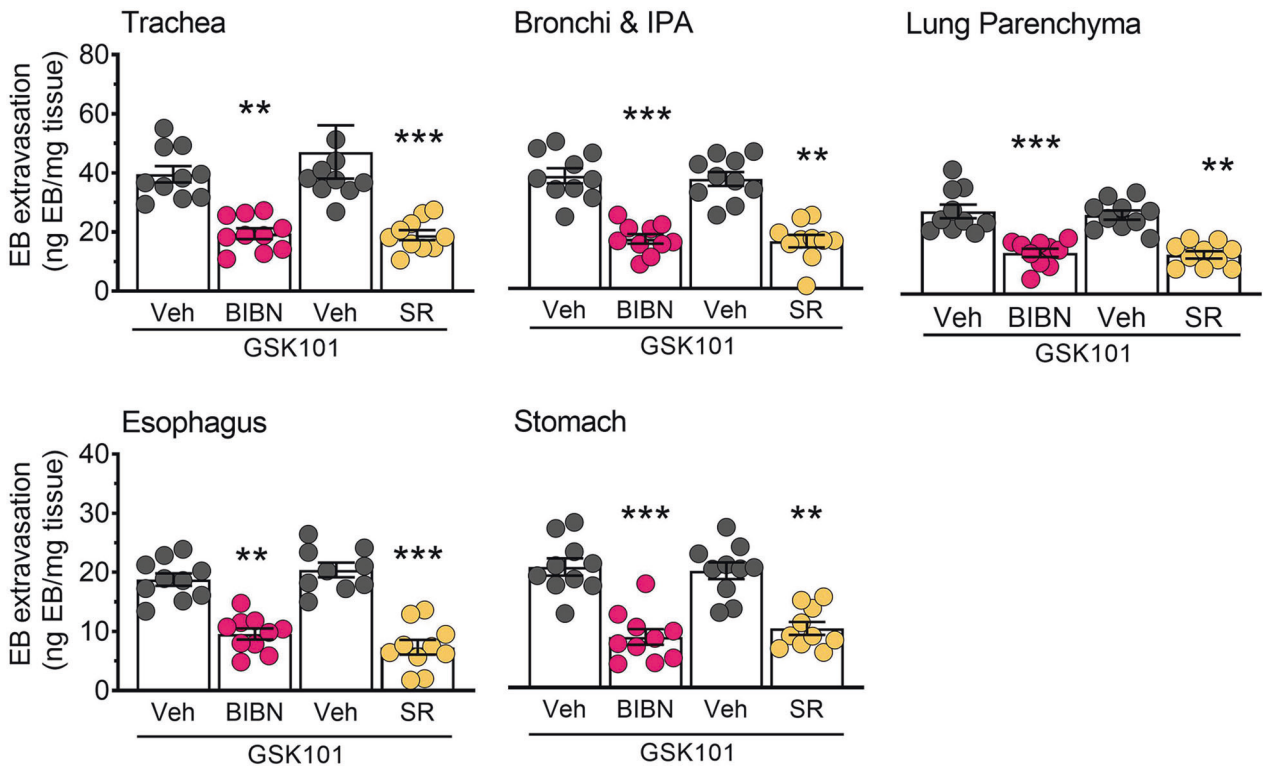

Fig. 6 TRPV4-induced edema is decreased by inhibition of CGRP or NK_1 receptors. Pre-treatment with the CGRP receptor antagonist BIBN4906 (BIBN; 1 mg/kg) or NK_1R antagonist SR140333 (SR; 1 mg/kg) significantly decreased tissue edema induced by GSK1016790A (100 µg/kg) compared to vehicle treatment in the airways and upper GI tract. Data are presented as mean ± S.E.M., $n = 9–10$ mice per group. $^{**}p < 0.01$; $^{***}p < 0.001$; One-way ANOVA and Dunnett's multiple comparisons test, significantly different compared to vehicle treated control (Veh).

chemically-induced acute lung injury [18, 21]. TRPV4 is also a mediator of sepsis-induced endothelial dysfunction and increased vascular permeability [56]. Consistent with this, 5-HT is also a potent vasoactive and signaling mediator and can promote disruption of cell–cell junctions at concentrations not much higher than those normally present under resting conditions [56, 57]. Here, we showed that 5-HT promotes pulmonary and esophageal plasma extravasation through a TRPV4-dependent mechanism. This also involves activation of NK_1R and the CGRP receptor, consistent with a putative neurogenic mechanism involving release of SP and CGRP from nerve fibers innervating the vasculature.

5-HT is mainly produced by enterochromaffin cells of the intestine, and is largely taken up and stored by platelets, or metabolized by the liver [57]. However, the lungs also play an important role in both 5-HT production and removal, and release of 5-HT by platelets may be important in the pathology of certain pulmonary diseases [57–60]. Additionally, 5-HT can be locally synthesized and released from peripheral arteries [61–63]. The 5-HT-TRPV4 signaling pathway may mediate a number of pathologies, including pulmonary hypertension, arterial smooth muscle proliferation, visceral hypersensitivity, and itch [8, 28, 30, 31]. Results of the present study suggest that the 5-HT receptor-TRPV4 axis could be an important pathway in pathologies, such as sepsis, where plasma 5-HT levels are known to be significantly elevated [64].

We have recently demonstrated that the potent and selective TRPV4 agonist, GSK101, caused dose-dependent extravasation in the airways and upper GI tract of mice, which was inhibited by the selective TRPV4 antagonist HC067 [20]. In contrast, GSK101 did not cause plasma extravasation in the bladder, heart, liver or kidney, suggesting that edema is not a general systemic effect of TRPV4 activation [20]. In the present study, we report that 5-HT induces plasma extravasation in the airways, esophagus and the stomach. Plasma extravasation induced by 5-HT was decreased by HC067 or TRPV4 deletion and limited to the tissues in which the TRPV4 activation caused edema, namely the airways and upper gut. These results support a role for TRPV4 in promoting 5-HT-induced plasma extravasation in the airways, esophagus and stomach. Extravasation in response to 5-HT was almost completely blocked by the TRPV4-specific

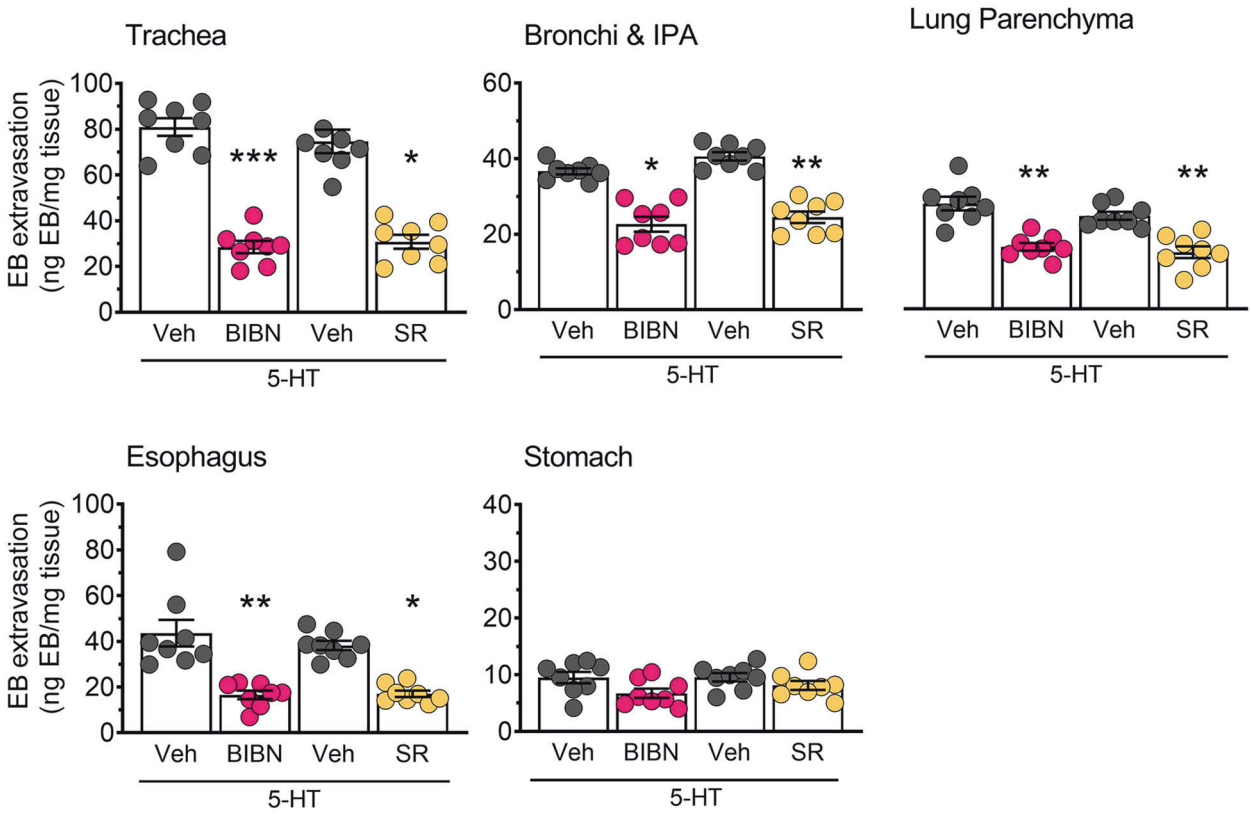

Fig. 7 Inhibition of CGRP or NK_1 receptors blocks 5-HT induced edema. Pre-treatment with the CGRP receptor antagonist BIBN4906 (BIBN; 1 mg/kg) or the NK_1R antagonist SR140333 (SR; 1 mg/kg) decreased plasma extravasation induced by 5-HT (100 µg/kg) in the airways and upper GI tract. Data are presented as mean ± S.E.M., $n = 9–10$ mice per group. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; One-way ANOVA and Dunnett's multiple comparisons test, significantly different compared to vehicle treated control (Veh).

inhibitor HC067. In contrast, the extent of GSK101-induced TRPV4-dependent vascular leak was markedly lower. Although this may be due to differences in the respective signaling pathways involved, it may also reflect the physicochemical properties of the ligands investigated and their relative bioavailability following systemic administration.

The release of neuropeptide transmitters from airway innervating nerves leads to inflammation and to vascular leak. This neurogenic response can also be initiated by exogenous irritants via airway nerves and may contribute to the development of airway pathologies [65–67]. We demonstrated that inhibitors of CGRP and SP receptors reduced TRPV4- and 5-HT-induced plasma extravasation in the airways and esophagus, supporting a mechanistic role for these neuropeptide receptors. It has been reported that CGRP does not cause microvascular leak in the airways and bladder of the guinea pig [68]. In contrast, CGRP has been reported to contribute to edema formation in mice [66] and rats [69], indicating potential species differences. Our results suggest that 5-HT-induced plasma extravasation in the airways and esophagus is mediated by activation of afferent nerves, requires TRPV4, and is likely to involve release of pro-inflammatory peptides (SP and CGRP) (Fig. 9).

Pre-clinically, TRPV4 plays important roles in pathological pulmonary edema and may therefore be a therapeutically useful target. Importantly, chronic treatment with a TRPV4 inhibitor in animal models did not affect osmoregulation or interfere with the activity of diuretics, which are often used to resolve edema in the clinic [18, 70–72]. Recently, a double-blind, placebo-controlled study using a selective TRPV4 antagonist reported that treatment with GSK2798745 resulted in a trend to improve pulmonary gas exchange in symptomatic patients with chronic heart failure [71, 73]. However, the use of inhibitors that directly target TRPV4 in pulmonary injury may be contraindicated by the role that TRPV4 plays in the complex signaling cascade that mediates hypoxic pulmonary vasoconstriction [74]. This mechanism helps to redistribute blood flow from poorly ventilated to more aerated lung areas, and inhibition of this response could be detrimental to patients with lung

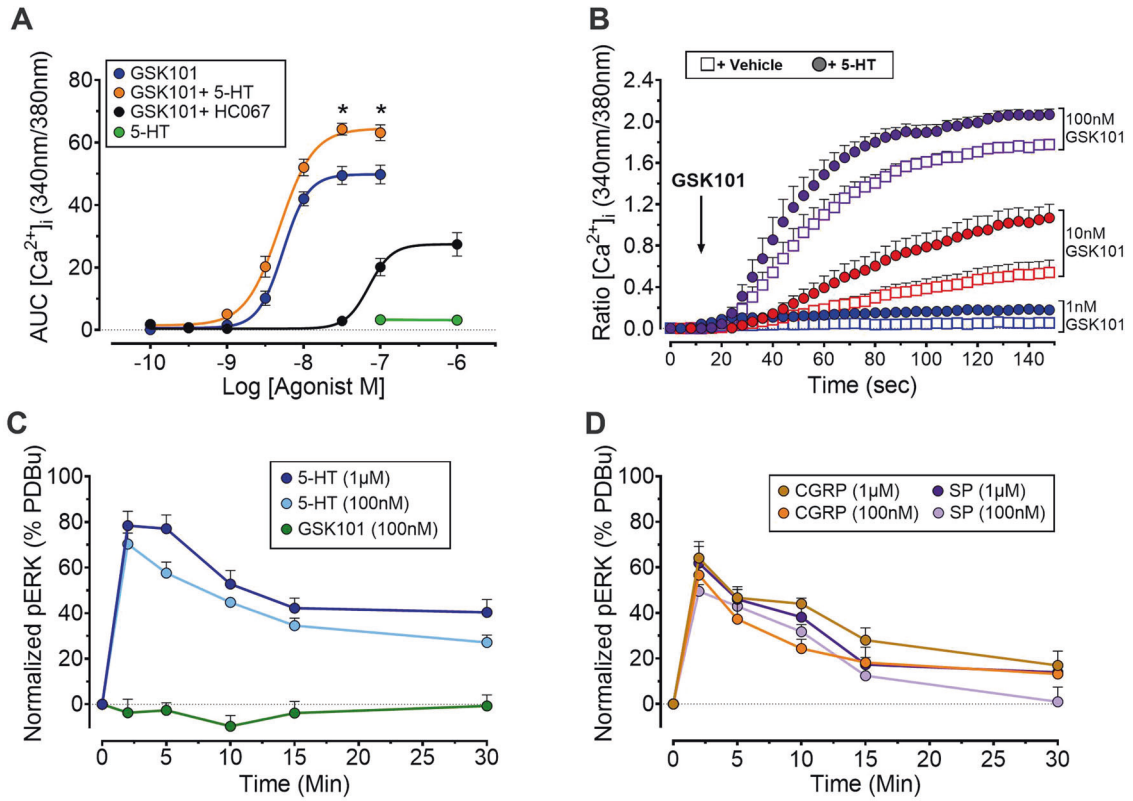

Fig. 8 Functional expression and interaction of TRPV4 and 5-HT receptors in HUVEC. A Pre-treatment with 5-HT augmented the magnitude (E_{max}) of responses to GSK101. GSK101-induced Ca^{2+} signaling was attenuated by the TRPV4 antagonist HC067. No change in $[Ca^{2+}]_i$ was detected following treatment with 5-HT. **B** Time traces demonstrating the effect of pre-treatment with 5-HT (open circle, 100 nM; 30 min) on GSK101-induced elevations in $[Ca^{2+}]_i$ (closed circles). **C**, **D** Elevated ERK phosphorylation (pERK) in response to treatment of HUVEC with 5-HT, GSK101, CGRP or SP. Data are presented as mean ± S.E.M., $n = 6$ technical replicates, pERK data are normalized to the positive control (PDBu, 1 μM).

disease [71, 73]. The benefits of TRPV4 antagonists for reducing pulmonary edema-associated lethality from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have also recently been proposed to outweigh the risks of contraindications such as these. Together, this highlights the need to further understand the relative cellular contribution of TRPV4 activity and also the upstream signaling mediators that lead to TRPV4 activation, to provide potential alternatives to these potent antagonists that directly inhibit TRPV4.

Our pharmacological data indicate that 5-HT_{2A} is the primary receptor subtype involved in promoting 5-HT-evoked plasma extravasation. Evidence supporting the in vivo requirement for TRPV4 activity is provided by the demonstration that 5-HT_{2A} mediated Ca^{2+} signaling is augmented by TRPV4 through a PLA_2- and PKC-dependent mechanism. Although our data support an indirect neurogenic mechanism of action involving enhanced neuropeptide release [23], the specific locations where 5-HT_{2A}-TRPV4 interactions occur (pre- or post-synaptic [75]) could not be definitively determined using available methodology. We cannot exclude a direct effect on endothelial cells as the HUVEC that we examined may not be the most suitable model for the microvasculature involved in 5-HT-dependent vascular leak as they are of a different origin and may not express the precise machinery required. Our results suggest that 5-HT and TRPV4 receptors are also expressed by endothelial cells and may cause protein leak via disruption of the vascular junctions in mice. In addition to expression by peptidergic afferent nerves [76] and vascular endothelial cells [77, 78], 5-HT receptors and TRPV4 are also expressed by immune cells, including macrophages [79–81]. Given the important immunomodulatory role of 5-HT, it is possible that the TRPV4-dependent effects of 5-HT on vascular permeability that we describe are mediated in part through immune cell activation. Future analysis to better define the relative contributions of 5-HT receptors and TRPV4 in endothelial and immune cells and on nerve endings of the different vascular beds would help to clarify the primary location of TRPV4-driven edema and the precise mechanisms involved.

In summary, we have established that TRPV4 mediates 5-HT-induced plasma extravasation in the airways and

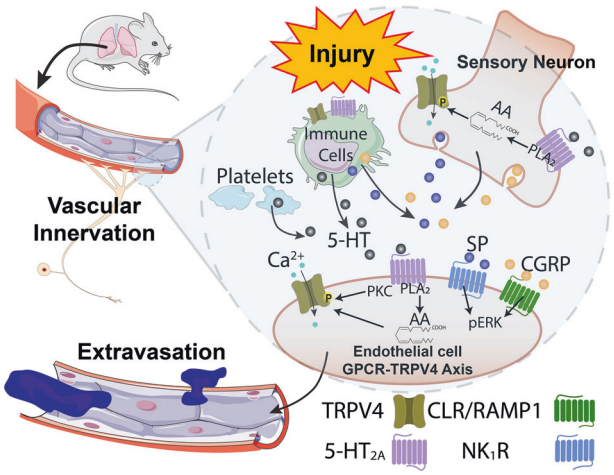

Fig. 9 Postulated neurogenic and direct mechanisms through which 5-HT receptors and TRPV4 may induce tissue edema. 5-HT activates 5-HT_{2A} receptor subtypes on afferent nerve terminals, immune cells, and vascular endothelial cells. Activation of peripheral sensory neurons or local immune cells promotes release of neuropeptides including SP and CGRP, activating NK_1R and CGRP receptors expressed by endothelial cells. Signaling downstream of these receptors leads to retraction of cell-cell junction proteins, leading to increased vascular leak and to tissue edema. Activation of 5-HT_{2A} sensitizes or activates TRPV4 through a PLA_2- and PKC-dependent mechanism, leading to enhanced neuropeptide release.

upper GI tract of mice through interaction with the 5-HT_{2A} receptor subtype. We have provided evidence to support an indirect, potentially neurogenic mechanism of action involving the neuropeptides SP and CGRP.

Data availability

Data presented in this study are available upon request from the corresponding authors.

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Author contributions *Participated in research design*: JSR, MSG, NWB, NAV, DPP, and PM. *Conducted experiments*: MSG, LKD, SP, FB, JSR, JGA, and SA. *Performed data analysis*: JSR, NAV, DPP, MSG, and PM. *Contributed to writing or critical assessment of the paper*: JSR, MSG, ABG, PR, PRG, SEC, NAV, DPP, and PM.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval Studies using mice were approved by the Animal Ethics Committees of RMIT and Monash Institute of Pharmaceutical Sciences.

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