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Modulation of the Gut Microbiota of type 2 Diabetic Mice by Sodium Butyrate Attenuates Ischemic Stroke Injury

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Abstract

Background: Type 2 diabetes (T2D) is a major comorbidity in patients with ischemic stroke that exacerbates brain injury. Recent studies have revealed that stroke injury can be influenced by the composition of the gut microbiota. However, the role of the gut microbiota of T2D patients in stroke injury and the potential benefits of manipulating the dysbiotic gut microbiota in T2D are unknown.

Methods: To modulate the gut microbiota profile, male diabetic (Db) mice and wild-type (Wt) mice were randomized to receive either sodium butyrate (SB) or sodium chloride (NaCl, Con) in drinking water. After 4 weeks, fecal samples were collected for microbiota analysis by 16S rRNA gene sequencing. We then performed fecal microbiota transplantation (FMT) and established middle cerebral artery occlusion (MCAO) model of stroke to investigate the causative relationship between the gut microbiota and stroke injury.

Results: We found that SB supplementation reduced food/water intake, fasting blood glucose levels and serum triglyceride (TG) levels in Db mice. Moreover, SB-treated Db mice displayed a different gut microbiota composition and higher levels of fecal butyrate than NaCl-treated Db mice. Twenty-four hours after cerebral ischemia/reperfusion, stroke mice that underwent FMT from SB-treated Db mice exhibited improvements in neurologic deficit scores and a smaller cerebral infarct volume than mice that underwent FMT from NaCl-treated Db mice. These improvements were accompanied by reductions in neuronal loss, apoptotic cell death, and microglial activation, increased levels of tight junction-related proteins and preservation of the endothelial glycocalyx in the ischemic brain.

Conclusions: This study suggests that the gut microbiota of T2D can be modified by SB to afford neuroprotection against ischemic stroke injury.

Background

Stroke is the second leading cause of death from cardiovascular disease, behind coronary heart disease, accounting for 17% of total deaths [1]. Type 2 diabetes (T2D) predisposes humans to ischemic stroke and is associated with poorer stroke outcomes, including poorer neurological outcomes and higher mortality, readmission rates, and stroke recurrence [2, 3]. Studies using murine models of these diseases have further confirmed this conclusion [4, 5]. However, the underlying mechanism by which T2D exacerbates stroke has not been fully elucidated.

In recent years, significant attention has been paid to the role of the gut microbiota in cardiovascular disease and metabolic disorders [6, 7]. Advances in microbial sequencing analysis have provided a wealth of information concerning the gut microbiota associated in T2D. Studies have revealed the alterations in the gut microbiota that occur in T2D; while there is a lack of consensus concerning which bacteria are altered significantly in T2D, a common observation is that the abundance of butyrate-producing bacteria is decreased in this condition [8–10]. Butyrate, a short-chain fatty acid (SCFA) that is naturally produced by bacterial fermentation of complex fiber in the colon and is also found in cheese

and butter [11], shows promising effects in obesity, diabetes and neurological disorders. Indeed, increased fiber consumption or butyrate supplementation has been shown to decease adiposity and improve insulin sensitivity [12–14]. Several lines of evidence have indicated that sodium butyrate (SB) exerts metabolic benefits against fat gain [15], diabetic inflammation [16], obesity [17], lipid metabolism [18] and steatohepatitis [19] by modulating the composition of the gut microbiota.

It has been reported that gut microbiota composition may have an impact on stroke outcome. Our previous work showed that dysbiosis of the gut microbiota is correlated with a worsened stroke outcome in patients [20]. Similar findings have been observed in mice. Singh et al. [21] demonstrated that the microbiota profile is altered after cerebral ischemia and that poststroke dysbiosis is associated with induction of the inflammatory response. Fecal microbiota transplantation (FMT) of a balanced microbiota after cerebral ischemia improves stroke outcome. Benakis et al. [22] discovered that gut microbiota disturbance leads to exacerbation of ischemic brain lesions by increasing intestinal proinflammatory T cells. Spychala et al. [23] showed that transplantation of the gut microbiota from young mice improves stroke outcome in aged mice, suggesting that targeting microbiota composition might exert beneficial effects in those with a high risk of stroke. A recent study demonstrated that fecal transplants from young mice contain much higher levels of SCFAs and related bacterial strains, which promote poststroke recovery [24].

Using db/db mice, a typical rodent model of T2D, we examined the effect of SB on diabetes-related parameters and the gut microbiota profile of T2D mice. Then, we subjected antibiotic-treated mice to FMT from diabetic (Db) mice or wild type (Wt) mice treated with or without SB. By using a middle cerebral artery occlusion (MCAO) model of stroke, we showed for the first time that the gut microbiota in T2D is causally linked to exacerbation of brain damage and can be modified by SB to afford neuroprotection against acute ischemic stroke injury.

Methods

Animal procedure and drug treatment

Six-week-old male db/db (C57BLKS/J-m+/+Lepr$^{db/db}$; Db) mice were obtained from GemPharmatech Co, Ltd (Nanjing, China), and age- and gender-matched C57BL/6J (Wt) mice were purchased from Guangdong Medical Lab Animal Center (Guangzhou, China). The experiments were approved by the Ethics Committee for Animal Care and Research of Zhujiang Hospital of Southern Medical University (Guangdong, China) following the National Guidelines (Guidelines on Administration of Laboratory Animals in China and Guidelines on the Humane Treatment of Laboratory Animals in China) and in accordance with the ARRIVE guidelines.

Mice were housed in an environment with controlled temperature and humidity on a 12-h:12-h light/dark cycle. After acclimatization for 1 week, the Wt (n = 10) and Db (n = 20) mice were randomized to receive one of the two types of drinking water for 4 weeks: (1) 0.1M sodium chloride (NaCl, Aladdin, Shanghai,

China) in distilled water as a control, or (2) 0.1M SB (Aladdin) in distilled water. This resulted in the following four groups: (1) Wt mice that received NaCl (Wt-Con group; n = 5), (2) Wt mice that received SB (Wt-SB group, n = 5), (3) Db mice that received NaCl (Db-Con group, n = 10) and (4) Db mice that received SB (Db-SB group, n = 10). After 4 weeks of intervention, the mice were placed in a clean cage with sterilized paper. After the mice defecated, the fecal samples were collected immediately in a sterilized centrifuge tube. The fecal samples were stored in a -80 °C freezer until analysis and transplantation.

For FMT, recipient C57BL/6 mice (n = 100) were randomly divided into four groups: (1) rWt-Con (n = 25), (2) rWt-SB (n = 25), (3) rDb-Con (n = 25) and (4) rDb-SB (n = 25) groups. Broad-spectrum antibiotics (1 g/L ampicillin, 1 g/L metronidazole, and 1 g/L neomycin sulfate, Sigma-Aldrich, California, USA) dissolved in drinking water were given ad libitum to the recipient mice for 14 consecutive days. The drinking water was renewed every 2 days. Fecal microbiota suspensions were prepared by diluting 1 g of fecal samples obtained from donor mice in 10 mL of sterile PBS and mixing, and then 0.2 mL of the suspension was intragastrically administered to each recipient mouse once daily for 14 consecutive days.

Cerebral ischemia model establishment

A cerebral ischemia model was established by inducing transient MCAO by intraluminal suture as previously described [21]. In brief, mice were anesthetized with isoflurane (induction: 2%; maintenance: 1.2%; in a 30:70 mixture of oxygen and nitrous oxide), and the body temperature was maintained at 37.0 ± 0.5 °C during surgery with a heating pad. An incision was made in the midline neck region, and the right common carotid artery and external carotid artery were exposed, isolated and ligated; the superior thyroid and occipital arteries were separated and cauterized with a preheated electrocautery device to prevent bleeding. Then, a monofilament (approximately 2 cm) was introduced into the external carotid artery and gently advanced into the internal carotid artery. After 60 min of occlusion, the filament was removed, and the ligature around the common carotid artery was removed to induce reperfusion. The wound was disinfected and sutured, and the mice were allowed to recover from anesthesia, returned to their home cages and provided free access to food and water.

Modified neurological severity score test

Neurological function was scored using the modified neurological severity score (mNSS) test [25]. The test was conducted 24 h of reperfusion. The mNSS is a composite of motor (muscle status and abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex tests. Neurological function was graded on a scale of 0–18 (normal score: 0; maximal deficit score: 18). The higher the brain injury severity score, the more severe the injury.

Triphenyltetrazolium chloride staining

After neurological function was scored, the mice were anesthetized with a lethal dose of sodium pentobarbital (0.1 g/kg) and perfused with phosphate buffer solution (0.01 M, pH = 7.4). The intact brain was dissected out, cut into 2-mm tissue slices, stained with 1% triphenyltetrazolium chloride (TTC, Sigma-Aldrich) for 15 min and immersed in 4% formaldehyde for fixation. Twenty-four hours later, the brain

slices were arranged in order and photographed. The cerebral infarct area was calculated using Image-Pro Plus software 6.0 (the red area indicates no infarction; the white area indicates infarction). The infarct area was calculated as the area of the nonischemic hemisphere minus the noninfarcted area of the ischemic hemisphere. The infarct volume was calculated as infarct area × thickness (2 mm). The percentage of cerebral infarction was calculated using the following formula: infarct volume/(the volume of the nonischemic hemisphere × 2) × 100% [26].

Gut microbiota analysis

Fecal pellets were collected from donor mice at the end of SB/NaCl intervention and from recipient mice before MCAO. Gut microbiota DNA was extracted using a DNA Isolation kit (MinkaGene, Guangzhou, China), and the concentration and purity of the DNA were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Electron Corporation, USA). The 16S rRNA gene was PCR-amplified using primer sets specific for the V3-V4 regions. The final PCR products were purified from unincorporated nucleotides and primers using the Qiaquick PCR Purification kit (Qiagen, Valencia, USA). The DNA concentrations of purified samples were normalized, and the DNA was sequenced using the Illumina MiSeq PE250 sequencer (Illumina, USA).

Extraction and quantification of SCFAs

Fecal pellets were collected from donor mice at the end of SB/NaCl intervention and from recipient mice before MCAO. Approximately 200 mg of feces was homogenized in 1 mL of ultrapure water that contained an internal standard of 2,2-dimethylbutyric acid. The homogenate was then centrifuged at 12,000 rpm for 10 min at 4 °C. The resulting supernatant was transferred to a new Eppendorf tube and mixed with 10 μL of 50% sulfuric acid, 0.5 g of sodium sulfate (Macklin, China), and 2 mL of analytically pure diethyl ether. The mixture was vortexed for 1 min and then centrifuged at 5000 rpm for 10 min at room temperature. The ether layer was finally collected for gas chromatography with mass selective detection (5977B GC/MSD, Agilent Technologies, Santa Clara, CA, USA). An HP-Free Fatty Acid Phase (HP-FFAP) capillary column (30 m length, 0.25 mm internal diameter, 19091F433, Agilent Technologies) was used for chromatographic separation, with helium as the carrier gas. The oven temperature was increasedby15 °C/min, from 90 to 180 °C. Gas chromatography mass spectrometry (GC/MS) data were collected and analyzed with MassHunter Workstation Software (Agilent Technologies). Final concentrations were calculated based on internal standards and are expressed as micromoles per gram of wet feces (μmol/g).

Electron microscopy

The animals were perfused through the abdominal aorta with a solution containing 2.5% glutaraldehyde, 2% paraformaldehyde, and 2% lanthanum nitrate, as described by Vogel et al. [27], to preserve the glycocalyx in brain tissues. After the brain tissues were embedded in epon resin and 120-nm sections were contrast-enhanced with 5% uranyl acetate in deionized water for 20 min and a solution containing 120 mmol/L sodium citrate, 80 mmol/L lead citrate and 160 mmol/L sodium for 2 min, the glycocalyx was photographed using a transmission electron microscope (EM 90; Zeiss, Oberkochen, Germany).

Determination of glycocalyx components (syndecan-1 and heparan sulfate (HS))

To measure the concentration of syndecan-1 and HS, blood samples were collected and centrifuged at 3000 × g for 10 min, and the clear supernatant was collected. Syndecan-1 concentrations were determined using an ELISA kit (Diaclone Research, Besancon, France). This kit used a solid phase monoclonal B-B4 antibody and a biotinylated monoclonal B-D30 antibody raised against syndecan-1. The concentrations of HS were determined using an ELISA kit (Seikagaku Corporation, Tokyo, Japan), which was based on two antibodies specific for HS-related epitopes.

Blood lipid and fasting blood glucose levels

At the end of SB/NaCl intervention, the serum levels of total-cholesterol (T-CHO) and triglyceride (TG) were quantified by kits following the manufacturer's protocols (Nanjing Jiancheng Biotech, China). After an overnight fast, blood glucose levels in tail vein blood were measured using a glucometer (ACCU-CHEK, Roche, USA).

Western blot analysis

The ipsilateral cortex around the infarct area was collected and stored at -80 °C until further use. The brain tissues were homogenized in RIPA lysis buffer (Beyotime Biotechnology, China) containing protease inhibitor PMSF (Beyotime Biotechnology) using a handheld homogenizer and incubated on ice for 20 min. The lysates were centrifuged at 12,000 × g for 20 min at 4 °C and the supernatants were transferred to fresh tubes. The protein samples were resolved on a 12% or 10% SDS-PAGE gel and electrotransferred onto a PVDF membrane. The membrane was blocked in 5% nonfat milk at room temperature for 1 h and then incubated with primary antibodies against Occludin (1: 1000; Abcam), Claudin-4 (1: 1000; Abcam) and β-actin (1:5000; Abcam) at 4 °C overnight. The membrane was washed in Tris-buffered saline (TBS) containing 0.1% Tween-20 and incubated with an HRP-conjugated goat anti-rabbit secondary antibody. The membrane was then visualized with an enhanced chemiluminescence system (Thermo Scientific, Rock-ford, IL). The band intensity was assessed by using Image Lab software.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). Statistical significance among the 4 groups was evaluated using one-way ANOVA with LSD post hoc test. SPSS 22.0 (IBM, Armonk, NY) and GraphPad Prism 5.0 (GraphPad, La Jolla, CA) software were employed for data analysis, and a p value < 0.05 was considered statistically significant. Mixed model ANOVA (within factor: time; between factor: treatment group) was used to analyze variables measured over time (i.e., body weight and blood glucose levels). Microbial community α- and β-diversity were calculated using Chao1, observed operational taxonomic units (OTUs), PD whole tree and Shannon indices (α-diversity) and weighted and unweighted distances (β-diversity) and were visualized by principal coordinate analysis (PCoA). Nonparametric Mann-Whitney and Kruskal–Wallis tests were used to determine the statistical significance of α-diversity measures and permutational MANOVA was used to evaluate differences in β-diversity. Microbial markers

based on differential abundance among the treatment groups were identified using ANOVA followed by post hoc Tukey's teat for multiple comparisons ($p < 0.05$) and linear discriminate analysis effect size (LEfSe) with an LDA > 1 and $q < 0.05$.

Results

Effect of SB on diabetes-related parameters

The diabetes-related parameters of the mice in the four groups are shown in Fig. 1. As expected, both groups of Db (Db-Con and Db-SB) mice consumed significantly more food and water and generally displayed higher body weights (Fig. 2a) than non-Db (Wt-Con and Wt-SB) mice. SB treatment had no effect on the food/water intake or body weight of the non-Db mice. However, SB significantly reduced the food intake of Db mice at weeks 3 and 4 (Fig. 2b) and reduced the water intake of Db mice at weeks 2, 3 and 4 (Fig. 2c). At baseline, fasting blood glucose levels were similar between mice of the same genotype that received SB or NaCl, and as expected, fasting blood glucose levels were markedly higher in both Db groups than in the non-Db groups. Hyperglycemia was exacerbated in Db mice during the intervention period. The Db-SB group exhibited lower fasting blood glucose levels than the Db-Con group after 2 weeks of SB treatment (Fig. 2d). However, there was no significant difference in fasting blood glucose levels between the non-Db groups. At the end of the 4-week intervention period, the Db groups displayed higher T-CHO and TG concentrations than the non-Db groups. There was no significant difference in T-CHO levels between the Db groups (Fig. 2e). However, the Db-SB group had a lower TG level than the Db-Con group (Fig. 2f). Taken together, these data indicate that SB reduced diabetes-related parameters in Db groups but did not have a significant effect on diabetes-related parameters in the non-Db groups.

Effects of SB on the gut microbiota profile and SCFA concentrations

To investigate whether SB affects the composition of the gut microbiota, total bacterial DNA was isolated from fecal samples from each mouse after 4 weeks of SB/NaCl intervention. The 16S rRNA gene was sequenced using the MiSeq platform. SB treatment did not affect the α-diversity Chao 1 index (Fig. 2a). However, according to PCoA, the gut microbiota of the Db groups differed significantly from those of the Wt groups (Fig. 2b). Additionally, there was a different in the gut microbiota composition between the Db groups (Fig. 2d) but not between the Wt groups (Fig. 2c). Taxonomic composition analysis revealed that Db-SB group mice had relatively higher abundances of butyrate-producing bacteria, including *Lachnospiraceae, Ruminococcaceae, Oscillospira, and Ruminococcus* than Db-Con mice (Fig. 2e). To further identify which bacterial taxa were distinct between the 2 groups, we performed LEfSe analysis and identified several species with significantly different abundances (Fig. 2f, g). Additionally, we found that members of *Christensenellaceae*, a family in the class *Clostridia* (e.g., *Christensenella* and *Christensenellaceae*) were enriched in the Db-SB group (Fig. 2g). Furthermore, the fecal concentrations of acetate, propionate, butyrate, valerate and total SCFAs were tested. The Db mice exhibited consistently

lower concentrations of SCFAs than the Wt mice (Fig. 3). SB treatment significantly increased the fecal butyrate concentration of Db mice (Fig. 3c).

Modulation of the gut microbiota of T2D mice by SB attenuated stroke injury

We depleted the microbiota in recipient mice by administering a cocktail of antibiotics for 14 days and found that α and β-diversity were drastically altered by antibiotic treatment (Supplementary Fig. 1a-f). Then, the recipient mice were orally inoculated with fecal suspensions from donor mice once daily for 2 weeks. We found that FMT had no effect on diabetes-related parameters between the 4 groups (Supplementary Fig. 2). After FMT, fecal pellets were collected for gut microbiota analysis. PCoA plots of unweighted UniFrac distances (Supplementary Fig. 1g, h) and α-diversity (Supplementary Fig. 3a) revealed that the microbiota of the recipients reflected the microbiota of the donors. Mice that received fecal microbiota from SB-treated Db (rDb-SB) mice exhibited a microbiota profile that was distinct from that of mice that received fecal microbiota from NaCl treated Db (rDb-Con) mice (Supplementary Fig. 3b, c). The abundances of butyrate-producing bacteria such as *Oscillospira*, *Lachnospiraceae* and *Ruminococcaceae* (Supplementary Fig. 3d) were higher in rDb-SB mice than in the rDb-Con mice. Following FMT, the recipient mice were subjected to MCAO for 1 h followed by reperfusion for 24 h. Neurological functional outcomes were measured by the mNSS test. The rDb-SB mice had lower mNSS scores (Fig. 4b) and a smaller infarct volume (Fig. 4c, d) than the rDb-Con mice. However, there were no significant differences in mNSS scores or infarct volume between rWt-Con mice and rWt-SB mice. Nissl staining was applied to investigate morphological changes in neurons, and we discovered that the rDb-Con group had more neuronal karyopyknosis, chromatolysis and shrinkage of cell bodies in the hippocampal CA1 region than the other groups (Fig. 4d-g). Moreover, the rDb-SB group exhibited less neuronal loss, microglial activation in the cortex and hippocampus (Fig. 5a, b) and apoptotic cell death in the hippocampus after stroke than the rDb-Con group (Fig. 5c, d). Taken together, these data suggest that the gut microbiota in T2D per se plays a critical role in exacerbating stroke outcome and that modulation of the gut microbiota of diabetic mice by SB alleviates stroke injury.

Modulation of the gut microbiota of T2D mice by SB attenuated stroke injury by protecting the blood-brain barrier

We further evaluated blood-brain barrier (BBB) damage after ischemic stroke. The results showed that the levels of the barrier-forming tight junction proteins Claudin-4 and Occludin were markedly higher in groups that were administered gut microbiota suspensions from SB-treated mice (Fig. 6a, b). The endothelial glycocalyx is considered an important component of the BBB. We assessed the cerebral capillary endothelial glycocalyx by electron microscopy. As shown in Fig. 6c, the rWt groups displayed a thick layer of glycocalyx, whereas only a residual glycocalyx was observed in the rDb-Con group, indicating serious endothelial glycocalyx degradation. On the other hand, the rDb-SB group had a relatively thicker layer of endothelial glycocalyx than the rDb-Con group (Fig. 6c). Serum levels of syndecan-1 and HS reflect the extent of glycocalyx degradation. In all groups, serum samples were

collected 24 h after reperfusion and used to assess the shedding of syndecan-1 and HS. Consistent with the electron microscopic results, the rDb-Con group exhibited higher shedding of syndecan-1 and HS than the rDb-SB group (Fig. 6d, e). However, no significant differences were observed in syndecan-1 levels or HS levels between the rWt-Con and rWt-SB groups.

Discussion

The primary findings of the present study were that SB modulated the gut microbiota profile of T2D mice and elevated the fecal concentration of butyrate, which alleviated stroke outcome independent from a T2D background. This protective effect was related to the enhancement of BBB integrity and preservation of the endothelial glycocalyx in the ischemic brain.

In this study, we found that the gut microbiota profile of Db mice was distinct from that of Wt mice. Several studies have shown that the gut microbiota might be an important contributor to the development of T2D. Ridaura et al. discovered that the obesity-associated metabolic phenotype can be transmitted from humans to mice by FMT [28]. In males with metabolic syndrome, FMT from lean male donors results in a significant improvement in insulin sensitivity, increased gut microbiota diversity and a remarkable increase in butyrate-producing bacteria [29]. However, in this study, FMT from Db mice had no effect on diabetes-related parameters, possibly due to the short period of transplantation. Studies by both Qin et al. [8] and Karlsson et al. [30] showed that T2D subjects exhibit reduced abundances of Clostridiales bacteria, such as *Roseburia* and *Faecalibacterium prausnitzii*, which are known as butyrate-producing bacteria. Thus, supplementation with butyrate might elicit favorable effects on diabetes-related parameters. In this study, we found that SB supplementation reduced food/water intake, fasting blood glucose levels and serum TG levels in Db mice. These data are consistent with those of Mollica et al., who found that oral administration of butyrate improves insulin sensitivity, and reduces serum TG levels and energy expenditure in diet-induced obese mice by enhancing mitochondrial function [31].

We also found that SB supplementation shaped the gut microbiota of Db mice, with SB-treated mice exhibiting a relatively higher abundance of butyrate-producing bacteria than NaCl-treated mice. Notably, in line with data published by Zhou et al. [19], we observed that SB treatment significantly increased the abundance of *Christensenellaceae*, a recently described family member of the phylum Firmicutes. *Christensenellaceae* have been shown to be enriched in individuals with a normal body mass index (BMI) compared to obese individuals with a BMI over 30 [32]. Moreover, increases in *Christensenellaceae* have been demonstrated following diet-induced weight loss [33]. A recent study found that a higher abundance of *Christensenellaceae* was positively associated with a lower cardiometabolic risk score in a cohort of 441 Colombians [34]. Moreover, *Christensenellaceae* has also been found to be associated with healthy glucose metabolism [35, 36]. However, the mechanism underlying its negative association with metabolic symptoms remains to be elucidated. Future studies focusing on *Christensenellaceae* might help gain insight for the treatment of T2D.

T2D is a well-known comorbidity that aggravates brain injury after ischemic stroke. The pathophysiological mechanisms by which T2D exacerbates brain injury after stroke have not been fully elucidated. In recent years, tremendous progress has been made in identifying the bidirectional interactions that occur between the gut and the brain, namely, the gut-brain axis. A series of provocative studies have suggested a prominent role for the gut microbiota in ischemic stroke [22–24]. Thus, we aimed to seek answers from the gut. A previous study revealed that *Clostridium butyrium* attenuates ischemic stroke injury in diabetic mice via modulation of the gut microbiota [37]. However, this study does not discount the effect of the blood glucose level on ischemic stroke injury, as treatment with *Clostridium butyrium* significantly alleviates hyperglycemia, which is a strong risk factor for poor stroke outcome. Admission hyperglycemia is associated with poorer stroke outcome possibly because it exacerbates ischemic injury by disturbing recanalization and worsening reperfusion injury [2]. Studies also suggest that inflammation may contribute to ischemic stroke concomitant with T2D [38]. Thus, we used FMT and MCAO models of stroke to address the causative relationship between the gut microbiota and stroke injury independent of the regulation of glycemia. As a result, mice that underwent FMT from SB-treated Db mice exhibited lower neurological deficit scores and a smaller cerebral infarct volume than mice that underwent FMT from NaCl-treated Db mice. Importantly, we found that the rDb-SB group had a higher abundance of butyrate-producing bacteria and a higher level of butyrate, which appear to be at least partially responsible for protection against ischemic brain injury, than the rDb-Con group. Benakis et al. found that intestinal IL-17-producing γδ T cells travel from the gut to the brain meninges following ischemic stroke to influence stroke injury [22]. Gut microbes have been demonstrated to induce $CD4^+$ T cells to differentiate into proinflammatory T cells or anti-inflammatory regulatory T cells [39–41]. Notably, SCFAs regulate leukocyte trafficking from the gut to other tissues, such as the uvea [42]. Moreover, SCFAs have been reported to regulate microglial homeostasis [43] and are important for the development and maintenance of the BBB [44]. In the current study, we observed that the rDb-SB group exhibited less microglial activation, neuronal loss and apoptosis in the ipsilateral cortex and hippocampus region of the ischemic hemisphere than the rDb-Con group. Our findings indicate that the gut microbiota in T2D per se has a great impact on ischemic stroke injury and can be influenced by SB treatment.

Ischemic stroke is often accompanied by BBB disruption. BBB dysfunction triggers vascular edema and creates an inflammatory environment, ultimately resulting in neuronal death and brain damage [45]. Therefore, maintenance of BBB integrity is thought to be key for protecting the brain from ischemia/reperfusion injury. A major finding of the present study was that modulation of the diabetic gut microbiota by SB attenuates cerebral ischemia by protecting the BBB. Some of the most important proteins that affect BBB permeability are tight junction proteins. Our data revealed that degradation of Occludin and Claudin-4 was relieved in the rDb-SB group compared with the rDb-Con group. Moreover, we observed that the rDb-Con group experienced the severest endothelial glycocalyx degradation and the highest serum concentrations of its components, i.e., the HS and syndecan-1. Interestingly, the endothelial glycocalyx in the rDb-SB group was mildly damaged. The endothelial glycocalyx, a gel-like layer that covers the vascular endothelial surface and floats into the lumen of the vessels, plays a critical role in vascular integrity and cardiovascular homeostasis [46]. In our previous report, we discovered that

endothelial glycocalyx degradation leads to increased BBB permeability in a rat model of asphyxia cardiac arrest [47]. Collectively, these data indicate that SB modulates the gut microbiota of T2D mice and that this modulatory effect affords neuroprotection against cerebral ischemia/reperfusion injury. However, future studies are necessary to determine the underlying mechanisms.

We acknowledge that this study has several limitations. First, although the Db mouse model used in the present study is the most widely used model of T2D, the monogenetic underpinnings of this model are not a perfect representation of T2D in humans. Different T2D mouse models, e.g., db/db mice and streptozotocin-induced diabetic mice, may exhibit different gut microbiome profile. Second, for FMT, we used the entire fecal content, which includes bacteria, fungi, viruses, metabolites from bacteria and undigested food. Therefore, it is not clear which component of the feces exerts effects against ischemic stroke injury. The elevated concentration of butyrate in the SB-treated group may have partially resulted from SB in the drinking water. Third, even though we confirmed that SB remodels the gut microbiota of Db mice to afford neuroprotection against cerebral ischemia injury, we did not explore the underlying mechanism. It is believed that intestine-derived T cells play a vital role in brain injury after stroke, and future studies investigating the immune status of T2D treated with SB might shed light on the underlying mechanism.

In conclusion, our findings demonstrate that SB is sufficient to shape the gut microbiota of Db mice and elevate the fecal concentration of butyrate, which alleviates diabetes-related symptoms. Restoration of the gut microbiota of T2D mice by SB can protect the BBB and reduce cerebral infarct volume. This study provides the first experimental evidence that the microbiota composition can be therapeutically exploited to protect against cerebral ischemia injury in patients with T2D. However, future studies are required to elucidate the underlying mechanism.

Abbreviations

T2D
type 2 diabetes; Db:diabetic mice; Wt/Con:wild type control mice; SB:sodium butyrate; NaCl:sodium chloride; FMT:fecal microbiota transplantation; MCAO:middle cerebral artery occlusion; TG:triglyceride; T-CHO:total-cholesterol; SCFA:short-chain fatty acid; mNSS:modified neurological severity score; TTC:triphenyltetrazolium chloride; GC-MS:gas chromatography-mass spectrometry; HS:heparan sulfate; PCoA:principal coordinate analysis; OTUs:taxonomic units; LEfSe:linear discriminate analysis effect size; BBB:blood-brain barrier; BMI:body mass index.

Declarations

Authors' contributions

JY, YH and HZ designed and supervised the study; HW and WS conducted the experiments; JL performed the FMT and GC-MS experiments; JZ and QW provided expertise in electron microscopy and ELISA

studies; XG and CT analyzed the data; HW, WS, JL and CT drafted and revised the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

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

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Figures

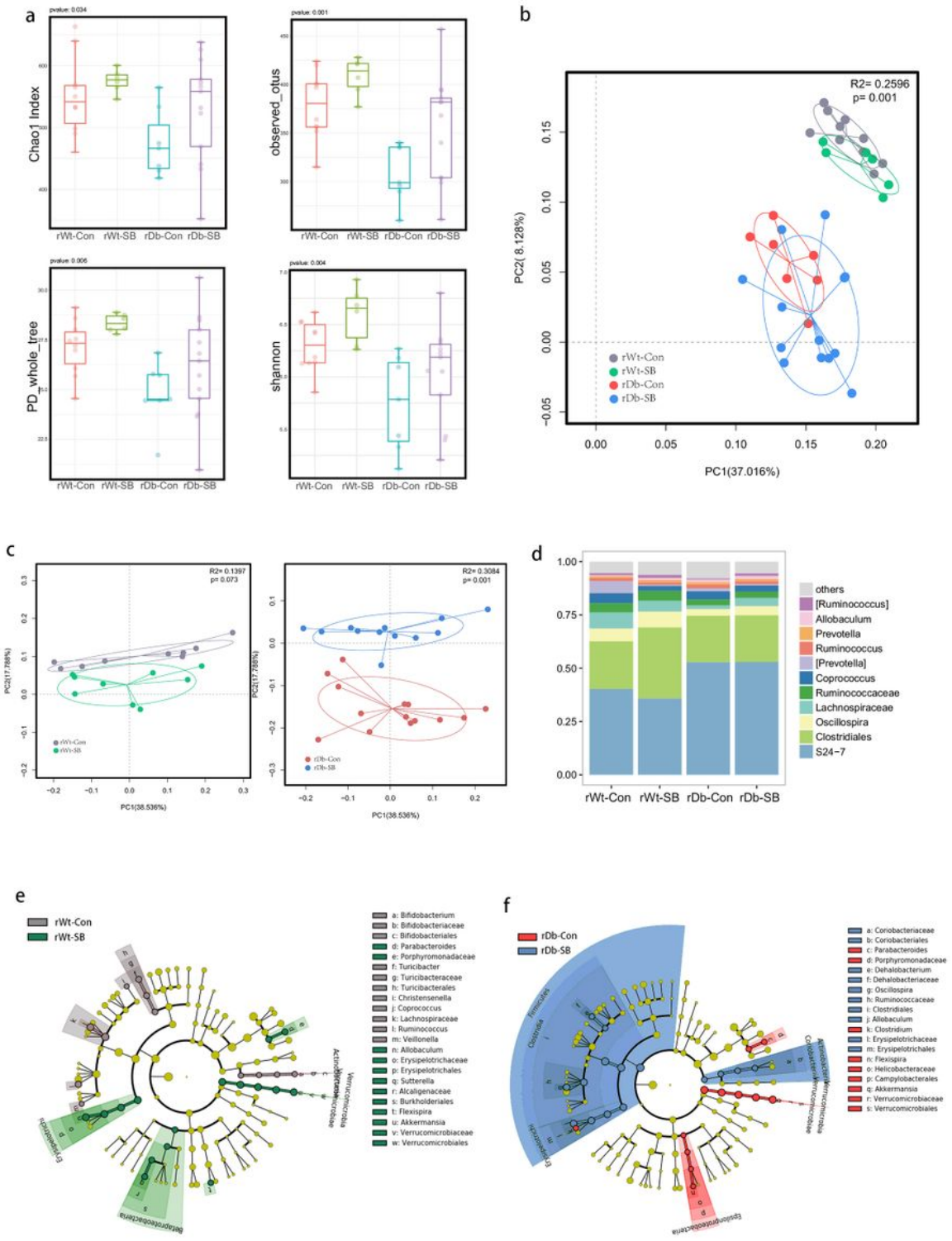

Figure 1

The effects of SB on diabetes-related parameters. (a) Study design for donor mice. After acclimatization for 1 week, Wt and Db mice received NaCl or SB in the drinking water for 4 weeks. Diabetes-related parameters were measured at different time point. Changes in body weight (b), food intake (c), water intake (d) and fast blood glucose levels (e) from weeks 0 to 4. T-CHO levels (f) and TG levels (g) at the

end of the 4-week intervention period. The data are expressed as the mean±SD; *p < 0.05 vs both Wt groups at the same time point; #p < 0.05, Db-Con vs Db-SB group at same time point; n=5-10/group.

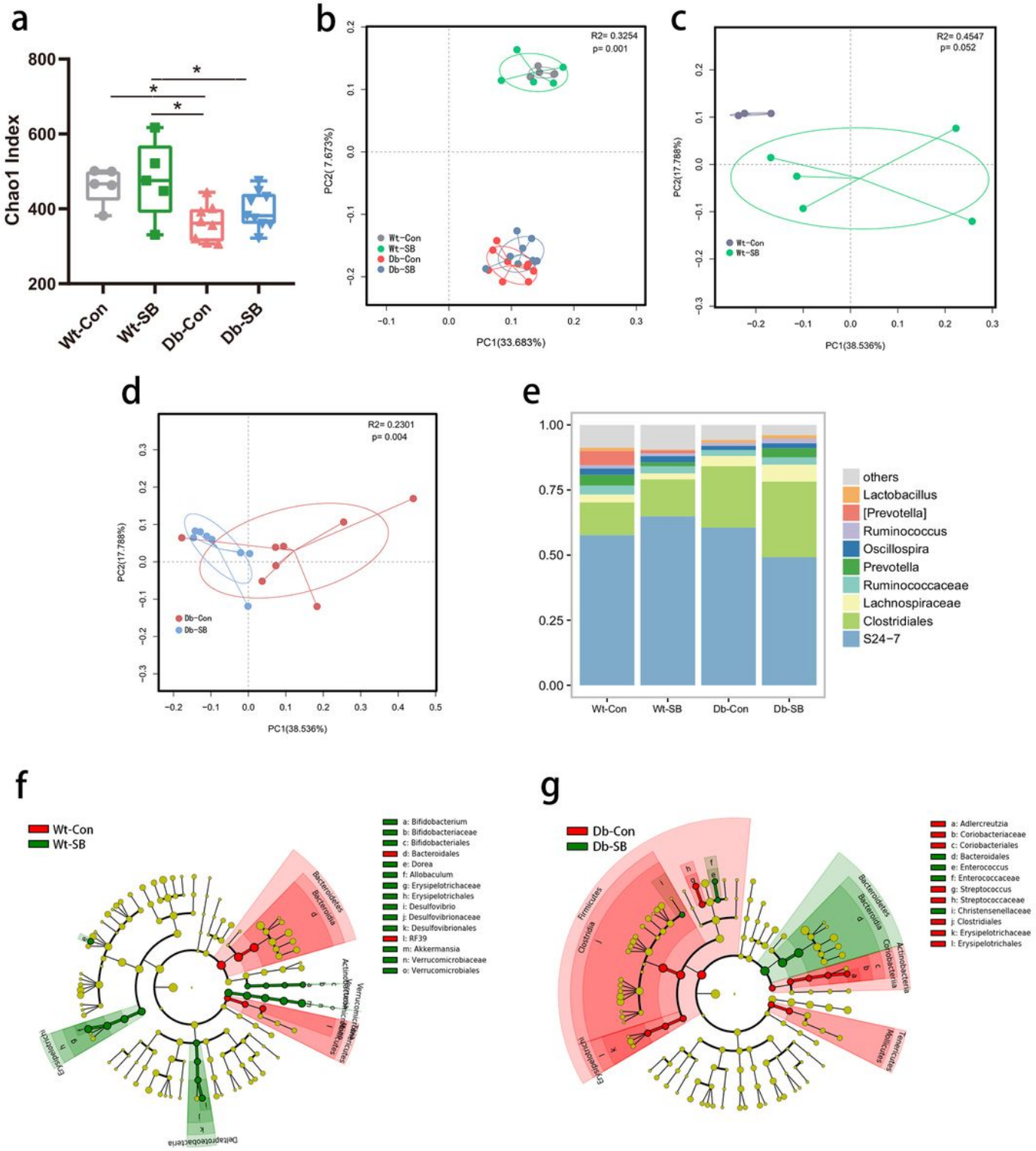

Figure 2

Gut microbiota profiles of mice. After 4 weeks of intervention, total bacterial DNA was isolated from fecal samples, and the 16S rRNA genes were sequenced. (a) Comparison of the α-diversity Chao 1 index between 4 groups. (b) PCoA plot of unweighted UniFrac distances. Each circle represents a single sample,

which are color-coded based on group. The eigenvalues principal coordinate (PC)1 and PC2 were 33.683% and 7.673%, respectively. (c) PCoA plot of weighted UniFrac distances between the Wt groups. (d) PCoA plot of weighted UniFrac distances between the Db groups. (e) Average relative abundances of predominant taxa at the genus level. Differentially abundant taxa between the Wt group (f) and Db group (g) identified by LEfSe analysis. * p<0.05; n=3-8/group.

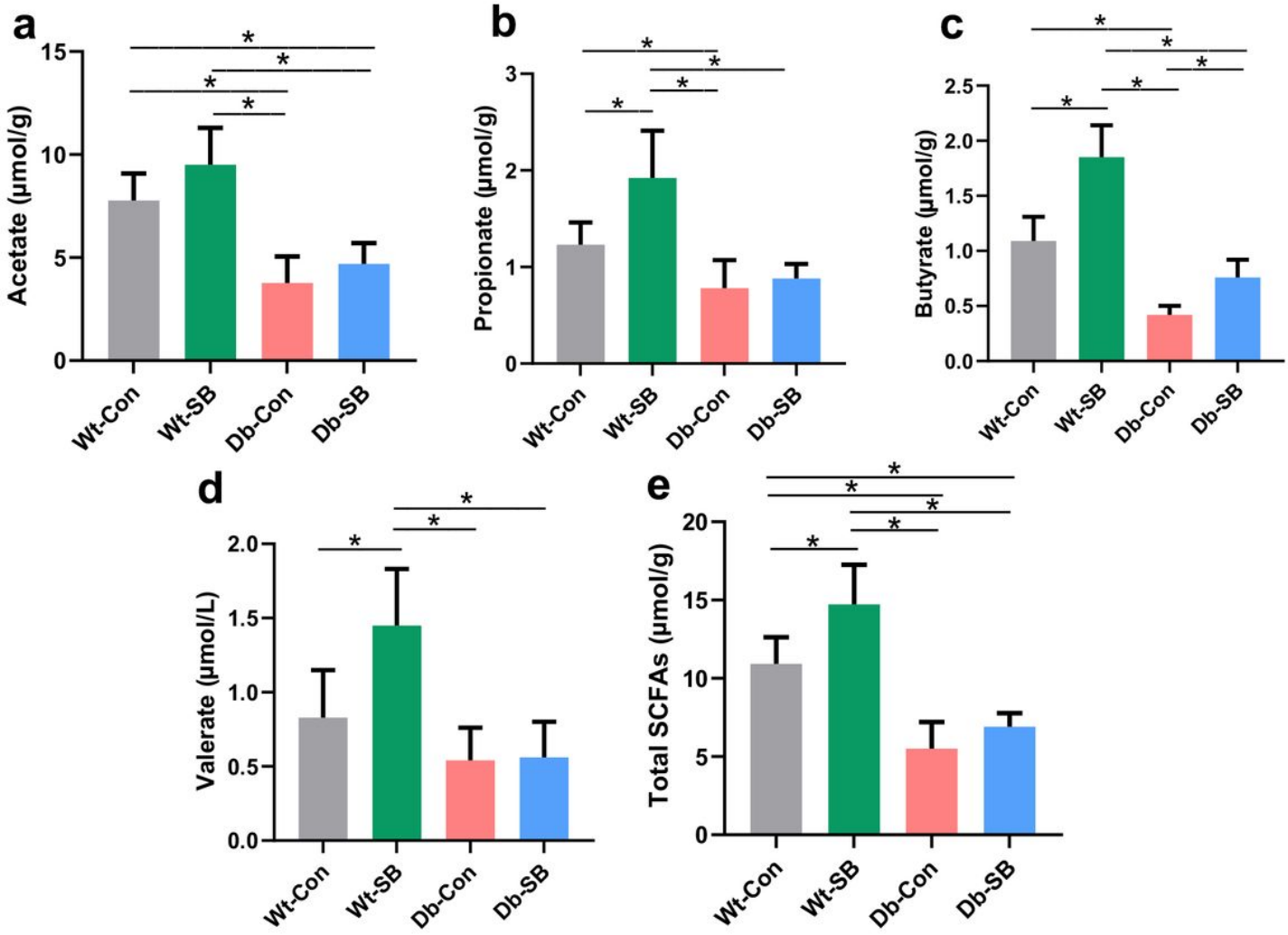

Figure 3

Fecal concentrations of SCFAs after 4 weeks of intervention. The data are expressed as the mean±SD; * p<0.05; n=5-8/group.

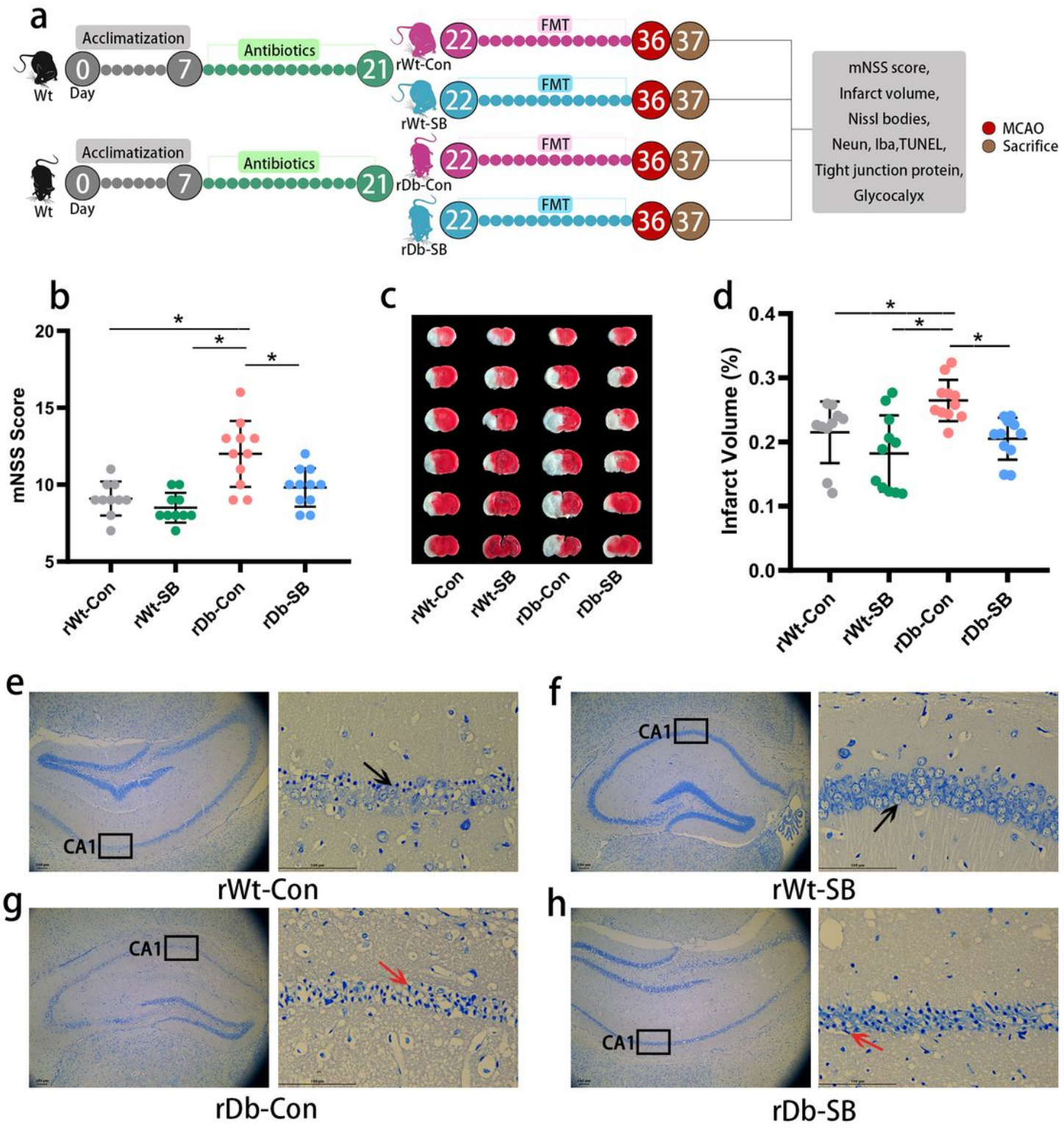

Figure 4

Modulation of the gut microbiota of T2D mice by SB attenuated stroke injury. (a) Study design for recipient mice. After acclimatization, the recipient mice were administered a cocktail of antibiotics for 14 days and then underwent FMT for an additional 14 days. After FMT, the recipient mice were subjected to MCAO for 1h and sacrificed after 24 h of reperfusion. (b) The mNSS test was performed before sacrifice. (c) Representative TTC staining of brain slices. (d) Percentage of cerebral infarct volume. (e-h)

Representative images of Nissl staining in the hippocampal CA1 region (scale bar=100 μm). The black arrows indicate clear and intact neurons with Nissl bodies uniformly distributed around the nuclei. The red arrows indicate damaged neurons with shrunken cytoplasm, loss of cell integrity and pyknosis of Nissl bodies. The data are expressed as mean±SD; * p<0.05; n=10-12/group.

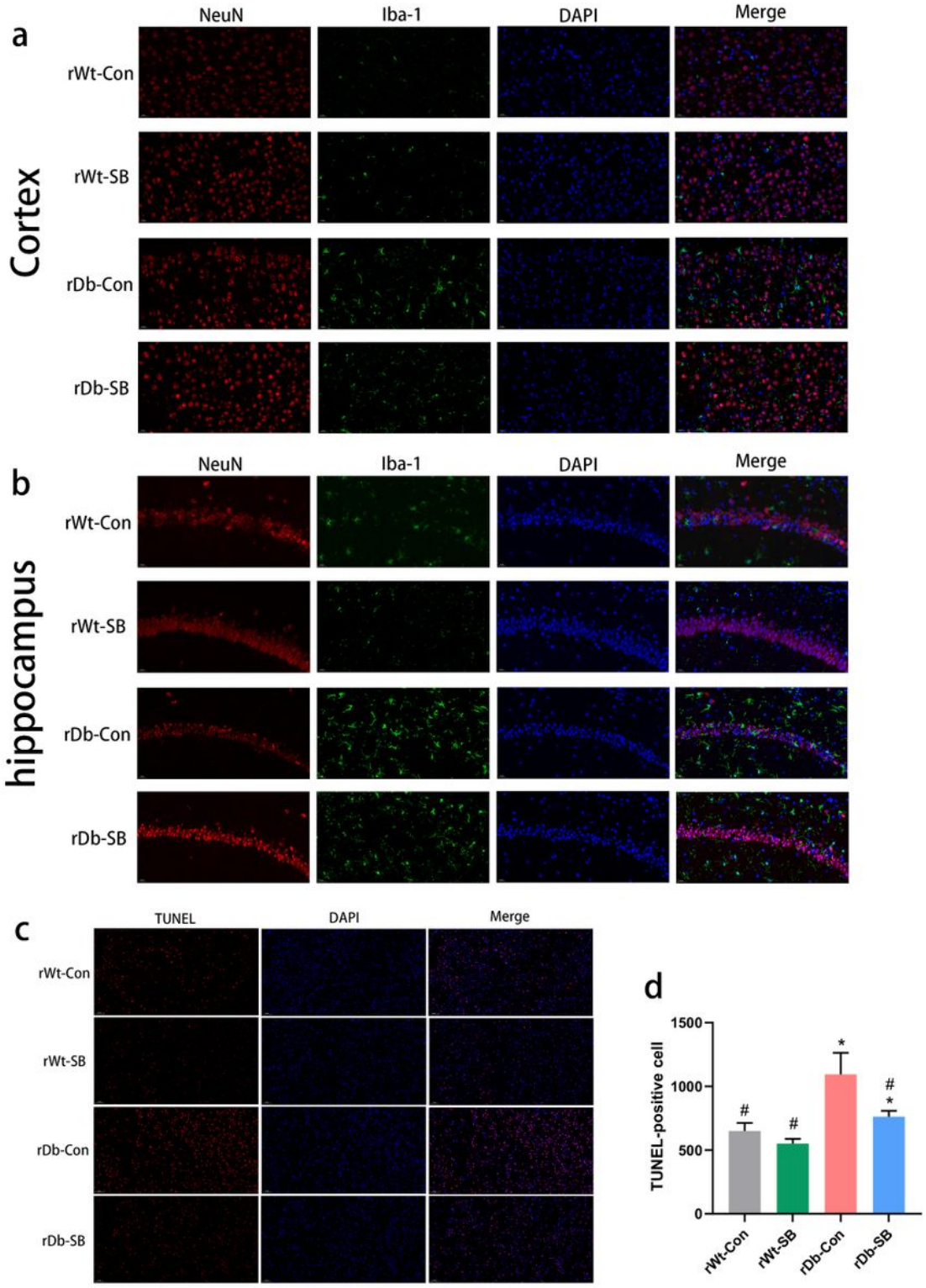

Figure 5

Modulation of the gut microbiota of T2D mice by SB reduced neuronal loss, microglial activation and apoptotic cell death after stroke. Double immunostaining for NeuN (neuronal marker) and Iba-1 (microglial marker) was performed in the peri-infarct cortex (a) and hippocampal CA1 region (b) 24 h after reperfusion. (c) Apoptotic neurons in the peri-infarct cortex were detected by TUNEL staining. (d) The number of apoptotic neurons in six random fields was determined using ImageJ. The data are expressed as the mean±SD; *p < 0.05 vs the rWt-Con group; #p < 0.05 vs the rDb-Con group; scale bar=20 μm; n=4-5/group.

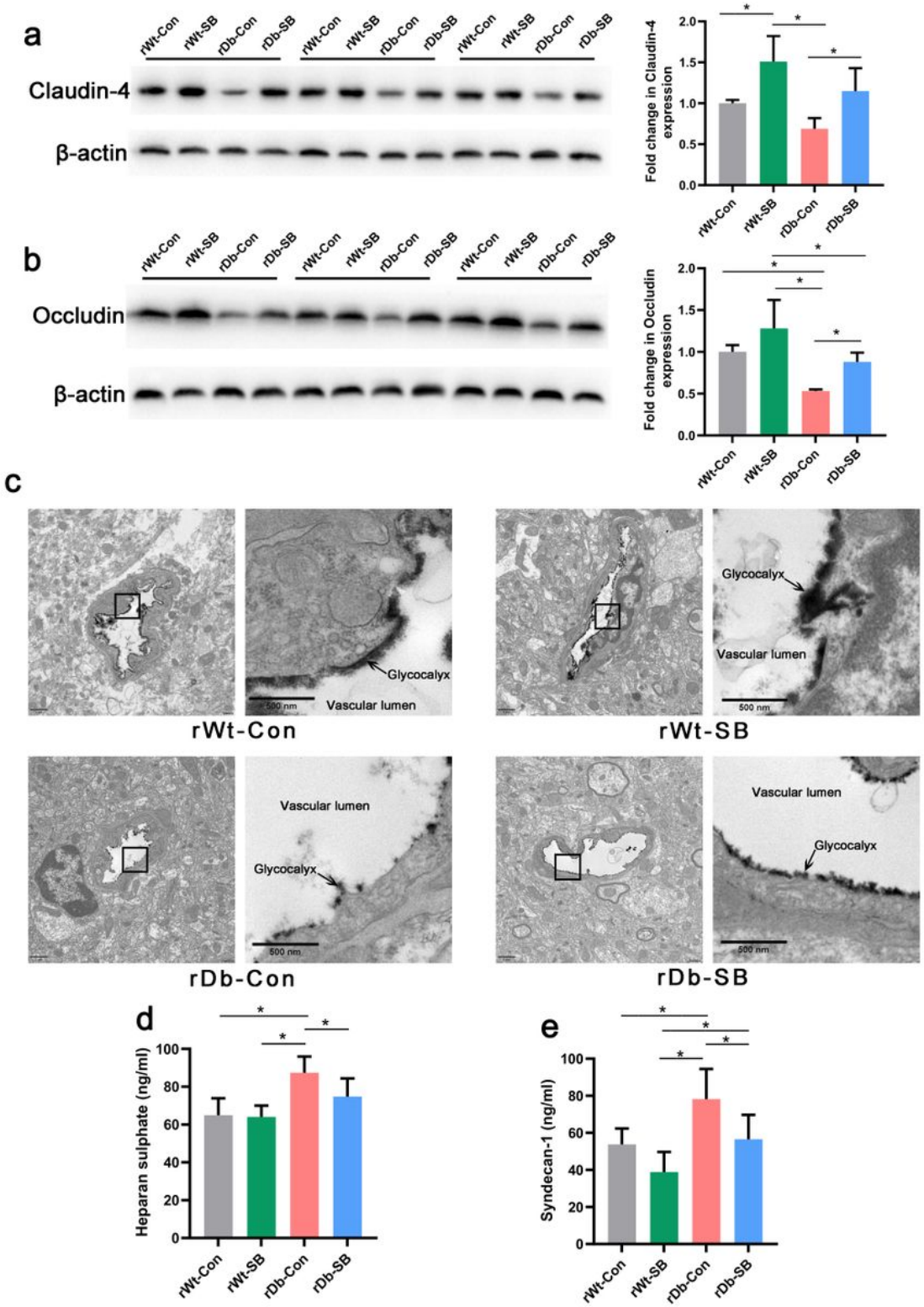

Figure 6

Tight junction protein expression and electron microscopy images of the cerebral capillary endothelial glycocalyx. Representative western blot pictures and normalized value of Claudin-4 (a) and Occludin (b) expression are shown. (c) Electron microscopy images of the cerebral capillary glycocalyx (scale bar=1 μm) and more detailed images of the cerebral capillary glycocalyx (scale bar=500 nm). Measurements of the components of the glycocalyx in plasma. (d) Syndecan-1 levels; (e) HS levels. The data are expressed as the mean±SD; * $p<0.05$; n=3-5/group.

Supplementary Files

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