5-Hydroxytryptamine 4 Receptor Agonist Attenuates Diabetic Enteric Neuropathy through Inhibition of the Receptor-Interacting Protein Kinase 3 Pathway

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Diabetes is a common disorder worldwide and may affect 536.6 million people in 2021.1 Diabetes is a chronic disorder that leads to several complications, including vascular disease, peripheral and autonomic neuropathies, retinopathy, and gastrointestinal (GI) tract dysfunctions, such as delayed gastric emptying, diarrhea, constipation, and abdominal pain, in up to 75% of diabetic patients, which have a negative impact on quality of life.2,3 Accumulating evidence indicates that GI tract dysmotility in diabetes arises from autonomic neuropathy, with reports of cholinergic denervation, sympathetic nerve damage, altered sympathetic function, vagal nerve dysfunction,4–6 and impairment of enteric neurons.7,8–10

One of the potential mechanisms underlying diabetic neuropathy is neuronal death. Previous studies have shown increased apoptosis in dorsal root ganglion neurons, superior cervical ganglion neurons, myenteric neurons, and nodose ganglion neurons in rats and cell culture models of diabetes.11 In addition, diabetes is a distinctive neurodegenerative disorder, which is considered one of the acquired demyelinating diseases. It is characterized by various changes in Schwann cells and peripheral axons.12 Recently, Schwann cell death, including apoptosis and pyroptosis, has

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been suggested as one of the main causes of pathogenesis of diabetic neuropathy.\textsuperscript{13–15} Necroptosis, defined as programmed necrotic cell death, results in neural loss primarily mediated by receptor-interacting protein kinase 1 (RIPK1), RIPK3, and mixed lineage kinase domain-like protein (MLKL).\textsuperscript{16} Evidence is accumulating that necroptosis-enhanced cell death regulates pathogenesis in some neurodegenerative diseases, including amyotrophic lateral sclerosis, Alzheimer disease, and Parkinson disease.\textsuperscript{17–20} However, it remains unclear whether RIPK3-mediated necroptosis contributes to neural loss in diabetes.

5- Hydroxytryptamine (5-HT), commonly known as serotonin, is a crucial regulator of GI tract functions, including motility, secretion, vasodilation, and sensation. 5-HT generally acts as either a neurotransmitter or a paracrine signaling molecule in the gut through various serotonin receptor types, among which the 5-HT\textsubscript{4} receptor (5-HT\textsubscript{4}R) is prominent.\textsuperscript{21,22} In the colon epithelium, 5-HT\textsubscript{4}R receptors are linked to 5-HT release, chloride secretion, and goblet cell degeneration.\textsuperscript{23} Recent investigations have explored the protective role of 5-HT\textsubscript{4}Rs in counteracting colitis and diabetes-induced mucous barrier dysfunction.\textsuperscript{24,25} Furthermore, activation of 5-HT\textsubscript{4}Rs expressed on enteric nerve terminals induces presynaptic facilitation of neurotransmitter release,\textsuperscript{26,27} as well as promotion of enteric neuronal survival and neurogenesis.\textsuperscript{28,29} This study aims to investigate the potential protective effect of a 5-HT\textsubscript{4}R agonist on enteric neuropathy caused by diabetes, which can contribute to motility disorders. The underlying mechanism may involve the inhibition of RIPK3-mediated necroptosis by activating 5-HT\textsubscript{4}R.

### Materials and Methods

#### Animals

RIPK3\textsuperscript{−/−}\textsuperscript{c} c57BL/6 mice were constructed by Cyagen Bioscience (Suzhou, China; number KOCMS180123LJ1). Wild-type (WT) c57BL/6 mice were purchased from Slac Laboratory Animal Company (Shanghai, China). The RIPK3\textsuperscript{−/−}\textsuperscript{c} and WT C57BL/6 mice were bred and housed in specific pathogen-free conditions with ad libitum access to water and food. The success of RIPK3 deletion in mice is shown in Supplemental Figure S1. All experimental protocols were approved by the Institutional Animal Care and Use Committees of Shanghai Jiao Tong University School of Medicine.

#### Induction of Type 1 Diabetes and 5-HT\textsubscript{4}R Agonist Treatment

Adult (age, 10 to 12 weeks) male mice were given a single i.p. injection of 150 mg/kg streptozotocin (STZ; number S0130; Sigma-Aldrich, St. Louis, MO) to establish type 1 diabetes, whereas nondiabetic mice were injected with 0.01 mol/L cold citrate buffer (pH 4.0). Mice with sustained hyperglycemia (>20 mmol/L) were used in the subsequent experiments. As previously documented,\textsuperscript{25,30} a 5-HT\textsubscript{4}R agonist, RS67333, was administered (i.p. injection) once every other day for six times, starting 2 weeks after STZ injection, at a dosage of 1 mg/kg (number 168986-60-5; Tocris, Ellisville, MO). The following experiments were performed 24 hours after the last administration of RS67333.

#### Determination of Colonic Motility

Mice were anesthetized with isoflurane 12 hours after fasting. A 3-mm glass bead was inserted 2 cm proximally to the anal opening using a glass rod lubricated with paraffin wax. The time between bead placement and bead expulsion was used to calculate the distal colonic transit time.\textsuperscript{31} Immunofluorescence

Immunofluorescence (IF) staining was performed on colon sections (15 \textmu m thick), or whole mount of muscularis externa consisting of myenteric plexus and longitudinal muscle layer. Before staining, the tissues were blocked with 10% normal goat serum in 0.05 mol/L buffered saline phosphate. Primary antibody against protein gene product 9.5 (PGP9.5), at a concentration of 1:500 (number 27053; Abcam, Cambridge, MA), was used. The images were taken using either fluorescent microscope (Leica DM2500; Leica Microsystems Limited, Buffalo Grove, IL) or fluorescence confocal microscopy (Leica TCS SP8 STED 3 ×; Leica Microsystems Limited) to examine PGP9.5 IF staining.

**RNAscope in Situ Hybridization Followed with IF Staining**

RNAscope\textsuperscript{in situ} hybridization was performed following the instructions from Advanced Cell Diagnostics Inc. (Newark, CA). The submucosal plexus was obtained by separating the submucosa from mucosa, and the myenteric plexus was obtained by separating the longitudinal muscle with the attached myenteric plexus from circular muscle. The whole mounts were subjected to protease plus iii treatment for 20 minutes and hybridized with RNAsecpe 5-HT\textsubscript{4}R probe (number 408241; Advanced Cell Diagnostics Inc.) for 2 hours in the HybEZ oven at 40°C. Signals were amplified using AMP-1, AMP-2, and AMP-3 reagents. Finally, the whole mounts were incubated with primary PGP9.5 antibody (1:500; Abcam).

### Whole Tissue Clearing and Immunostaining

The distal colon was removed and opened longitudinally. The colon was fixed in 4% paraformaldehyde overnight and washed for 1 hour six times with phosphate-buffered saline. Partial colon was separated into mucosa and muscularis. Then, the tissues, including mucosa, muscularis, and intact colon, were treated with reagents known as clear, unobstructed brain imaging cocktail to clear the tissue for

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imaging. The tissues were then incubated with primary PGP9.5 (1:500; Abcam) or HuC/D (1:200; number A-21272; Invitrogen, Waltham, MA) antibody for 48 hours at 37°C. After the incubation, the tissues were viewed using a two-photon laser-scanning microscope (FVMPE-RS; Olympus, Tokyo, Japan). The images were further processed using Imaris 9.0 (Bitplane) to reconstruct three-dimensional images.

**Fluorescent Quantification**

Integrated intensity fluorescent signals were measured using ImageJ software version 1.53K Java 1.8.0 (NIH, Bethesda, MD; http://imagej.nih.gov/ij). The percentage change in IF signal was calculated with the nondiabetic group’s mean set at 100.

**Western Blot Analysis**

Mucosa, muscularis, and intact colon were homogenized with lysis buffer: 20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride. In addition, the buffer also contained protease inhibitor cocktail and phosphatase inhibitor from Sigma (St. Louis, MO). The blots were then incubated with primary antibodies, including PGP9.5 (1:1000; Abcam), caspase-9 (1:1000; number 185719; Abcam), caspase-3 (1:1000; number 184719; Abcam), RIPK3 (1:200; number 62344; Abcam), phosphorylated RIPK3 (pRIPK3; 1:1000; number 195117; Abcam), or MLKL (1:200; number 194699; Abcam). Finally, horseradish peroxidase–conjugated secondary antibody (1:3000; Bio-Rad Laboratories, Hercules, CA) was added in the experiment.

**Statistical Analysis**

The data were presented as means ± SD and then statistically analyzed using GraphPad Prism version 9.0 (GraphPad software, La Jolla, CA). One-way analysis of variance, followed by Tukey test and t-test, analyzed variables from different groups. \( P < 0.05 \) was considered statistically significant.

**Results**

The Development of Neural Loss in the Colon of Diabetic Mice

To explore the developmental characteristics of diabetic neuropathy in the colon, the neural changes were evaluated through PGP9.5 IF staining of colon sections from STZ-induced type 1 diabetic mice. In nondiabetic mice, the PGP9.5-immunoreactive (IR) nerve fibers were continuously and regularly situated around the crypts, resembling meshes in the mucosa. Two weeks after diabetes induction, the mesh-like nerve fibers were somewhat discontinuous around the crypts in the mucosa. By 4 weeks after induction, line-like or dot-like nerve fibers were commonly distributed in the mucosa. The distribution pattern of ganglia or nerve fibers in muscularis did not change with time following induction of diabetes. Statistically, there was a significant decrease in the density of PGP9.5-IR nerve fibers or ganglia in either mucosa or muscularis 4 weeks after diabetes induction (Figure 1).

5-HT₄R Agonist Attenuates Diabetes-Induced Dysmotility and Neural Loss in the Colon

Previous studies have discovered that 5-HT₄R is present in the enteric nervous system (ENS) of the intestine. To verify the localization of 5-HT₄Rs in colon ENS, RNase in situ hybridization for 5-HT₄R and subsequent IF staining for PGP9.5 were performed from whole mounts that contained submucosal and myenteric plexus, separately. 5-HT₄R mRNA was present on certain neuronal cells expressing PGP9.5, in both submucosal and myenteric plexuses of the colon (Figure 2, A and B).

To determine the impact of a 5-HT₄R agonist on diabetic neuropathy in the colon, RS67333 (1 mg/kg) was administered once every other day for six occasions, beginning 2 weeks after STZ injection. The colon motility was assessed by measuring bead excretion time 24 hours after the last injection and subsequently examined neural changes by testing IF staining of PGP9.5 in the colon. Notably, administration of 5-HT₄R agonist unaltered diabetes-evoked reduction in body weight and increase in blood glucose levels (Figure 2, C and D).

As expected, diabetic mice exhibited a significantly longer time for bead excretion compared with nondiabetic mice. However, diabetic mice treated with RS67333 showed a much shorter time for bead excretion in comparison to diabetic-control mice (Figure 2E). More important, the use of a 5-HT₄R agonist alleviated diabetes-induced neural loss, as evidenced by the reduction of PGP9.5-IR nerve fibers in the mucosa, PGP9.5-IR ganglia in muscularis from colon sections, or myenteric plexus from whole mounts (Figure 2, F and G).

To depict the overall perspective of the protection induced by the 5-HT₄R agonist against colon neural disruption caused by diabetes, entire tissues, including mucosa, muscularis, and intact colon, were cleared and subjected to IF staining for detecting PGP9.5 or HuC/D. From the side views of intact colon walls, the distribution of PGP9.5-IR nerve fibers or ganglia was observed. Comparing diabetic and nondiabetic mice, the innervation in the colon appeared relatively sparse in the former, but the innervation appeared more pyknotic in diabetic mice subjected to the 5-HT₄R agonist treatment (Figure 3A). In nondiabetic mice, the mucosa had dense, mesh-like PGP9.5-IR nerve fibers. These mesh-like nerve fibers were not concentrated in diabetic mice. However, the 5-HT₄R agonist treatment restored the dense, mesh-like nerve fibers in...
diabetic mice (Figure 3C). Similarly, the densities of PGP9.5-IR ganglia and HuC/D-IR neurons in the myenteric plexus were lower in diabetic mice than in nondiabetic mice. Nevertheless, the densities of PGP9.5-IR ganglia and HuC/D-IR neurons were reestablished in diabetic mice after 5-HT₄R agonist treatment (Figure 3E). By clear tissue and IF staining analysis, the 5-HT₄R agonist did abate diabetes-induced neural loss in the colon (Figure 3, B, D, and F). Quantitatively, Western blot analysis confirmed that 5-HT₄R agonist treatment attenuated diabetes-induced increase in protein levels of PGP9.5 (Figure 3, G and H).

5-HT₄R Agonist Alleviates Diabetic Neuropathy of the Colon through Inhibition of Necroptosis-Related Signal Pathways

Myenteric neuronal apoptosis has been shown to contribute to diabetes-induced loss of enteric neurons. However, little is known about whether neuronal necroptosis occurs in diabetes. To investigate whether necroptosis is involved in colon neural loss in diabetes and to determine if 5-HT₄R agonist is capable of inhibiting key molecular signals that regulate necroptosis, Western blot analysis was used to detect expression of RIPK3, MLKL, and their phosphorylation in the colon of diabetic mice while administering RS67333. At the same time, the effects of RS67333 on the expression of caspase-9 and caspase-3, essential apoptotic regulators, were examined in the colon of diabetic mice. After inducing diabetes, an increase in the expression of these proteins was observed. However, RS67333 did not alter the elevation in their expression (Figure 4, A–C). Likewise, diabetes led to a significant increase in expression of RIPK3, pRIPK3, and MLKL in the colon. Interestingly, once diabetic mice were treated with RS67333, RIPK3, pRIPK3, or MLKL expression was reduced in the colon (Figure 4, D–H). Unfortunately, detection of pMLKL was unsuccessful, potentially because of the limitation of antibody (data not shown).

Figure 1  Changes in colon innervation during development of diabetes. A and C: Representative photomicrographs of immunofluorescence staining with protein gene product 9.5 (PGP9.5) antibody (green) and DAPI counterstain (blue) in mucosa (A) and muscularis (C) of the colon sections. The areas with white dotted lines represent muscularis. B and D: Quantitative analysis of PGP9.5 expression in mucosa and muscularis of the colon. Data are given as means ± SD (B and D). n = 6 per group (B and D). *P < 0.05, **P < 0.001, one-way analysis of variance, followed by Tukey test. Scale bar = 100 μm (A and C). D-2W, 2 weeks after diabetes induction; D-4W, 4 weeks after diabetes induction; ND, nondiabetic.
Figure 2  Effects of 5-hydroxytryptamine 4 receptor (5-HT₄R) agonist on diabetes-induced hypomotility and neural loss in the colon of mice. A and B: Representative confocal photomicrographs of RNAscope with 5-HT₄R probe visible with green dots, immunofluorescence (IF) staining with protein gene product 9.5 (PGP9.5) antibody (red), and DAPI counterstain (blue) in submucosal and myenteric plexuses of colon from nondiabetic mice. The white boxed areas show the magnified areas indicated by the boxed areas to the right. C and D: Effects of 5-HT₄R agonist on diabetes-induced changes in body weight and blood glucose in mice. E: Effects of 5-HT₄R agonist on diabetes-induced colonic hypomotility in mice. F: Representative confocal microscopy images of IF staining with PGP9.5 antibody (green) and DAPI counterstain (blue) in mucosa, muscularis, and myenteric plexus of the colon. The areas with white dotted lines represent muscularis. G: Effects of 5-HT₄R agonist on diabetes-induced changes in PGP9.5 expression in mucosa, muscularis, and myenteric plexus (MP) of the colon in mice. Data are given as means ± SD (C–E and G), n = 6 per group (E); n = 3 to 6 per group (G). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001, one-way analysis of variance, followed by Tukey test. Scale bar = 25 μm (A, B, and F). D, diabetic.
To validate that the mediation process takes place through RIPK3, changes in MLKL and PGP9.5 expression, as well as colon motility, were examined in diabetic mice with RIPK3 knockout. Initially, Western blot analysis showed minimal RIPK3 expression in both the mucosa and muscularis of nondiabetic and diabetic RIPK3−/− mice, indicating successful deletion of RIPK3. Furthermore, the effect of RIPK3 deletion on MLKL expression was assessed. As shown in Figure 5, A through F, the density of MLKL immunoblotting bands in the mucosa and muscularis of diabetic WT mice was significantly greater than that in nondiabetic WT mice. However, there was no significant
difference in MLKL density between nondiabetic and diabetic RIPK3\(^{-/-}\) mice. Remarkably, diabetes-induced elevation in MLKL expression was notably reduced by RIPK3 deletion in the mucosa and muscularis. Furthermore, the functional impacts of deleting RIPK3 on diabetes-induced neuropathy and dysmotility in the colon were determined. To achieve this, the current study employed IF staining to examine PGP9.5 expression and conducted a bead excretion test to evaluate colonic transit. In comparison to nondiabetic WT mice, those mice with diabetes showed substantial declines in PGP9.5-IR nerve fibers and ganglia in the mucosa and myenteric plexus 4 weeks after diabetes induction. However, diabetes-induced neural loss in mucosa and myenteric plexus was considerably attenuated when RIPK3 was deleted (Figure 5, G–J).

Similarly, STZ-treated WT mice showed significant delay in bead excretion after 4 weeks, whereas the delay in bead excretion induced by STZ was significantly reduced in RIPK3-deficient mice (Figure 5K).

Discussion

The current study revealed progressive neural loss in the colon of diabetic mice. Intriguingly, administration of a 5-HT\(_4\)R agonist led to a reduction in colonic hypomotility and nerve fibers and ganglia loss in the colon of diabetic mice. Notably, the 5-HT\(_4\)R agonist inhibited the up-regulation of RIPK3 and MLKL, the necroptotic machinery, and even phosphorylation of RIPK3 in the colon of diabetic mice.
Figure 5  Role of receptor-interacting protein kinase 3 (RIPK3) in diabetes-induced neural loss and hypomotility in the colon of mice. A and B: Representative immunoblotting bands of RIPK3 and mixed lineage kinase domain-like protein (MLKL) in mucosa and muscularis of the colon from wild-type (WT) and RIPK3−/− mice with or without diabetes. C–F: Quantitative analysis of RIPK3 and MLKL expression in mucosa and muscularis of the colon. RIPK3 deletion significantly blocked diabetes-induced MLKL up-regulation. G and H: Representative confocal photomicrographs of immunofluorescence staining with protein gene product 9.5 (PGP9.5) antibody (green) and DAPI counterstain (blue) in colon mucosa and myenteric plexus from WT and RIPK3−/− mice with or without diabetes. I and J: Quantitative analysis of PGP9.5 expression in mucosa and myenteric plexus (MP) of the colon. RIPK3 deficiency alleviated diabetes-induced neural loss in both the mucosa and myenteric plexus of the colon. K: Lack of RIPK3 attenuated diabetes-induced colonic hypomotility. Data are given as means ± SD (C–F and I–K). n = 3 per group (C–F); n = 3 to 6 per group (I and J); n = 6 per group (K). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001, one-way analysis of variance, followed by Tukey test. Scale bar = 50 μm (G and H). ND, nondiabetic.
Moreover, diabetic mice lacking RIPK3 displayed recovery from neural loss, colonic hypomotility, and MLKL up-regulation in the colon.

GI tract dysfunction related to diabetes, such as gastroparesis and constipation, has been extensively studied but requires further investigation as effective clinical treatments are lacking. An earlier report demonstrated that 5-HT₄R agonist tegaserod improved gastric emptying rate in db/db mice. Unfortunately, this drug has been withdrawn from most markets because of undesirable adverse effects. Recently, a novel selective partial agonist for 5-HT₄R, minapride, has emerged as a potential treatment for constipation. Another 5-HT₄R agonist, prucalopride, has been shown to accelerate gastric emptying in healthy individuals but is not effective in patients with diabetic gastroparesis. Hence, more effective prokinetic agents are required to treat GI tract dysfunctions in diabetic patients. This study evaluated the use of RS67333, a potent and highly selective 5-HT₄R agonist, to treat diabetic mice. Results showed that chronic RS67333 treatment apparently reduced diabetes-induced colonic hypomotility, possibly because of the activation of 5-HT₄Rs in the ENS. RNA analysis via RNAseq detected 5-HT₄R mRNA in the submucosal and myenteric plexuses of the colon, supporting the finding in the sentence above. Given the observed decline of endogenous 5-HT in diabetes in our preliminary experiments (data not presented), the use of exogenous 5-HT₄R agonist may serve as a compensatory effect to improve GI tract function in diabetes.

A report shows that in guinea pigs, 5-HT₄Rs are highly concentrated in the myenteric plexus of the gastric antrum and corpus. When these receptors are stimulated, acetylcholine will be released, producing prokinetic effects. This study aimed to determine if activating 5-HT₄R could protect the ENS against diabetes and improve GI tract motility function. Previous studies have suggested that GI tract dysmotility is linked to autonomic neuropathy, with evidence of damage to sympathetic nerve or altered function, and vagal nerve dysfunction or cholinergic denervation of the GI tract. More recent studies have uncovered a connection between GI tract motility dysfunction and enteric neuropathy. This study showed that in diabetic mice induced by STZ, there was gradual neural damage in both the mucosa and muscularis of the colon, with significant changes by 4 weeks after injection. Treatment with a 5-HT₄R agonist starting 2 weeks after the onset of diabetes significantly ameliorated neural loss, as observed by a decrease in PGP9.5-IR nerve fibers and/or ganglia in the mucosa and muscularis of colon sections or whole mounts of myenteric plexus. Whole tissue clearing and immunostaining for PGP9.5 or HuC/D to observe the overall changes in colon innervation were performed. Three-dimensional imaging proved more vividly that diabetes disrupted fine and tight mesh-like nerve fibers in the mucosa and neural network in the muscularis. However, treating diabetic mice with a 5-HT₄R agonist rescued the pattern of spatial distribution of nerve fibers and ganglia in the colon. Furthermore, quantitative assay by Western blot analysis confirmed the above effects induced by 5-HT₄R agonist. These findings suggest that an agonist of 5-HT₄R may play a protective role in diabetic neuropathy, particularly in the ENS.

Next, this study investigated how 5-HT₄R agonist alleviated diabetic neuropathy in the colon. Diabetic neuropathy is a complicated condition with multifactorial mechanisms. Several studies have indicated a connection between apoptosis and enteric neuron decline in diabetes. The increased protein levels of caspase-9 and caspase-3 in the colon 4 weeks after STZ injection were shown in the current study, aligning well with prior studies. However, these levels remained unchanged when RS67333 was provided to diabetic mice. Unfortunately, the antibody used here recognized full-length, uncleaved caspase-3, likely because of its limitations. Nevertheless, 5-HT₄R agonist exhibited no influence on diabetes-induced up-regulation of caspase-9, the apoptosis initiator, indicating absence of anti-apoptotic action from 5-HT₄R agonist.

On the other hand, there is limited understanding of the function of necroptosis in diabetic neuropathy. Signaling through tumor necrosis factor receptor 1, toll-like receptors, and certain other receptors can trigger necroptosis, a form of regulated necrosis in the absence of caspase-8 activity. This process is initiated by RIPK1, RIPK3, and MLKL, which together form necrosomes. Following phosphorylation, MLKL binds to the cell membrane and causes rupture, driving inflammatory responses. To date, growing evidence indicates that neuronal necroptosis contributes to the pathogenesis of neurodegenerative disorders. A report showed that inhibiting RIPK1 pharmacologically delays axonal degeneration of cultured dopaminergic and cortical neurons. RIPK3-/- and MLKL-/- mice exhibit decreased degeneration of dopaminergic neurons, improving motor function in preclinical Parkinson disease models. Necroptosis has also been found in the brains of patients with Alzheimer disease and may be connected to the pathologic features of the disease, such as neuronal death and neuroinflammation. Active MLKL triggers more extensive neuronal death and worsens cognitive deficits in mice with Alzheimer disease. In mouse models of Alzheimer disease, blockade of necroptosis inhibits Aβ accumulation and improves cognitive function. The current study proved that diabetes led to increased expression of RIPK3, pRIPK3, and MLKL, pivotal proteins that regulate necroptosis, in the colon. Notably, prolonged administration of the 5-HT₄R agonist RS67333 demonstrably hindered the elevation of RIPK3, pRIPK3, and MLKL induced by diabetes in the colon. Furthermore, eliminating RIPK3 gene inhibited the up-regulation of MLKL substrate in both mucosa and muscularis of the colon in diabetes. Crucially, the absence of RIPK3 gene prevented neural loss, comprising nerve fibers in the mucosa and nerve cells in the myenteric plexus, as well as colonic hypomotility. These findings suggest a
strong correlation between enteric neuronal necroptosis and dysmotility and neural loss in the colon in diabetes. Activation of 5-HT₄R by RS67333 may relieve colonic motility dysfunction and enteric neuropathy by inhibiting enteric neuronal necroptosis mediated by RIPK3.

Recognized as a G-protein–coupled receptor, 5-HT₄R engages the cellular cAMP/protein kinase A pathway on activation. This triggers an enzyme called protein kinase A to phosphorylate cAMP response element binding protein. 13 This study proposes that 5-HT₄R agonists might inhibit neuro-inflammation by stimulating this cAMP/protein kinase A–cAMP response element binding protein signaling pathway, thereby lowering diabetes-linked increases in RIPK3, pRIPK3, and MLKL. Moreover, it is conceivable that 5-HT₄R agonist could protect against diabetic enteric neuron loss by improving mucosa barrier function. 14 Thus, further research is needed on understanding the specific mechanism through which 5-HT₄R agonists impact RIPK3.

In summary, the current study identifies 5-HT₄R as a regulator of necroptosis signaling pathways in diabetic neuropathy. Targeting 5-HT₄R may be a therapeutic strategy for treating diabetic neuropathy in the GI tract.

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Author Contributions

Y.C., Y.K., and G.Z. performed the experiments and analyzed the data; J.W. and Y.W. performed some experiments; W.R. contributed to the discussion and revised the manuscript; and G.Z. and H.H. were responsible for study design, supervision, and wrote/revised the manuscript. All authors approved the final manuscript. G.Z. is the guarantor of this study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Disclosure Statement

None declared.

Supplemental Data

Supplemental material for this article can be found at http://doi.org/10.1016/j.ajpath.2024.01.006.

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