Butyrate alleviates cognitive impairment by improving gut mucosal barrier function and blocking neuroinflammatory signaling in LDLR−/− mice

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Research Article

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Abstract

Background

Short-chain fatty acids (SCFAs), the main metabolites produced by bacterial fermentation of dietary fiber, possess neuroactive properties. The present study aimed to explore whether SCFAs-butyrate can repress neuroinflammation and alleviate cognitive impairment via improving gut mucosal barrier function and mediating tryptophan metabolism in LDLR\(^{−/−}\) mice.

Methods

LDLR\(^{−/−}\) mice were fed an atherosclerotic diet with 1.25% cholesterol and 40% calories from fat and concomitantly treated by intragastric administration with either normal saline as model group or 400mg/kg/day sodium butyrate for 8 weeks.

Results

For gut barrier function, butyrate increased expression of tight junction protein, and inhibited intestinal inflammatory pathways by targeting the toll-like receptor 4 signaling and the nod-like receptors domain containing 3 (NLRP3) inflammasome. Subsequently, butyrate reduced not only the serum lipopolysaccharide level but also its capability to cross the blood-brain barrier, leading to the suppression of tau phosphorylation and the improvement of neuroinflammation by inhibiting the increase in reactive microglia and astrocytes, and blocking NLRP3 inflammasome activation in the cortex and hippocampus. Final behavioral tests indicated that butyrate delayed cognitive impairment via decreasing latency to the platform and lengthening swimming distance in the target quadrant. Nevertheless, butyrate showed no effect on serotonin, indoleacetic acid, L-kynurenine, niacinamide, and their metabolites, indicating that the observed effects on cognitive function are unrelated to the tryptophan metabolism.

Conclusion

In summary, the present study provided the novel experimental evidence that butyrate played a neuroprotective role by targeting the NLRP3 inflammasome. The reduction of neuroinflammation may be related to the improvement of gut mucosal barrier function and the decrease of the penetration of intestinal endotoxin from the gut to the central nervous system.

Background

The bidirectional communications between the brain and the gut, called the microbiota-gut-brain axis, have opened novel avenues for the prevention and treatment of neurodegenerative disease. During gut
dysbiosis caused by a poor diet, dysfunctional gut-brain axis signaling leads to increased neuroinflammation, as well as imbalanced metabolism and cognitive impairment [1]. Previous animal study from our laboratory has demonstrated that oat fiber can modulate the microbiota-gut-brain axis via producing more metabolites short-chain fatty acids (SCFAs), and ameliorate cognitive impairment caused by a high fat/cholesterol diet [2]. SCFAs, as the most important and pleiotropic functional components of microbe-to-host signaling, are the mainly end-products during diet digestion and fermentation of partially and non-digestible polysaccharides by the gut microbiota [3, 4]. A growing body of studies reveals that SCFAs exhibit critical roles in the maintenance of health, which not only participate in the pathophysiology of cardiovascular disease via altering gut microbiota but also regulate cardiovascular disease-associated syndromes [5]. A recent study demonstrated that SCFAs, especially butyrate, can regulate central nervous system processes through direct and indirect means and ultimately shape behavior and cognitive function [6, 7]. Considering aging with cognitive decline as a most important risk factor affecting cardiovascular disease [8], the effect of dietary fiber and it metabolites SCFAs on cognitive impairment associated with cardiovascular disease has attracted more and more concern.

The molecular mechanisms of SCFAs on mediating cognitive behavior and brain function involve many aspects, ranging from metabolic effects to receptor signaling and enzymatic inhibition [6, 7, 9]. Most importantly, the related signaling mechanism may be the activation of G-protein-coupled receptors (GPRs) particularly GPR41, GPR43, and GPR109A [10], which play a crucial role by improving gut integrity and decreasing the translocation of bacterial lipopolysaccharide (LPS) into blood-brain barrier (BBB) [11]. Recently, the nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, which is highly located in microglia and involved in the process of neuroinflammation, is expected to be a potential target in neurodegenerative diseases [12, 13]. However, the possible neuroprotective mechanism by which SCFAs mitigates cognitive impairment by targeting NLRP3 inflammasome has not been illustrated by now. In addition, SCFAs receptors, GPR109A is identified as a niacin receptor; and tryptophan which is an essential amino acid and a precursor for niacin synthesis in the host liver is involved in neurol and psychological activities, and tryptophan metabolism as a therapeutic target has been implicated in a variety of neurodegenerative diseases [14]. Whether SCFAs can indirectly act on tryptophan metabolism through activating GPR109 is worth exploring.

There has been some evidence that cognitive impairment may be associated with modifiable cardiovascular risk factors and atherosclerosis in the general population [15, 16]. Cognitive impairment is considered as a consequence of atherosclerosis of extracranial or intracranial vessels, or as the independent but convergent disease processes with atherosclerosis sharing common pathophysiological pathways [17]. The LDL receptor knock-out (LDLR−/−) mice are an established atherosclerotic model with cognitive dysfunction, which are characterized by a reduction of spatial learning and memory ability [18]. Studies has shown that LDLR−/− mice were more prone to cognitive impairment when feeding a high-fat and high-cholesterol diet [18–20]. Therefore, the present study employed an established animal model of atherosclerosis with cognitive impairment and explored the following items: (1) whether SCFAs-butyrate as a selective cognitive enhancer ameliorated atherosclerosis-related cognitive impairment by enhancing
gut integrity and decreasing circulating LPS from bloodstream to the neural tissue? (2) whether butyrate protected from neuroinflammation by inhibiting NLRP3 inflammasome signaling? (3) Could butyrate affect tryptophan metabolism via activating SCFAs receptors-GPR109A in LDLR−/− mice fed a high-fat/cholesterol diet?

Materials And Methods

Ethics statement

The animal protocol for the present study was approved by the local Institutional Animal Welfare Committee (Permission No. SUDA20211206A03). All experimental schemes were conducted in accordance with the Institutional Guidelines in the Care and Use of Experimental Animals of the Ministry of Science and Technology (Beijing, China). All experimental protocols were made to minimize the sample size and to relieve the suffering of mice.

Animals Processing

Male LDLR−/− mice and C57BL/6 wild-type mice with the same genetic background, seven-week-old and weighting 20 ± 2 g, were purchased from GemPharmatech Co., Ltd. (Nanjing, China) and raised in a barrier facility with a 12/12 h light/dark period at room temperature (22 ± 2°C). After arriving at Laboratory Animal Research Centre, the animals were acclimatized for one week with free access to sterile water and a standard laboratory diet with 10% calories from fat. Then, sixteen LDLR−/− mice were given an atherosclerotic diet with 1.25% cholesterol and 40% calories from fat (XT108C, Jiangsu Xietong Pharmaceutical Bio-engineering Co., Ltd., Nanjing, China) for an 8-week-long feeding period, and were randomly divided into two groups: a model group receiving saline solution (Mod, n = 8) and a treatment group receiving sodium butyrate (SB, n = 8). The animal model of atherosclerosis with cognitive impairment has been successfully established in our laboratory [2]. C57BL/6 mice continued to be fed with a standard laboratory diet, which served as a normal control in behavioral analysis and other examinations (NC, n = 8). Animals from the SB group were treated by gavage with 400mg/kg/d sodium butyrate (Sigma-Aldrich, St. Louis, MO, USA) five days a week according to Du’s report and our pre-experiment [21], and mice from the Mod group and NC group were treated by gavage with the same concentration of normal saline. During the eight-week feeding, all mice had free access to food and sterile water. The formulation and composition of diets were summarized in Supplementary Table S1. The time schedule for the animal experimental study was shown in Fig. 1.

Behavioral Test

After feeding for 7 weeks, a MWM test was performed for examining butyrate on cognitive behavior. During the entire experimental test, the lights were turned off to minimize the light stimuli on the activity performance of mice. Briefly, in a continuous 5-day oriented navigation test, mice were put into a circular
black pool to find a hidden platform (1.0 cm below the water surface), which was fixed in one of the four quadrants during the training. The trial was terminated once the animals arrived at the hidden platform within 60 s; otherwise the mice would be artificially taken to the platform to stay for 20 seconds and then removed from the pool. The time that a mouse reached the submerged platform was recorded through a video camera to evaluate spatial learning ability. On the sixth day, the platform was taken away for the spatial probe test. The number of mice crossing the platform, the swimming time spent in the target quadrant and the swimming distance were recorded and analyzed to evaluate the spatial memory ability. Throughout the experiment, Supermaze tracking software (Shanghai Xinsoft Information Technology Co., Ltd., Shanghai, China) was performed for data collection and analysis.

**Samples Collection**

At the end of the experiments, blood samples from fasting mice were collected; serum was separated for the measurement of circulating LPS level. The length of colon tissue was measured using the same straightedge after dissecting the mice. Subsequently, purpose samples from sacrificed mice were immediately harvested for subsequent tests. Briefly, after washing with ice phosphate buffer saline, the parietal-temporal cortex and hippocampus from the fresh hemi-brain, and colon tissues were dissected and stored in an ultralow temperature refrigerator. The other hemispheres and a part of distal colon tissue fixed in 4% paraformaldehyde were used for immunofluorescence staining or hematoxylin and eosin (H&E) staining. Cecum content was taken in a sterile environment for further tryptophan metabolism via untargeted metabolomics analysis.

**Colonic Histopathological Analyses**

The distal colon tissues fixed in 4% paraformaldehyde, were dehydrated and embedded in paraffin. Then a series of slices were stained by the H&E staining method and examined under light microscopy (Olympus BX-50, Olympus Optical, Tokyo, Japan). Five discontinuous sections were randomly selected from colon tissue (n = 5 in each group) for histopathological analysis and scoring.

**Serum LPS Analyses**

The concentration of circulating LPS was measured by an Elisa method following the manufacturer’s guidelines (Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, China). The coefficient of variation (CV) for intra-assay precision was less than 9%.

**Measurement Of Colonic Tight Junction Proteins And Inflammatory Cytokines**
Concentrations of tight junction proteins including zonula occludens-1 (ZO-1), claudin-3 and occludin, and inflammatory cytokines including tumor necrosis factor-alpha (TNF-), interleukin (IL)-1β and IL-10 from colon tissues were determined by commercial Elisa kits (Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, China; Elabscience Biotechnology Co., Ltd., Wuhan, China). In brief, fresh colon samples were homogenized and the supernatants were separated via centrifugation at 10,000g for 15min. The protein concentration of the supernatant was quantified through the BCA protein assay (Beyotime Institute of Biotechnology, Nantong, China). An equal concentration of the supernatant was detected through the Elisa method. The coefficient of variation (CV) for intra-assay precision was less than 9%-11%.

**Western Blot Analyses**

First, colon tissues, isolated parietal cortex and hippocampus were cleaved in tissue lysate. Protein concentration from tissue supernatant was quantified through the BCA protein assay (Beyotime Institute of Biotechnology, Nantong, China). Second, equal protein (30 µg) was separated via sodium dodecyl sulfate-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane by electrophoretic transfer method. Third, the membrane was blocked with 5% skim milk at room temperature and then incubated overnight at 4 °C with tight junction proteins including ZO-1, claudin-3 and occludin, toll-like receptor 4 (TLR4) signaling pathway including TLR4, myeloid differentiation primary response 8 (MyD88), TNF receptor-associated factor 6 (TRAF6), TIR-domain-containing adapter-inducing interferon-β (TRIF), receptor-interacting protein (RIP) and IκB kinase β (IKKβ), NLRP3 inflammasome signal factors including nuclear factor-kappa B (NF-κB) p65, NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), cysteinyl aspartate specific proteinase-1 (Caspase-1), IL-1β and IL-18, SCFA receptors including GPR41, GRP43 and GPR109A, Aβ1-42 and p-Tau ser 396/ser 404, and neuroinflammation-related markers including glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule 1 (IB 1). The primary antibodies mentioned above were purchased from Abcam (Cambridge, MA, USA), ImmunoWay Biotechnology Company (Plano, TX, USA), or Cell Signaling Technology (Danvers, MA, USA). Final, membranes were incubated with secondary antibodies for 1 h at room temperature. And the protein bands were detected using an enhanced chemiluminescence ECL Detection Systems (EMD Millipore). β-actin or tubulin were used as the internal control.

**Immunofluorescence Double Staining And Analyses**

The paraffin-embedded brain tissues were sliced and stained via immunofluorescence double labeling of GFAP and IB 1 according to previous description by the authors. Briefly, after blocking in 3% BSA solution for 30 min, the embedded sections were incubated with a primary antibody mixture including mouse anti-GFAP (1:500, Servicebio) and rabbit anti-IB 1 (1:500, Wako) overnight at 4 °C. After removing excess primary antibodies with PBS solution, sections were incubated with the respective secondary
antibodies (for GFAP, Streptavidin Alexa Fluor 555 conjugate, 1:1000, Invitrogen; for IBA1, Alexa Fluor 488 goat anti-rabbit, 1:500, Invitrogen) for 1 h at room temperature. The sections were counterstained with DAPI (Invitrogen) and images were obtained with Pannoramic Midi (3D Histech Ltd, Budapest, Hungary). The positive area (µm²) and average fluorescence intensity of the target area of each section were quantified with the Area Quantification FL V2.1.2 module in Halo V3.0.311.314 (Indica Labs, USA) software.

Tryptophan Metabolism Via Untargeted Metabolomics Analyses

Approximately 48 metabolites from mice cecum content were detected and quantified via ultra-performance liquid chromatography (Agilent 1290 II, Agilent Technologies, Germany) coupled to Quadrupole-TOF mass spectrometry (5600 Triple TOF Plus, AB SCIEX, Singapore) (UPLC-MS) according to the reported reference [22]. Briefly, samples mixed with ice-cold 80% methanol, were ground using an abrader at 5000 rpm and centrifuged for 10 min at 12000 rpm. The supernatants were dried using SpeedVac (Genevac miVac, Tegent Scientific Ltd.) and the dried extracts were redissolved with 1% acetonitrile for UPLC-MS analysis. Information-dependent acquisition mode was used for MS/MS analyses of the metabolites, and data acquisition and processing were performed using Analyst® TF 1.7.1 Software (AB Sciex, Concord, ON, Canada). All detected ions were collected using MarkerView 1.3 (AB Sciex, Concord, ON, Canada) into Excel database in the format of two-dimensional matrix. This determination was performed by LipidALL Technologies Co., Ltd. (Beijing, China), and data of the secondary mass spectrometry were extracted via PeakView 2.2 software (AB Sciex, Concord, ON, Canada) and compared with HMDB, Metabolites database, METLIN and standard substance. The identified metabolomics data were analyzed using R language method.

Statistical Analyses

All data were presented as the mean ± standard error of the mean (SEM). The significance of variables among different groups was carried out with one-way ANOVA followed by a Tukey’s post hoc test. Statistical analysis was performed using SPSS 22.0 statistical software (IBM, New York, USA) and GraphPad Prism (version 6.03, GraphPad Software, San Diego, CA). A p value < 0.05 was considered at statistical significance.

Results

Butyrate on gut mucosal barrier and inflammatory factors

Compared to the C57BL/6 mice of NC group, the length of the LDLR−/− mice's colon tissue was shortened in the Mod group (Fig. 2A, p < 0.05). Butyrate slightly increased the length of colon tissue, however there was no statistical difference between SB group and Mod group. For colonic histopathological analysis, no pathological change was observed in the NC group (Fig. 2B). Conversely, connective tissue hyperplasia,
local mucosal damage and a small amount of inflammatory cell infiltration were exhibited in the Mod group. Although no significant differences in histopathological score were identified in both SB and Mod groups, butyrate properly ameliorated the histopathological damage of colon tissues.

The results from inflammatory cytokines showed the intestinal IL-1β, IL-10 and TNF-, and serum LPS concentration significantly increased in the Mod group compared to the NC group (Fig. 2C, 2D, p < 0.05). Interestingly, butyrate increased the levels of tight junction proteins including ZO-1 and claudin-3 from colon homogenate (Fig. 2E, p < 0.05), accompanied by an improvement of colonic inflammatory response via decreasing TNF- level (Fig. 2C, p < 0.05), and a reduced circulating LPS level (Fig. 2D, p < 0.05). Butyrate had no effect on colonic inflammatory factors IL-1β and IL-10. Consistent with the colonic homogenate results, western blot analysis showed butyrate raised the protein expressions of tight junction proteins including ZO-1, claudin-3 and occludin (Fig. 2F, p < 0.05).

**Butyrate on colonic inflammatory pathways and SCFAs receptors**

To explore the specific mechanisms by which butyrate acts on intestinal inflammation, we examined the relative expression of inflammatory factors involved in TLR4 signaling pathway (Fig. 3A) and NLRP3 inflammasome pathway (Fig. 3B) from the colon tissue. Compared to the Mod group, butyrate decreased the relative levels of TLR4, MyD88, TRAF6, TRIF, RIP and IKKβ, and NF-κB p65, NLRP3, ASC, Caspase-1 and IL-1β (p < 0.05). In addition, the expression of metabolite-sensing receptors GPR41, GPR43, GPR109A, was significantly activated in the SB group, compared to those in the Mod group (Fig. 3C, p < 0.05).

**Butyrate on cognitive behavior**

MWM test was used for evaluating the potential of butyrate on the spatial learning and memory ability of experimental animals. For LDLR−/− mice from Mod group, animals presented an elevated escape latency to the platform and a reduced swimming time and distance to find the hidden platform, compared to C57BL/6 mice from the NC group. After treatment, butyrate effectively reversed the increment in escape latency to the platform during navigation trials (Fig. 4A). For spatial probe trials, butyrate effectively improved long-time spatial learning and memory ability via elevating the number of platform crossing (Fig. 4B), as well as increasing swimming time spent in the target zone (Fig. 4C) and swimming distance (Fig. 4D), compared to the Mod group (p < 0.05). The representative characteristic pattern of the swimming paths demonstrated that LDLR−/− mice from the SB group remained for a longer period in the quadrant of the platform (Fig. 4E).

**Butyrate on Aβ and tau phosphorylation from cortex and hippocampus**

Aβ plaque deposition and abnormal phosphorylation of Tau protein are two major risk factors for the development of cognitive impairment. As shown in Fig. 5, butyrate decreased the protein expression of p-Tau ser396 in the cortex (p < 0.05), and also reduced the relative level of p-Tau ser396 & ser404 in the hippocampus (p < 0.05), compared to the Mod model group, while butyrate didn't change the protein level of Aβ1–42 in either cortex or hippocampus.
Butyrate on integrity of blood-brain barrier and SCFA receptors from cerebral cortex

In order to explore the effect of butyrate on the integrity of BBB, tight junction proteins including ZO-1 and occludin from the cerebral cortex were measured by the western blot method. Compared to the Mod group, butyrate reversed the destroyed BBB by elevating the levels of tight junction proteins including ZO-1 and occludin (Fig. 6A, p < 0.05), which was caused and aggravated by an atherosclerotic diet with 1.25% cholesterol and 40% calories from fat in LDR−/− mice. In addition, GPRs as SCFA receptors can act on the central nervous system by regulating neuroinflammation reaction. SCFA receptors including GPR41 and GRP43 from the cerebral cortex were increased after butyrate treatment for eight weeks, compared to the Mod group (Fig. 6B, p < 0.05), while butyrate had no effect on SCFA receptors GPR109A from cerebral cortex tissue.

Butyrate on neuroinflammation-related markers

In the pathological process of neurodegenerative diseases, microglia and astrocytes are the primary effectors of neuroinflammation, with GFAP and IB 1 as biomarkers, respectively. For immunofluorescence double staining, the increment in reactive microglia and astrocytes caused by a high fat/cholesterol diet in both cortex and hippocampus of LDLR−/− mice, was inhibited by butyrate according to quantitative analysis based on the positive area and the average intensity (Fig. 7A-D). Furthermore, in the cortex (Fig. 7E), butyrate reduced the relative expression of GFAP (p < 0.05) but did not affect IB 1; in the hippocampus (Fig. 7F), GFAP and IB 1 were significantly decreased in the SB group, compared to the Mod group (p < 0.05). The result was in line with immunofluorescence double labeling.

Butyrate on NLRP3 inflammasome pathway in both cortex and hippocampus

Aberrant activation of NLRP3 inflammasome signal has been implicated in contributing to the pathology of neurodegenerative diseases. Compared to the Mod group, in the cortex, butyrate decreased the relative expression of NF-κB p65, NLRP3, ASC, Caspase-1, IL-1β and IL-18 (Fig. 8A, p < 0.05); in the hippocampus, butyrate reduced the levels of NF-κB p65, NLRP3, ASC, IL-1β and IL-18 (Fig. 8B, p < 0.05).

Butyrate on tryptophan metabolism

Tryptophan metabolism has been implicated in a variety of neurodegenerative diseases by regulating neuronal function and intestinal homeostasis. The results from untargeted metabolomics analysis indicated that butyrate treatment did not change the peak areas of intestinal metabolites from LDLR−/− mice, including L-Tryptophan, indoleacetic acid, serotonin, 5-Hydroxyindoleacetic acid, 3-Indolepropionic acid, 1H-Indole-3-carboxaldehyde, indole-3-methyl acetate, indoleacetic acid, 3-Indolehydracrylic acid, L-Kynurenine and niacinamide, compared to the Mod group (Fig. 9).

Discussion
The aging population in the world is rapidly expanding, and so much is the age-related cognitive decline that has become a major public health concern [23]. Cognitive impairment associated with cardiovascular disease and atherosclerosis may be prevented and its progression may be retarded because cardiovascular disease and atherosclerosis are mostly modifiable [24, 25]. Thus, exploring novel preventive approaches for cognitive impairment associated with cardiovascular disease and atherosclerosis is important and has attracted increasing interest. The present study provided several potentials of butyrate for mitigating cognitive impairment in LDLR$^{-/-}$ mice fed an atherosclerotic diet with 1.25% cholesterol and 40% calories from fat (Fig. S1). First, it improved gut mucosal barrier function via raising the levels of tight junction proteins, and alleviated intestinal inflammation via blocking the TLR4/MyD88/NF-κB signaling and inhibiting the NLRP3 inflammasome activation, thus leading to reduced translocation of intestinal endotoxins (e.g. LPS) to the circulation. Second, it weakened Aβ plaque deposition and abnormal phosphorylation of Tau protein via inhibiting neuroinflammatory response in both cortex and hippocampus. Third, it restored the damaged BBB via strengthening the levels of tight junction proteins and upregulating SCFAs receptors to further limiting the LPS from bloodstream to the neural tissue. These aforementioned effects on LDLR$^{-/-}$ mice were independent of the tryptophan metabolism.

The gut mucosal barrier is the first line of defense that prevents intestinal bacterial toxins (e.g. LPS) from invading the intestinal tissues and causing intestinal mucosal injury [26]. The impairment of gut barrier function caused by a high-fat diet increased gut permeability and enhanced the penetration of gut microbiota-derived LPS into the circulation. LPS-related inflammation promotes the development of atherosclerosis progression, which in turn may impair cognitive function centrally [27]. Elevated bacterial LPS in circulation is a trigger to TLR4-mediated pro-inflammatory cascade when interacting with adaptor molecule MyD88, and to NLRP3 inflammasome activation by suppressing NF-κB [28, 29]. Our study showed that butyrate treatment improved gut permeability and kept the integrity of the gut barrier by upregulating the expression of tight junction proteins, e.g., ZO-1, claudin-3 and occludin; meanwhile, butyrate inhibited TLR4 signaling activation through targeting MyD88-dependent pathway and blocking NF-κB pathway in our experimental model of atherosclerosis and cognitive declination. In addition, butyrate activated the G-protein-coupled receptors (GPRs) from colon tissues, including GPR41, GPR43, and GPR109A, which can improve the intestinal inflammation and then delay cognitive impairment by gut–brain signalling [30, 6].

Epidemiological studies suggested that high circulating cholesterol level can cause arterial stiffness and cognitive decline in the elderly population [31, 32]. Animal studies showed that a diet rich in fat and cholesterol can aggravate the cerebral neuroinflammatory response and exacerbate cognitive impairment of LDLR$^{-/-}$ mice [18–20]. SCFAs-butyric acid is a common bacterial metabolic byproduct, which is considered a key messenger in gut-brain communication, and it plays a vital role in the pathogenesis of the neurodegenerative disease [5, 6]. From a physiological standpoint, the present study revealed that butyrate prevented cognition from declination which is characterized by a lower spatial learning and memory ability in LDLR$^{-/-}$ mice. From a biochemical view, butyrate alleviated neuroinflammatory reaction
and decreased tau protein phosphorylation. Neuroinflammation as a double-edged sword plays a crucial role in the progression of neurodegenerative disease, especially in the activation of microglia [33]. The NLRP3 inflammasome as an intracellular molecular sensor of microglia, is activated by signals that represent the hallmarks of neurodegenerative diseases [12, 13, 34], and such activation would exacerbate the disease progression via a pro-inflammatory chain reaction. Butyrate treatment inhibited the increment of GFAP that is a biomarker of reactive astrocytes and the activation of microglia by decreasing the level of IBA1. The molecular mechanism of butyrate on inflammatory factors was related to the blocking of the NLRP3 inflammasome signal pathway, resulting in a reduction of cytokines IL-1β and IL-18 in both cortex and hippocampus. Aberrant activation of NLRP3 inflammasome can aggravate cognitive impairment by causing Aβ deposition [35]. Despite a slight inhibition of amyloid plaque deposition, butyrate had no effect on the protein expression of Aβ, however, butyrate obviously lowered the aggregation of hyperphosphorylated tau via decreasing the protein levels of p-Tau ser396 and ser404. A recent study demonstrated that NLRP3 inflammasome activation can induce tau hyperphosphorylation and aggregation via targeting tau kinases in an IL-1β-dependent manner [36].

An intact BBB is essential for brain health. LPS is a proinflammatory component of Gram-negative bacterial cell wall, which can disrupt the structure of BBB permeability, and aggravated neuroinflammatory reaction in neurodegenerative disease [37]. Interestingly, butyrate reduced BBB permeability via reducing circulating LPS and raising tight junction proteins ZO-1 and occludin levels. In addition, butyrate activated SCFAs receptors GPR41, GPR43 and GPR109A, which play an important role in neuroinflammation and microbiota-gut-brain signal regulation [5, 6]. We presumed an atherosclerotic diet with 1.25% cholesterol and 40% calories from fat caused the disruption of BBB and increased the permeability through triggering intestinal inflammation with a high circulating bacteria-derived LPS level [38], while butyrate can reverse this process through inhibiting LPS-induced intestinal inflammation, and ameliorate cognitive function and mitigate neuronal damage through decreasing neuroinflammatory response and enhancing the activation of SCFAs receptors.

Tryptophan metabolism, through the serotonin/5-hydroxytryptamine (5-HT) pathway and the kynurenine (Kyn) pathway and microbial transformation of tryptophan to indolic compounds, is involved in the regulation of brain function and intestinal homeostasis, constituting common therapeutic target for neurodegenerative disease [39, 40]. Clinical evidence demonstrated that a reduced 5-HT as a serotonergic neurotransmitter was related to cognitive deficits in aging, Alzheimer's disease and also other neurodegenerative disorders [41], in which gut-derived serotonin may interact with gut-brain axis signaling [42]. As for Kyn pathway, a range of evidence suggests that alterations in the balance between neuroactive Kyn metabolites may play a role in neurodegenerative diseases [39]. However, the present study found that butyrate for a period of 8 weeks treatment was unable to influence the tryptophan metabolism, and it also had no effect on cerebral GPR109A as a niacin receptor. This explained why butyrate failed on tryptophan metabolism, nevertheless, whether a longer supplementation or a higher dosage may target tryptophan metabolism via regulating intestinal microflora is worth exploring in the future.
Taken together, based on our new experimental evidence that butyrate improved gut mucosal barrier function and alleviated intestinal inflammatory reaction, and reduced the translocation of intestinal toxins from the gut to circulation, and then to the central nervous system. Immediately, butyrate played a neuroprotective role via restoring the damaged BBB and attenuating the neuroinflammatory response via targeting NLRP3 inflammasome. The present study concludes that the progression retardation of cognitive impairment in a mouse model of atherosclerosis by butyrate is also attributed from another mechanism that is neuronally indirect and independent.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a CARD</td>
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<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
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<tr>
<td>Caspase-1</td>
<td>Cysteinyl aspartate specific proteinase-1</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>GPRs</td>
<td>G-protein-coupled receptors</td>
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<tr>
<td>IB 1</td>
<td>Ionized calcium-binding adaptor molecule 1</td>
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<td>IKKβ</td>
<td>IκB kinase β</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response 8</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NLRP3</td>
<td>The nod-like receptor family pyrin domain containing 3</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor-interacting protein</td>
</tr>
<tr>
<td>SCFAs</td>
<td>Short-chain fatty acids</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor-associated factor 6</td>
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<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
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<tr>
<td>ZO-1</td>
<td>Zonula occludens-1</td>
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**Declarations**

**Author contributions**
S.H. performed study design. R.S., H.G., H.J. and S.H. performed mice experiments. R.S., W.Z. and S.H. analyzed data, discussed experimental results, and provided important intellectual content throughout the study. R.S. and S.H. wrote the paper with input and approval from all authors. W.Z. revise the manuscript. All authors read and approved the submitted manuscript.

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**Availability of data and materials**

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

All animal experiments were approved by the local Institutional Animal Welfare Committee (Permission No. SUDA20211206A03), and conducted in accordance with the Institutional Guidelines in the Care and Use of Experimental Animals of the Ministry of Science and Technology (Beijing, China). All experimental protocols were made to minimize the sample size and to relieve the suffering of mice.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing financial interest.

**References**


Figures

![Simplied chart of the present experimental design.](image)

**Figure 1**

Simplified chart of the present experimental design.
Figure 2

Effects of butyrate on gut mucosal barrier and inflammatory factors. A, Colon length ($n = 8$ mice per group). B, Representative images of H&E staining of the colon tissues from each group at 20× resolution. C, Inflammatory cytokines including TNF-α, IL-1β and IL-10 from the colon tissue via an Elisa method ($n = 8$ mice per group). D, Serum LPS level via an Elisa method ($n = 8$ mice per group). E, Tight junction proteins including ZO-1, claudins and occludin from the colon tissue via an Elisa method ($n = 8$ mice per group). F, Tight junction proteins including ZO-1, claudin-3 and occludin from the colon tissue by Western blot method ($n = 4$ mice per group), and tubulin was served as normalization standard. Data are presented as the mean ± SEM. *$p < 0.05$ versus NC group; # $p < .05$ versus Mod group.
Figure 3

Butyrate on intestinal inflammatory pathways and SCFA receptors. A, TLR4 signaling pathway, including TLR4, MyD88, TRAF6, TRIF, RIP and IKKβ. B, NLRP3 inflammasome pathway, including NF-κB p65, NLRP3, ASC, Caspase-1, IL-1β and IL-18. C, SCFA receptors, including GRP41, GRP43 and GRP109A. β-actin or tubulin was served as normalization standard. Data are presented as the mean ± SEM (n = 4 mice per group). Representative bands are presented on the top of the bar. *p < 0.05 versus NC group; # p < 0.05 versus Mod group.
Figure 4

Cognitive behavior measurement using the Morris Water Maze test. A, Mean escape latency to the hidden platform on the first 5 training days of the navigation test. B, Number of crossing the platform location. C, Swimming time in the target quadrant during probe trials. D, Swimming distance in the target quadrant during probe trials. E, Representative swimming trajectories of mice from each group. Data are presented as the mean ± SEM (n = 8 mice per group). *p < 0.05 versus NC group; # p < 0.05 versus Mod group.

Figure 5
Butyrate on Aβ and Tau phosphorylation. Protein levels of Aβ1-42 and p-Tau ser 396 & ser 404 in the cortex (A) and hippocampus (B) via Western blot method while β-actin served as a normalization reference. Data are presented as the mean ± SEM (n = 4 mice per group). Representative bands are presented on the top of the bar. *p < 0.05 versus NC group; # p < 0.05 versus Mod group.

![Bar graphs showing protein levels of Aβ1-42 and p-Tau](image)

**Figure 6**

Butyrate on tight junction proteins and SCFA receptors from the cerebral cortex. Protein levels of tight junction proteins including ZO-1 and occludin (A), and SCFA receptors including GPR41, GPR43 and GPR109A (B) through western blot method, while tubulin served as a normalization reference. Data are presented as the mean ± SEM (n = 4 mice per group). Representative bands are presented on the top of the bar. *p < 0.05 versus NC group; # p < 0.05 versus Mod group.
Figure 7

Butyrate on neuroinflammation-related markers. Immunofluorescent double staining for GFAP (red) and IBA1 (green) in both cortex with 100 μm for scale bar (A) and hippocampus with 500 μm for scale bar (B). Quantification of the relative positive area (%) and average fluorescence intensity was analyzed in both cortex (C) and hippocampus (D) (n = 3 mice per group). Protein levels of GFAP and IBA1 were determined in both cortex (E) and hippocampus (F) via the western blot method (n = 4 mice per group), while β-actin served as a normalization reference. Data are presented as the mean ± SEM. Representative bands are presented on the top of the bar. *p < 0.05 versus NC group; # p < 0.05 versus Mod group.
Figure 8

Butyrate on NLRP3 inflammasome pathway in both cortex (A) and hippocampus (B). Factors involved in NLRP3 inflammasome signal were measured via western blot method, which included NF-κB p65, NLRP3, ASC, Caspase-1, IL-1β and IL-18, while β-actin served as a normalization reference. Data are presented as the mean ± SEM (n = 4 mice per group). Representative bands are presented on the top of the bar. *p < 0.05 versus NC group; # p < 0.05 versus Mod group.
Figure 9

Butyrate on tryptophan metabolism. A, PCA_scree and heat map of average tryptophan metabolites. B, Comparison of tryptophan metabolites. Data are presented as the mean ± SEM (n = 5 mice per group).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- westernfigure.pdf
- FigureS1.png
- TableS1.docx