Original Article

Activation of the 5-hydroxytryptamine 4 receptor ameliorates tight junction barrier dysfunction in the colon of type 1 diabetic mice

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Abstract

Hyperglycemia drives dysfunction of the intestinal barrier. 5-Hydroxytryptaine 4 receptor (5-HT$_4$R) agonists have been considered therapeutics for constipation in clnic. However, the roles of 5-HT$_4$R activation in mucosa should be fully realized. Here, we investigate the effects of 5-HT$_4$R activation on diabetes-induced disruption of the tight junction (TJ) barrier in the colon. Not surprisingly, the TJ barrier in diabetic mice with or without 5-HT$_4$R is tremendously destroyed, as indicated by increased serum fluorescein isothiocyanate (FITC)-dextran and decreased transepithelial electrical resistance (TER). Simultaneously, decreased expressions of TJ proteins are shown in both wild-type (WT) and 5-HT$_4$R knockout (KO) mice with diabetes. Notably, chronic treatment with intraperitoneal injection of a 5-HT$_4$R agonist in WT mice with diabetes repairs the TJ barrier and promotes TJ protein expressions, including occludin, claudin-1 and ZO-1, in the colon, whereas a 5-HT$_4$R agonist does not improve TJ barrier function or TJ protein expressions in 5-HT$_4$R KO mice with diabetes. Furthermore, stimulation of 5-HT$_4$R inhibits diabetes-induced upregulation of myosin light chain kinase (MLCK), Rho-associated coiled coil protein kinase 1 (ROCK1), and phosphorylated myosin light chain (p-MLC), which are key molecules that regulate TJ integrity, in the colonic mucosa of WT mice. However, such action induced by a 5-HT$_4$R agonist is not observed in 5-HT$_4$R KO mice with diabetes. These findings indicate that 5-HT$_4$R activation may restore TJ integrity by inhibiting the expressions of MLCK, ROCK1 and p-MLC, improving epithelial barrier function in diabetes.

Key words 5-HT$_4$R, diabetes, tight junction barrier, MLCK, ROCK1

Introduction

Hyperglycemia, a characteristic of the metabolic disease known as diabetes, is linked to a variety of dysfunctions. As an autoimmune T-cell-mediated condition, type 1 diabetes (T1D) is associated with disorders of the immune system, microbiota, and mucosal barrier in the gut [1]. Emerging evidence indicates that T1D-induced hyperglycemia results in the disruption of the intestinal barrier and risk of enteric infection through reprogramming epithelial cells [2].

The intestinal mucosa acts as a barrier between the host and luminal contents. The physical epithelial barrier can effectively prevent pathogens, toxins, antigens, and other harmful molecules from entering the lumen [3]. Increased intestinal permeability is a sign of epithelial dysfunction, which is linked to the disruption of tight junctions (TJs), the primary factor determining paracellular permeability [4,5]. The TJ consists of multiple protein complexes, including claudins and occludin, transmembrane proteins, and zona occludens (ZOs), linking transmembrane and cytoplasmic proteins [6]. Reduced expression of ZO-1 in *db/db* mice associated with intestinal dysfunction has been reported [2].

Peripheral 5-hydroxytryptamine (5-HT) produced by enterochromaffin cells plays an important role in motor, secretory and sensory

functions in the gut by activating several 5-HT receptors [7]. Recently, close attention has been paid to the importance of the mucosal 5-HT$_4$ receptor (5-HT$_4$R). Stimulation of mucosal 5-HT$_4$R evokes 5-HT release, goblet cell degranulation and Cl$^-$ secretion, and activation of mucosal 5-HT$_4$R can produce antinociceptive action [8]. The activation of epithelial 5-HT$_4$R can reduce inflammation by promoting epithelial cell proliferation and wound healing [9]. 5-HT$_4$R agonist protects the colon against diabetes-induced bacterial translocation by triggering mucin 2 production [10]. Therefore, it is reasonable to speculate that activation of 5-HT$_4$R may restore disruption of TJ barrier in diabetes.

It is generally accepted that phosphorylation of myosin light chain (p-MLC) induces contraction of the perijunctional actomyosin ring, which leads to the internalization of TJ proteins and disruption of TJ barrier. MLC kinase (MLCK) and Rho-associated coiled coil protein kinase (ROCK) can phosphorylate MLC directly [11]. Melatonin has been shown to ameliorate intestinal epithelial permeability in diabetic rats by inhibiting either MLCK or ROCK signaling [12]. Furthermore, PKA has been linked to the downregulation of MLC phosphorylation through the inhibition of MLCK and ROCK signaling in endothelial barrier function [13,14]. 5-HT$_4$R is a G protein-coupled receptor which activates the intracellular cAMP-PKA substrate when stimulated.

In the present study, we investigated whether 5-HT$_4$R activation ameliorates diabetes-driven TJ barrier dysfunction by inhibiting MLC phosphorylation mediated by either MLCK or ROCK1.

Materials and Methods
Animals
Adult ($\sim$10 week) male 5-HT$_4$R knockout (KO) and age-matched wild-type (WT) C57BL/6 mice were used in the current study. The 5-HT$_4$R KO mice (GeneBank Accession Number: NM_008313.4; Ensembl: ENSMUSG00000026322) were purchased from Cyagen Biosciences Inc. (Guangzhou, China) and bred at the Animal Facility of Basic Medical Sciences, Shanghai Jiao Tong University. The success of 5-HT$_4$R KO in mice is shown in Supplementary Figure S1. The animals were housed in a temperature-controlled room (25°C) with specific pathogen-free conditions and *ad libitum* access to water and food. All experimental protocols were approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine (A-2022-051).

Induction of diabetes and experimental design
Mice were injected intraperitoneally with streptozotocin (STZ; 150 mg/kg; Sigma, St Louis, USA) dissolved in 0.01 M cold citrate buffer (pH 4.0) to establish type 1 diabetes. Nondiabetic mice were given the same vehicle volume. Blood glucose was tested weekly using the glucose-oxidase test strip and a reflectance meter (Roche Diagnostics GmbH, Clarecastle, Ireland). Animals showing sustained hyperglycemia were used for the subsequent experiments. After 2 weeks of STZ injection, a highly selective 5-HT$_4$R agonist RS67333 (1 mg/kg; Tocris, Ellisville, USA) was intraperitoneally administered once every other day six times based on our preliminary experiment and a previous study [15]. The following experiments were performed 24 h after the last injection.

Measurement of serum FITC-dextran
On the test day, the mice were fasted for 4 h, and then 4 kDa fluorescein isothiocyanate (FITC)-dextran (440 mg/kg) was administered by gavage. Blood was collected from the heart 3 h after gavage, centrifuged at 10,000 g for 10 min and left to stand overnight at 4°C. The fluorescence of serum FITC-dextran was measured using a fluorescence plate reader (Gene Company Limited, Shanghai, China) with 485 nm excitation and 535 nm emission.

Ussing chamber experiment
Transepithelial electrical resistance (TER) was measured with a P 2300 Ussing chamber system (Warner Instruments, Holliston, USA) according to the manufacturer's instructions. In brief, Eusebio chambers were calibrated, and the flat sheet of distal colon excised from mice was immediately mounted onto the chamber pin. Voltage clamp recordings were performed. Tissues were maintained at 37°C in oxygenated (95% O$_2$ with 5% CO$_2$) Krebs solution (120 mM NaCl, 5.9 mM KCl, 1.2 mM NaH$_2$PO4$_2$, 1.2 mM MgSO$_4$, 15.4 mM NaHCO$_3$, 2.5 mM CaCl$_2$, and 11.5 mM glucose) throughout the recording period [16].

Immunofluorescence (IF) microscopy
Mice were transcardially perfused with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde (PFA) under deep anesthesia. The distal colons were removed, postfixed in 4% PFA and embedded with OCT. The sections (10 μm) were blocked with 10% normal goat serum in 0.05 M PBS and were incubated with one of the following primary antibodies at 4°C overnight: 1) rabbit anti-occludin (1:1500; GB111401; Servicebio, Shanghai, China); 2) rabbit anti-claudin-1 (1:500; GB11032; Servicebio); and 3) rabbit anti-ZO-1 (1:1500; GB111402; Servicebio). The following day, sections were incubated with goat anti-rabbit Alexa Fluor 488 secondary antibody (1:1000; Molecular Probes-Invitrogen, Eugene, USA) at room temperature for 1 h. The sections were viewed under a fluorescence microscope (Leica DM2500; Leica, Wetzlar, Germany), and digital images were captured using Leica Application Suite version 4.3 (Leica). Integrated density was measured to evaluate fluorescent signals using ImageJ (http://rsb.info.nih.gov/ij/).

Western blot analysis
The distal colon was longitudinally opened, and the mucosa was removed by cell scraping. The mucosa was homogenized with lysis buffer (20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1 mM EDTA, 1 mM PMSF), and protease inhibitor cocktail and phosphatase inhibitor. Equal amounts of protein were separated on a 10% Tris-glycine gel and then transferred to a PVDF membrane (Merck Millipore, Darmstadt, Germany). Blots were incubated with one of the following antibodies: 1) rabbit anti-occludin (1:2000; #91131; Cell Signaling, Beverly, USA); 2) rabbit anti-claudin-1 (1:2000; T56872; Abmarts, Berkeley Heights, USA); 3) rabbit anti-MLCK (1:500; GB113358; Servicebio); 4) rabbit anti-ROCK1 (1:500; GB111691; Servicebio); and 5) rabbit anti-pMLC (1:500; TA8618; Abmarts), followed by incubation with HRP-conjugated secondary antibody (1:2000; #1706515; Bio-Rad, Hercules, USA). The density of specific bands was measured with ImageJ and was normalized against a loading control (β-actin).

Statistical analysis
Data are presented as the mean ± standard error and then statistically analyzed using GraphPad Prism version 9.0 (GraphPad, La Jolla, USA). The percentage change in either serum FITC-dextran

or immunofluorescent signal was quantified when the mean value in the nondiabetic group was considered 100. One-way or two-way ANOVA with post hoc Tukey's multiple comparisons test was performed as appropriate. $P < 0.05$ was considered statistically significant.

Results

5-HT₄R activation attenuates diabetes-induced dysfunction in the TJ barrier

To determine whether 5-HT₄R activation affects TJ barrier function in diabetic mice, we detected serum FITC-dextran and TER levels in the distal colon upon chronic treatment with the 5-HT₄R agonist RS67333 (1 mg/kg) beginning 2 weeks after STZ injection. The TJ barrier was destroyed, as indicated by higher serum FITC-dextran levels in both WT and 5-HT₄R KO mice with diabetes than in non-diabetic mice. Treatment with chronic RS67333 significantly reduced the diabetes-induced increase in serum FITC-dextran in WT mice. To confirm that RS67333-mediated protection occurs via the 5-HT₄R receptor, experiments were performed in mice with

genetic deletion of 5-HT₄R. As expected, the protective effect of RS67333 on the TJ barrier against diabetes was not observed in mice lacking 5-HT₄R (Figure 1A). In addition, TER decline in the distal colon, which is a direct index of a disrupted TJ barrier, was observed in both WT and 5-HT₄R KO mice with diabetes. The 5-HT₄R agonist alleviated diabetes-induced TER decline in the distal colon in WT mice, but this effect was absent in 5-HT₄R KO mice, further providing evidence that 5-HT₄R activation protects the TJ barrier against diabetes (Figure 1B–D). However, RS67333 did not affect diabetes-induced changes in body weight and blood glucose in either WT or 5-HT₄R KO mice (Table 1).

5-HT₄R activation inhibits diabetes-induced downregulation of TJ proteins in the colon

To investigate whether 5-HT₄R-mediated protection from diabetes-driven TJ barrier disruption is involved in 5-HT₄R-promoted expression of TJ proteins, we examined the effects of a 5-HT₄R agonist on occludin, claudin-1, and ZO-1 expressions in the diabetic colon by IF staining and western blot analysis.
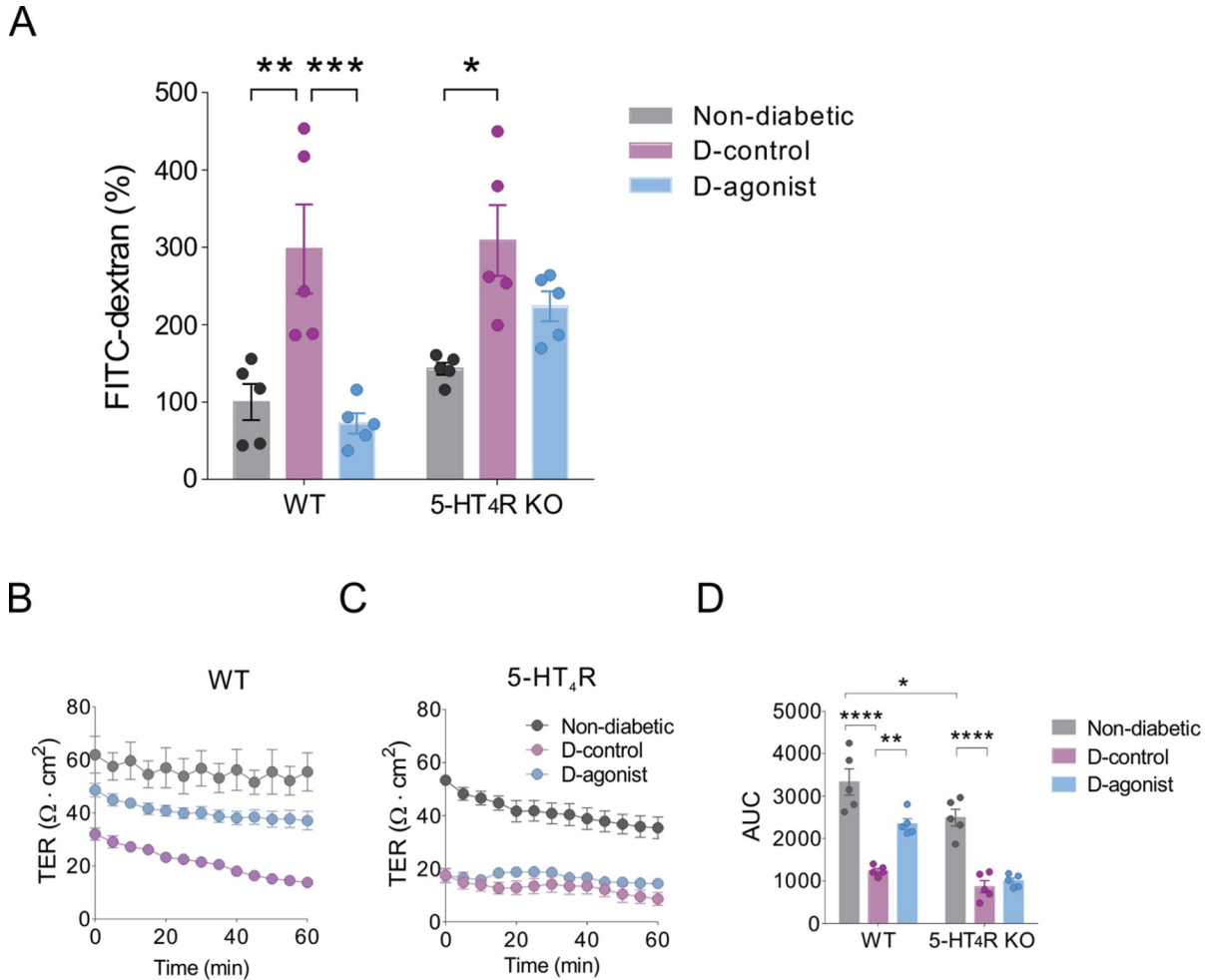

Figure 1. Activation of 5-HT₄R alleviates diabetes-induced dysfunction of the tight junction barrier in mice (A) Effects of a 5-HT₄R agonist on serum FITC-dextran 4 weeks after STZ injection in WT and 5-HT₄R KO mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, two-way ANOVA with Tukey's multiple comparisons test ($n = 5$ mice for each group). (B–D) Effects of a 5-HT₄R agonist on distal colon TER 4 weeks after STZ injection in WT and 5-HT₄R KO mice. Ussing chamber recordings from WT and 5-HT₄R mice with nondiabetic, diabetic-control and diabetic-5-HT₄R agonist treatments are shown in (B,C). The area under the curve (AUC) for the statistical analysis of TER is shown in (D). *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, two-way ANOVA with Tukey's multiple comparison test ($n = 5$ mice for each group). D: diabetic.

Table 1. Effects of a 5-HT$_4$R agonist on diabetes-induced alterations in body weight and blood glucose levels in WT and 5-HT$_4$R KO mice

Group	Body weight (from day 0)			Blood glucose (mM)		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
WT Nondiabetic	100	105.2 ± 2.18	111.3 ± 2.05	9.1 ± 0.26	10.2 ± 0.31	9.0 ± 0.19
WT D-control	100	82.3 ± 4.32[****]	82.4 ± 1.99[****]	9.1 ± 0.42	28.6 ± 1.06[****]	32.9 ± 0.45[****]
WT D-agonist	100	80.4 ± 3.55	81.3 ± 2.15	10.5 ± 0.45	29.1 ± 0.97	32.8 ± 0.40
KO Nondiabetic	100	106.5 ± 5.17	110.9 ± 5.64	9.1 ± 0.30	8.7 ± 0.23	9.4 ± 0.41
KO D-control	100	84.4 ± 1.42[****]	83.1 ± 2.94[****]	10.2 ± 0.36	27.6 ± 1.67[****]	31.5 ± 1.06[****]
KO D-agonist	100	83.8 ± 2.32	82.6 ± 2.86	8.8 ± 0.4	25.6 ± 2.03	31.9 ± 1.38

[****]$P < 0.0001$, vs nondiabetic.

As shown in nondiabetic mice, occludin IF staining, characterized by a band-like pattern, was evenly localized on the lateral membrane of epithelial cells in the colon. On the other hand, weak staining and uneven occludin distribution were observed in mice with diabetes. Notably, the dense staining pattern of occludin was repaired by the 5-HT$_4$R agonist in mice with diabetes (Figure 2A). Expression of occludin in the colon was downregulated by diabetes in both WT and 5-HT$_4$R KO mice. The 5-HT$_4$R agonist restored occludin expression in diabetic WT mice, while it failed to exert such an effect on diabetic mice with 5-HT$_4$R deletion. Even in nondiabetic mice, occludin expression was lower in 5-HT$_4$R KO mice than in WT mice (Figure 2B,C).

IF staining revealed that claudin-1 was continuously localized on the lateral membrane of epithelial cells, particularly concentrated in the apical part, in the colon of nondiabetic mice. However, the scattered distribution of claudin-1 was observed in colon epithelial cells in diabetic mice. Strikingly, continuous distribution partially reappeared in diabetic mice treated with the 5-HT$_4$R agonist (Figure 3A). The quantitative change in IF staining of claudin-1 was similar to that of occludin in both WT and 5-HT$_4$R KO mice (Figure 3B,C).
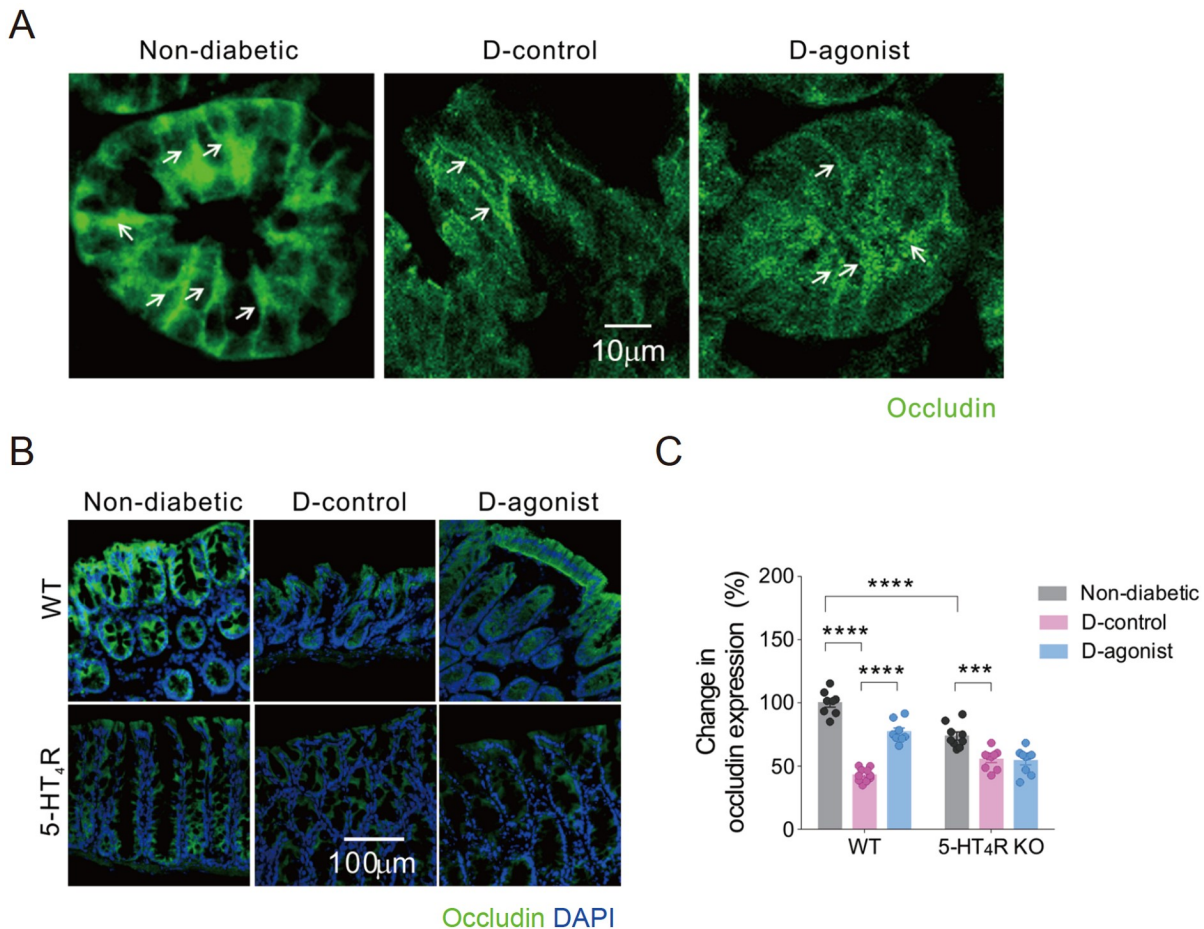

Figure 2. Activation of 5-HT$_4$R interferes with diabetes-induced decrease in immunofluorescence intensity of occludin in the colon of mice (A) Representative photomicrographs of IF staining of occludin in colon epithelial cells from WT mice, indicated by white arrows. Scale bar: 10 μm for all three images. (B) Representative photomicrographs of IF staining of occludin (green) with DAPI (blue) in colon sections of WT and 5-HT$_4$R KO mice. Scale bar: 100 μm for all six images. (C) Quantification of the IF intensity of occludin. ***$P < 0.001$, ****$P < 0.0001$, two-way ANOVA with Tukey's multiple comparisons test ($n = 7$–10 sections from 3–4 mice for each group). D: diabetic.
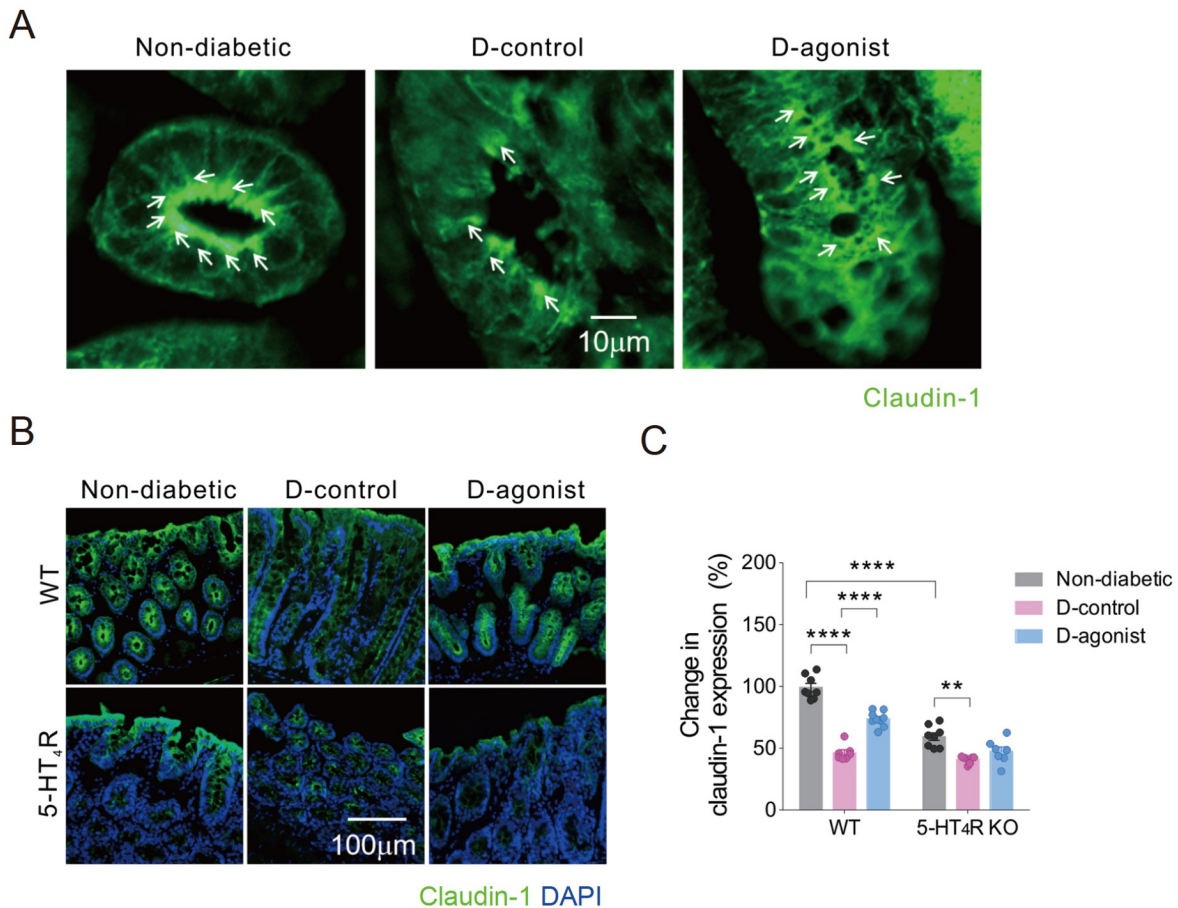
Figure 3. Activation of 5-HT$_4$R restricts diabetes-induced decrease in immunofluorescence intensity of claudin-1 in the colon of mice (A) Representative photomicrographs of IF staining of claudin-1 in colon epithelial cells from WT mice, indicated by white arrows. Scale bar: 10 μm for all three images. (B) Representative photomicrographs of IF staining of claudin-1 (green) with DAPI (blue) in colon sections of WT and 5-HT$_4$R KO mice. Scale bar: 100 μm for all six images. (C) Quantification of the IF intensity of claudin-1. **$P < 0.01$, ****$P < 0.0001$, two-way ANOVA with Tukey's multiple comparisons test ($n = 7$–10 sections from 3–4 mice for each group). D: diabetic.

Thick line-like ZO-1 staining was regularly distributed throughout the lateral membrane of epithelial cells in the colon of diabetes-free mice. Thin line-like ZO-1 staining was irregularly localized on the lateral membrane of epithelial cells in the colon of mice with diabetes. Compared to diabetic control mice, a greater amount of thin-line ZO-1 staining was regularly distributed in colon epithelial cells in diabetic mice treated with the 5-HT$_4$R agonist (Figure 4A). Quantitative changes in IF staining of ZO-1 were similar to those of occludin and claudin-1 in both WT and 5-HT$_4$R KO mice (Figure 4B,C).

Western blot analysis showed that occludin and claudin-1 protein levels in colonic mucosa were lower in diabetic mice than in nondiabetic mice with or without 5-HT$_4$R. As expected, stimulation of 5-HT$_4$R by administration of its agonist enhanced occludin and claudin-1 protein levels in colonic mucosa in WT mice with diabetes, but the agonist did not block diabetes-induced decreases in occludin and claudin-1 protein levels in colonic mucosa in 5-HT$_4$R KO mice (Figure 5).

5-HT$_4$R activation inhibits diabetes-triggered upregulation of MLCK, ROCK1 and pMLC in the colon

To determine whether the 5-HT$_4$R-mediated restoration of TJ proteins in the colonic mucosa in diabetes is related to the recovery of TJ integrity, we performed western blot analysis to examine the protein levels of MLCK, ROCK1, and p-MLC in the colonic mucosa after chronic treatment with the 5-HT$_4$R agonist RS67333. In WT mice, the protein levels of MLCK, ROCK1, and p-MLC in the colonic mucosa were increased by STZ but not by vehicle. Chronic treatment with RS67333 dramatically inhibited diabetes-induced upregulation of MLCK, ROCK1, and pMLC in colonic mucosa compared to the control. To confirm that such effects induced by RS67333 occur via 5-HT$_4$R, protein levels in mice lacking 5-HT$_4$R were further tested. STZ still led to increases in the protein levels of MLCK, ROCK1, and p-MLC in the colonic mucosa compared to vehicle treatment, but RS67333 did not block the diabetes-induced increases in the protein levels of MLCK, ROCK1, or p-MLC in the colonic mucosa of 5-HT$_4$R KO mice (Figure 6).

Discussion

Intestinal homeostasis is required for the physiological function of the intestinal epithelial barrier [17,18]. Intestinal barrier dysfunction has been associated with a broad range of diseases, including inflammatory bowel disease [19], celiac disease [20] and nonalcoholic fatty liver disease [21], and is also correlated with systemic disorders of the outside intestine, such as diabetes [2], cancer [22] and neurodegeneration [23]. T1D induces intestinal barrier dys-
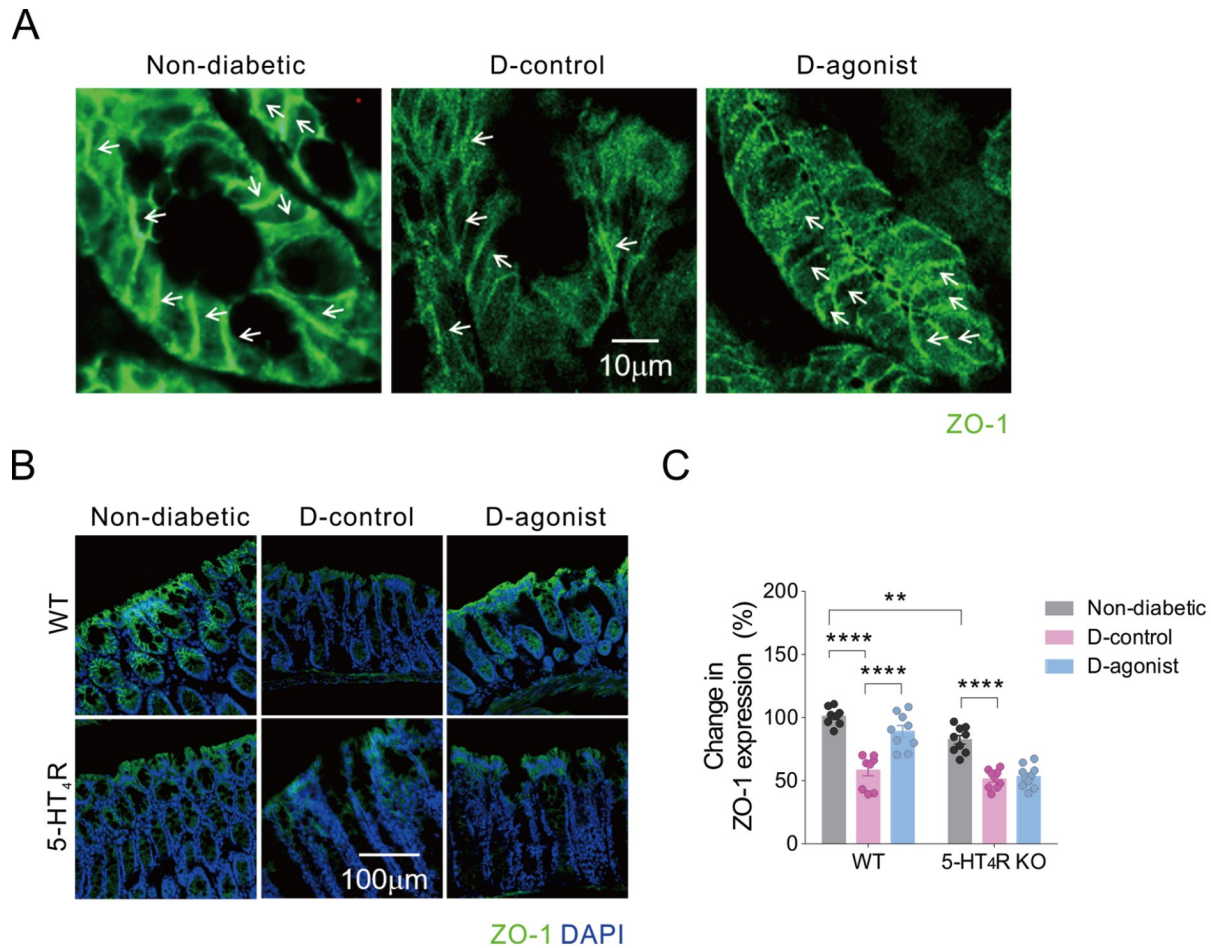
Figure 4. Activation of 5-HT$_4$R impedes diabetes-induced decrease in immunofluorescence intensity of ZO-1 in the colon of mice (A) Representative photomicrographs of IF staining of ZO-1 in colon epithelial cells from WT mice, indicated by white arrows. Scale bar: 10 μm for all three images. (B) Representative photomicrographs of IF staining of ZO-1 (green) with DAPI (blue) in colon sections of WT and 5-HT$_4$R KO mice. Scale bar: 100 μm for all six images. (C) Quantification of the IF intensity of ZO-1. $**P < 0.01$, $****P < 0.0001$, two-way ANOVA with Tukey's multiple comparison test ($n = 7$–10 sections from 3–4 mice for each group). D: diabetic.

function, developing both commensal bacterial penetration [10] and pathogen infection [2]. In the current study, increased serum FITC-dextran and decreased TER, considered paracellular permeability dysfunction, were observed in STZ-induced diabetes. Our findings are consistent with the above studies where T1D develops intestinal barrier disruption.

The intestinal epithelium plays a complex role as a semipermeable barrier that supports the absorption of nutrients while preventing the translocation of harmful luminal contents into the body [17]. An interplay between structural components and molecular interactions in the intestinal epithelium maintains intestinal integrity to achieve such a "contradictory" role. The epithelial barrier consists of two major selective permeability pathways, one of which is the paracellular pathway that is regulated primarily by TJs and adherens junctions. TJs consist of multiple protein complexes of transmembrane, cytoplasmic plaque, cytoskeletal, and signaling proteins [6]. Occludin is the first identified transmembrane TJ protein [24]. Defects in barrier function are absent in mice lacking occludin [25], while enhanced TJ permeability occurs in epithelial monolayers with *occludin* knockdown [26]. The current study showed that occludin expression in the colon detected by both immunofluorescence staining and western

blot analysis was decreased by STZ, confirming that T1D leads to occludin downregulation in the colon. Claudins, also transmembrane proteins, were initially identified in chicken liver junctional fractions [27]. Water and macromolecules will be lost when claudin-1, considered "sealing claudins", is deleted [28]. Claudin-1 expression in the colon assessed by either immunofluorescence staining or western blot analysis was shown to be decreased by STZ in our study, indicating that T1D also results in the downregulation of claudin-1 in the colon. ZOs, multidomain scaffolding proteins, form a complex linking transmembrane and cytoskeletal proteins [29]. A previous study indicated that ZO-1, rather than ZO-2 or -3, plays a more important role in controlling TJ assembly [30]. Although the protein levels of ZO-1 could not be detected by western blot analysis due to the limitations of the antibody utilized in the current study, immunofluorescence staining results demonstrated that ZO-1 expression in the colon was reduced by STZ, suggesting that T1D also induces ZO-1 downregulation in the colon. Taken together, increased epithelial permeability and downregulation of occludin, claudin-1 and ZO-1 were observed in diabetic mice, indicating that T1D induces TJ barrier disruption.

The consensus is that 5-HT$_4$R, one of the 5-HT receptors, is widely distributed in the gastrointestinal (GI) tract, including enteric
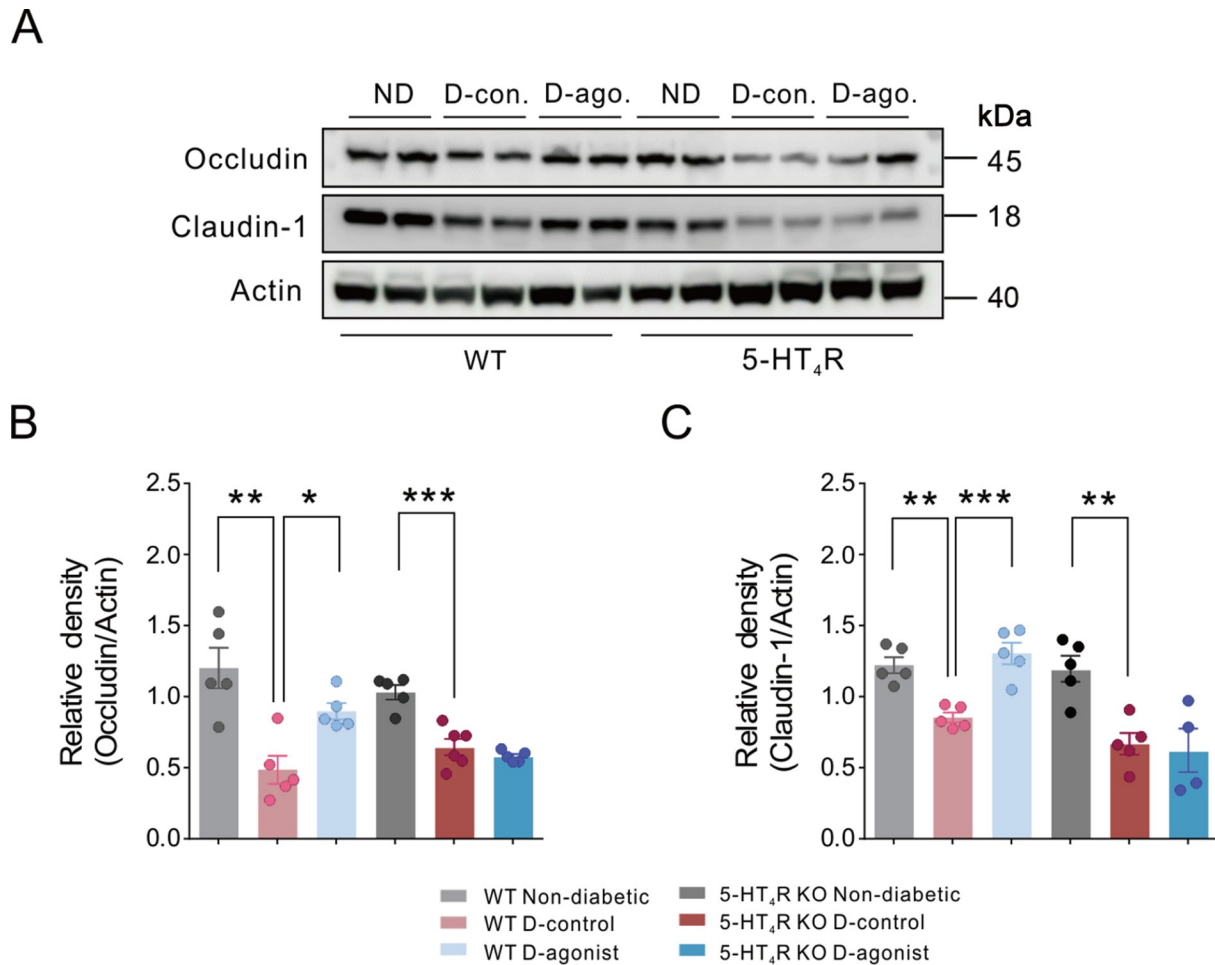
Figure 5. Activation of 5-HT$_4$R limits diabetes-induced decrease in protein levels of occludin and claudin-1 in colonic mucosa in mice
(A) Representative occludin and claudin-1 immunoblots in colonic mucosa from WT and 5-HT$_4$R KO mice. ND: nondiabetic; D-con.: diabetic-control; D-ago.: diabetic-agonist. (B,C) Quantification of occludin and claudin-1 immunoblots. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one-way ANOVA with Tukey's multiple comparisons test ($n = 4$–6 mice for each group).

neurons [31] and epithelial cells [8–10]. 5-HT$_4$R agonists have generally been used to treat constipation due to their prokinetic actions [32]. In particular, a 5-HT$_4$R agonist has been used to treat gastroparesis in a murine model of diabetes [33] but also in patients with diabetic gastroparesis [34]. Recent studies highlight the protective actions of 5-HT$_4$Rs expressed in epithelial cells. Epithelial 5-HT$_4$R-mediated enhancement of wound healing processes is related to increased cell proliferation/migration and decreased cell apoptosis [9,35]. Although it has been reported that diabetes results in a decline in the endogenous ligand 5-HT [36], STZ cannot influence 5-HT$_4$R expression in colon epithelial cells [10]. In addition, protection against the penetration of commensal bacteria into the mucosa against diabetes occurs by promoting mucin 2 production when 5-HT$_4$R is stimulated [10]. Here, we reported that activation of 5-HT$_4$R stimulated by RS67333 alleviated increased epithelial permeability, characterized by higher serum FITC-dextran and lower TER in diabetic mice compared to nondiabetic mice. In addition, RS67333 may inhibit T1D-driven downregulation of TJ proteins, including occludin, claudin-1, and ZO-1. However, these effects induced by RS67333 failed to be achieved in mice lacking 5-HT$_4$R. The protective effects of 5-HT$_4$R activation were independent of glucose control because RS67333 was not capable of altering

diabetes-induced changes in body weight and blood glucose. These results suggest that 5-HT$_4$R activation can ameliorate T1D-induced TJ barrier disruption, expanding the above-mentioned knowledge about the protective actions of epithelial 5-HT$_4$R.

5-HT$_4$R is a Gs protein-coupled receptor that specifically activates adenylyl cyclase to induce intracellular cAMP formation and subsequent activation of protein kinase A (PKA) [37]. PKA has been linked to downregulation of MLC phosphorylation through inhibition of MLCK and ROCK signaling in endothelial barrier function [13,14]. In the current study, the 5-HT$_4$R agonist RS67333 inhibited diabetes-induced upregulation of MLCK, ROCK1, and p-MLC in the colon, but the agonist failed to produce such an effect when *5-HT$_4$R* was knocked out, indicating that 5-HT$_4$R-induced activation of PKA may lead to inhibition of MLCK/ROCK1 expression and downstream phosphorylation of MLC.

It is well known that phosphorylation of MLC leads to perijunctional actomyosin ring contraction, disrupting TJ protein assembly and TJ barrier integrity [4]. MLCK is a Ca^{2+}-calmodulin-activated serine/threonine kinase that phosphorylates MLC to promote actomyosin ring contraction [6]. MLCK1 is preferentially localized within the perijunctional actomyosin ring [38]. It has been reported that *MLCK1* knockdown decreases TJ permeability to
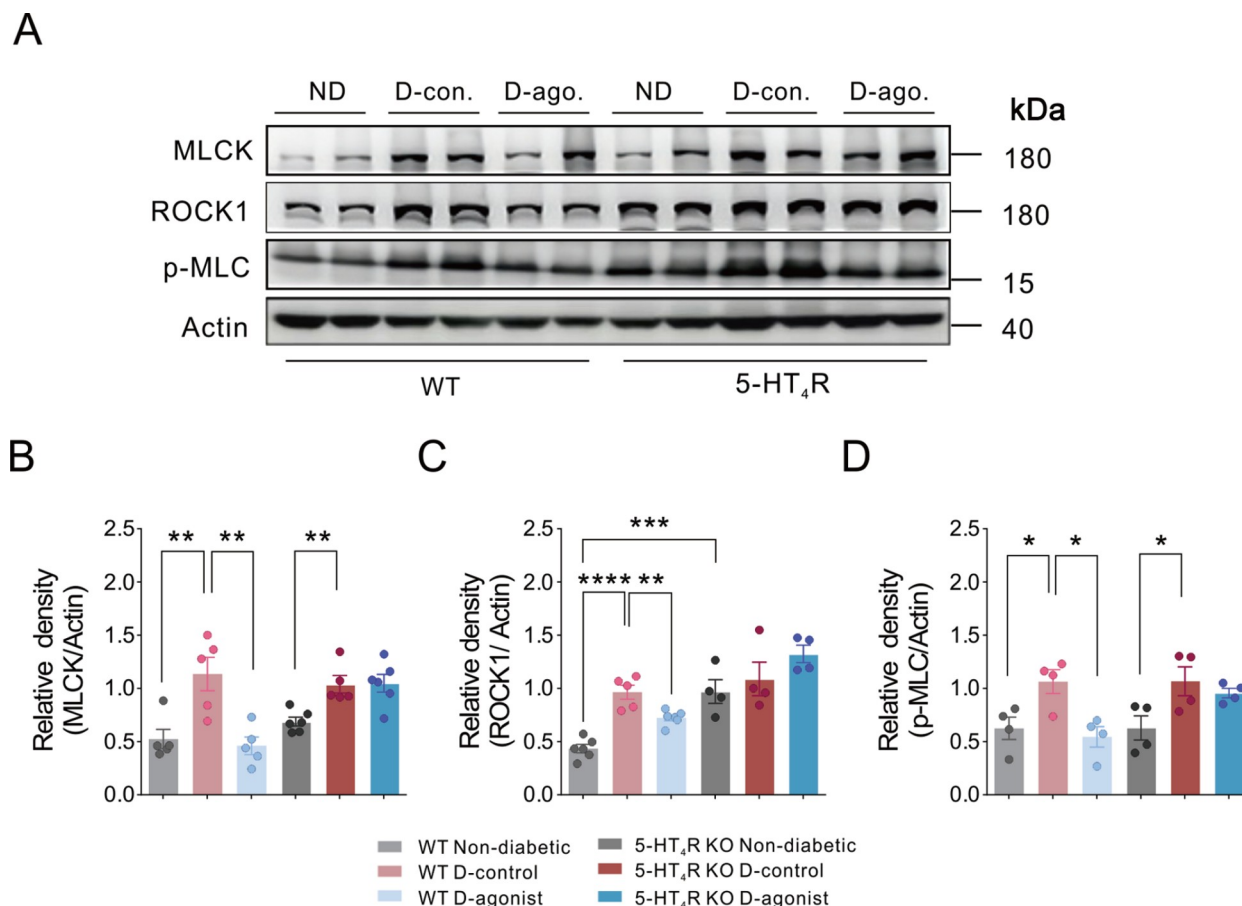
Figure 6. **Activation of 5-HT$_4$R suppresses diabetes-driven increase in the protein levels of MLCK, ROCK1 and p-MLC in the colonic mucosa of mice** (A) Representative immunoblots of MLCK, ROCK1 and p-MLC in colonic mucosa from WT and 5-HT$_4$R KO mice. ND: nondiabetic; D-con.: diabetic-control; D-ago.: diabetic-agonist. (B–D) Quantification of MLCK, ROCK1 and p-MLC immunoblots. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, one-way ANOVA with Tukey's multiple comparisons test ($n = 4$–6 mice for each group).

regulate barrier function [39]. MLC phosphorylation can also be directly impacted by ROCKs, which are downstream effectors of GTP-binding Rho proteins and regulate perijunctional actomyosin ring dynamics [40,41]. ROCK1 is widely distributed in the GI tract, liver, and lung [42]. Proinflammatory cytokines cause dysfunction of the intestinal TJ barrier via either MLCK1 or ROCK1, leading to increased intestinal permeability [43,44]. The current study showed that T1D led to the downregulation of TJ proteins and upregulation of MLCK, ROCK1, and p-MLC, suggesting that disrupted TJ assembly induced by MLCK/ROCK1-p-MLC signaling pathways may contribute to the downregulation of TJ proteins in the colon in diabetes. However, the limitation of the current study is that we did not provide direct evidence that 5-HT$_4$R activation protects the TJ barrier against diabetes by limiting the MLCK/ROCK1-pMLC signaling pathways.

Emerging evidence indicates potential therapeutic targeting of MLCK- and ROCK-mediated pathways [45,46], but inhibitors targeting either MLCK or ROCK have some limitations. For example, systemic toxicity is associated with smooth muscle MLCK inhibition and lower selectivity. Consequently, therapeutic approaches to inhibit epithelial MLCK or ROCK are not currently clinically available. However, 5-HT$_4$R agonists have been used in clinics. Intriguingly, our current study demonstrated that 5-HT$_4$R activation not only inhibits the diabetes-induced upregulation of MLCK and

ROCK1 but also restores the downregulation of TJ proteins in the colon in diabetes. These data suggest that 5-HT$_4$R may become a potential therapeutic target to regulate MLCK or ROCK for the treatment of GI disorders related to intestinal barrier in clinic.

In summary, this study demonstrated that a 5-HT$_4$R agonist alleviated T1D-induced disruption of the TJ barrier, decreased the expressions of TJ proteins, including occludin, claudin-1, and ZO-1, and increased the expressions of MLCK, ROCK1, and p-MLC. However, such effects induced by the 5-HT$_4$R agonist fail to occur in diabetic mice with 5-HT$_4$R deletion. Our results suggest that 5-HT$_4$R activation protects the TJ barrier against diabetes, possibly by limiting the MLCK/ROCK1-pMLC signaling pathways (Figure 7).

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.
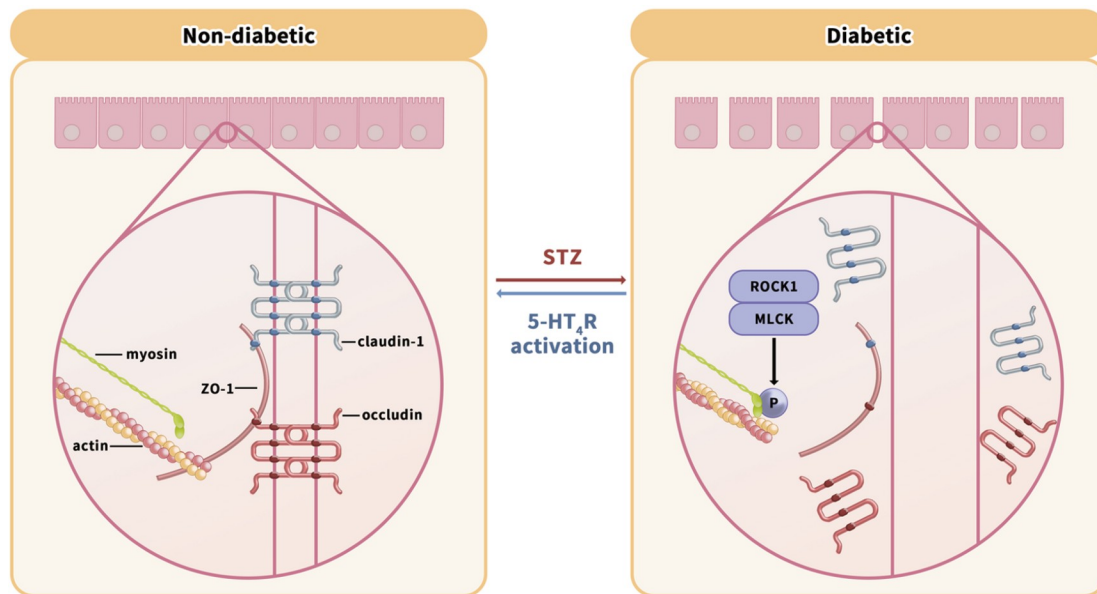
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Figure 7. Schematic diagram of the role of 5-HT$_4$R activation in the alleviation of diabetes-induced dysfunction of TJ barrier The potential protection of 5-HT$_4$R activation in the TJ barrier from diabetes is mediated by inhibition of diabetes-induced upregulation of MLCK/ROCK1-p-MLC pathways, which promotes the contraction of the actomyosin ring, leading to disruption of TJ integrity and downregulation of TJ proteins.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Mønsted MØ, Falck ND, Pedersen K, Buschard K, Holm LJ, Haupt-Jorgensen M. Intestinal permeability in type 1 diabetes: an updated comprehensive overview. *J Autoimmunity* 2021, 122: 102674

2. Thaiss CA, Levy M, Grosheva I, Zheng D, Soffer E, Blacher E, Braverman S, *et al.* Hyperglycemia drives intestinal barrier dysfunction and risk for enteric infection. *Science* 2018, 359: 1376–1383

3. Turner JR. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 2009, 9: 799–809

4. Suzuki T. Regulation of intestinal epithelial permeability by tight junctions. *Cell Mol Life Sci* 2013, 70: 631–659

5. Lee SH. Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. *Intest Res* 2015, 13: 11

6. Odenwald MA, Turner JR. The intestinal epithelial barrier: a therapeutic target? *Nat Rev Gastroenterol Hepatol* 2017, 14: 9–21

7. Spohn SN, Mawe GM. Non-conventional features of peripheral serotonin signalling—the gut and beyond. *Nat Rev Gastroenterol Hepatol* 2017, 14: 412–420

8. Hoffman JM, Tyler K, MacEachern SJ, Balemba OB, Johnson AC, Brooks EM, Zhao H, *et al.* Activation of colonic mucosal 5-HT4 receptors accelerates propulsive motility and inhibits visceral hypersensitivity. *Gastroenterology* 2012, 142: 844–854

9. Spohn SN, Bianco F, Scott RB, Keenan CM, Linton AA, O'Neill CH, Bonora E, *et al.* Protective actions of epithelial 5-hydroxytryptamine 4 receptors in normal and inflamed colon. *Gastroenterology* 2016, 151: 933–944.e3

10. Han C, Geng Q, Qin J, Li Y, Yu H. Activation of 5-hydroxytryptamine 4 receptor improves colonic barrier function by triggering mucin 2 production in a mouse model of type 1 diabetes. *Am J Pathol* 2022, 192: 876–886

11. Jin Y, Blikslager AT. The regulation of intestinal mucosal barrier by myosin light chain kinase/rho kinases. *Int J Mol Sci* 2020, 21: 3550

12. Yang X, Zou D, Tang S, Fan T, Su H, Hu R, Zhou Q, *et al.* Ameliorative effect of melatonin against increased intestinal permeability in diabetic rats: possible involvement of MLCK-dependent MLC phosphorylation. *Mol Cell Biochem* 2016, 416: 23–32

13. Tang S, Tang H, Su H, Wang Y, Zhou Q, Zhang Q, Wang Y, *et al.* Glucagon-like peptide-1 attenuates endothelial barrier injury in diabetes via cAMP/PKA mediated down-regulation of MLC phosphorylation. *Biomed Pharmacother* 2019, 113: 108667

14. Li X, Fetter R, Schwabe T, Jung C, Liu L, Steller H, Gaul U. The cAMP effector PKA mediates moody GPCR signaling in *Drosophila* blood–brain barrier formation and maturation. *eLife* 2021, 10: e68275

15. Agrawal L, Vimal SK, Shiga T. Role of serotonin 4 receptor in the growth of hippocampal neurons during the embryonic development in mice. *Neuropharmacology* 2019, 158: 107712

16. Thomson A, Smart K, Somerville MS, Lauder SN, Appanna G, Horwood J, Sunder Raj L, *et al.* The Ussing chamber system for measuring intestinal permeability in health and disease. *BMC Gastroenterol* 2019, 19: 98

17. Choi W, Yeruva S, Turner JR. Contributions of intestinal epithelial barriers to health and disease. *Exp Cell Res* 2017, 358: 71–77

18. Vancamelbeke M, Vermeire S. The intestinal barrier: a fundamental role in health and disease. *Expert Rev Gastroenterol Hepatol* 2017, 11: 821–834

19. Johansson MEV, Gustafsson JK, Holmén-Larsson J, Jabbar KS, Xia L, Xu H, Ghishan FK, *et al.* Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut* 2014, 63: 281–291

20. Heyman M, Abed J, Lebreton C, Cerf-Bensussan N. Intestinal permeability in coeliac disease: insight into mechanisms and relevance to pathogenesis. *Gut* 2012, 61: 1355–1364

21. Miele L, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, Mascianà R, *et al.* Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 2009, 49: 1877–1887

22. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, Taniguchi K, *et al.* Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* 2012, 491: 254–258

23. Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE,

Challis C, *et al.* Gut microbiota regulate motor deficits and neuroin-flammation in a model of parkinson's disease. *Cell* 2016, 167: 1469–1480. e12

24. Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. Occludin: a novel integral membrane protein localizing at tight junctions.. *J Cell Biol* 1993, 123: 1777–1788

25. Saitou M, Furuse M, Sasaki H, Schulzke JD, Fromm M, Takano H, Noda T, *et al.* Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol Biol Cell* 2000, 11: 4131–4142

26. Buschmann MM, Shen L, Rajapakse H, Raleigh DR, Wang Y, Wang Y, Lingaraju A, *et al.* Occludin OCEL-domain interactions are required for maintenance and regulation of the tight junction barrier to macromolecular flux. *Mol Biol Cell* 2013, 24: 3056–3068

27. Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol* 1998, 141: 1539–1550

28. Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, *et al.* Claudin-based tight junctions are crucial for the mammalian epidermal barrier. *J Cell Biol* 2002, 156: 1099–1111

29. Shen L, Weber CR, Raleigh DR, Yu D, Turner JR. Tight junction pore and leak pathways: a dynamic duo. *Annu Rev Physiol* 2011, 73: 283–309

30. Tsukita S, Katsuno T, Yamazaki Y, Umeda K, Tamura A, Tsukita S. Roles of ZO-1 and ZO-2 in establishment of the belt-like adherens and tight junctions with paracellular permselective barrier function. *Ann New York Acad Sci* 2009, 1165: 44–52

31. Poole DP, Xu B, Koh SL, Hunne B, Coupar IM, Irving HR, Shinjo K, *et al.* Identification of neurons that express 5-hydroxytryptamine 4 receptors in intestine. *Cell Tissue Res* 2006, 325: 413–422

32. Mawe GM, Hoffman JM. Serotonin signalling in the gut—functions, dysfunctions and therapeutic targets. *Nat Rev Gastroenterol Hepatol* 2013, 10: 473–486

33. crowell M, mathis C, schettler V, yunus T, lacy B. The effects of tegaserod, a 5-HT4 receptor agonist, on gastric emptying in a murine model of diabetes mellitus. *Neurogastroenterol Motil* 2005, 17: 738–743

34. Andrews CN, Woo M, Buresi M, Curley M, Gupta M, Tack J, Wilsack L, *et al.* Prucalopride in diabetic and connective tissue disease-related gastroparesis: randomized placebo-controlled crossover pilot trial. *NeuroGastroenterol Motil* 2021, 33: e13958

35. Park CJ, Armenia SJ, Zhang L, Cowles RA. The 5-HT4 receptor agonist prucalopride stimulates mucosal growth and enhances carbohydrate absorption in the ileum of the mouse. *J Gastrointest Surg* 2019, 23: 1198–1205

36. Cićin-Sain L, Jernej B. Reduction of gastrointestinal serotonin in alloxan-diabetic rats: reversal by 5-hydroxytryptophan treatment. *Behav Brain Res* 1996, 73: 285–288

37. Kumar JS, Mann J. PET tracers for serotonin receptors and their applications. *Cent Nerv Syst Agents Med Chem* 2014, 14: 96–112

38. Cunningham KE, Turner JR. Myosin light chain kinase: pulling the strings of epithelial tight junction function. *Ann New York Acad Sci* 2012, 1258: 34–42

39. Clayburgh DR, Rosen S, Witkowski ED, Wang F, Blair S, Dudek S, Garcia JGN, *et al.* A differentiation-dependent splice variant of myosin light chain kinase, MLCK1, regulates epithelial tight junction permeability. *J Biol Chem* 2004, 279: 55506–55513

40. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, *et al.* Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* 1996, 273: 245–248

41. Walsh SV, Hopkins AM, Chen J, Narumiya S, Parkos CA, Nusrat A. Rho kinase regulates tight junction function and is necessary for tight junction assembly in polarized intestinal epithelia. *Gastroenterology* 2001, 121: 566–579

42. Hartmann S, Ridley AJ, Lutz S. The function of rho-associated kinases ROCK1 and ROCK2 in the pathogenesis of cardiovascular disease. *Front Pharmacol* 2015, 6: 276

43. Graham WV, He W, Marchiando AM, Zha J, Singh G, Li HS, Biswas A, *et al.* Intracellular MLCK1 diversion reverses barrier loss to restore mucosal homeostasis. *Nat Med* 2019, 25: 690–700

44. Segain JP, Raingeard de la Blétière D, Sauzeau V, Bourreille A, Hilaret G, Cario-Toumaniantz C, Pacaud P, *et al.* Rho kinase blockade prevents inflammation via nuclear factor κB inhibition: evidence in Crohn's disease and experimental colitis. *Gastroenterology* 2003, 124: 1180–1187

45. Lin H, Cadete VJJ, Sawicka J, Wozniak M, Sawicki G. Effect of the myosin light chain kinase inhibitor ML-7 on the proteome of hearts subjected to ischemia–reperfusion injury. *J Proteomics* 2012, 75: 5386–5395

46. Paleček J, Zweigerdt R, Olmer R, Martin U, Kirschning A, Dräger G. A practical synthesis of Rho-Kinase inhibitor Y-27632 and fluoro derivatives and their evaluation in human pluripotent stem cells. *Org Biomol Chem* 2011, 9: 5503–5510