NG2-Glia Cause Diabetic Blood-Brain Barrier Disruption by Secreting MMP-9

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**Highlights**

- Increased number of NG2-glia in the hippocampus of db/db mice.
- Abnormal structure and function of the hippocampal BBB of db/db mice.
- Activation of Wnt/β-catenin signaling in NG2-glia in the hippocampus of db/db mice.
- Increased secretion of MMP-9 by NG2-glia in the hippocampus of db/db mice.
- Elevated MMP-9 levels are associated with the activation of Wnt/β-catenin signaling.

**Conclusion**

NG2-glia can disrupt the blood-brain barrier in diabetic mice by activating Wnt/β-catenin signaling, upregulating MMP-9, and degrading tight junction proteins.

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Background: Disorders of the blood-brain barrier (BBB) arising from diabetes mellitus are closely related to diabetic encephalopathy. Previous research has suggested that neuron-glia antigen 2 (NG2)-glia plays a key role in maintaining the integrity of the BBB. However, the mechanism by which NG2-glia regulates the diabetic BBB remains unclear.

Methods: Type 2 diabetes mellitus (T2DM) db/db mice and db/m mice were used. Evans-Blue BBB permeability tests and transmission electron microscopy techniques were applied. Tight junction proteins were assessed by immunofluorescence and transmission electron microscopy. NG2-glia number and signaling pathways were evaluated by immunofluorescence. Detection of matrix metalloproteinase-9 (MMP-9) in serum was performed using enzyme-linked immunosorbent assay (ELISA).

Results: In T2DM db/db mice, BBB permeability in the hippocampus significantly increased from 16 weeks of age, and the structure of tight junction proteins changed. The number of NG2-glia in the hippocampus of db/db mice increased around microvessels from 12 weeks of age. Concurrently, the expression of MMP-9 increased in the hippocampus with no change in serum. Sixteen-week-old db/db mice showed activation of the Wnt/β-catenin signaling in hippocampal NG2-glia. Treatment with XAV-939 improved structural and functional changes in the hippocampal BBB and reduced MMP-9 secretion by hippocampal NG2-glia in db/db mice. It was also found that the upregulation of β-catenin protein in NG2-glia in the hippocampus of 16-week-old db/db mice was significantly alleviated by treatment with XAV-939.

Conclusion: The results indicate that NG2-glia can lead to structural and functional disruption of the diabetic BBB by activating Wnt/β-catenin signaling, upregulating MMP-9, and degrading tight junction proteins.

Keywords: Astrocytes; Blood-brain barrier; Diabetes mellitus; Endothelial cells; Oligodendrocyte precursor cells; Oligodendroglia; Pericytes

INTRODUCTION

Diabetes is a metabolic disorder characterized by an increasing incidence year after year, exhibiting a notable correlation with the emergence of numerous nervous system diseases such as vascular dementia, cerebrovascular disease, anxiety, and depression. The underlying mechanism may be associated with the abnormal structure and function of the blood-brain barrier (BBB) due to pathological remodeling of brain microvessels caused by diabetes [1]. Diabetic encephalopathy is commonly understood to be a condition marked by structural and functional abnormalities of the central nervous system as a result of diabetes, with the most characteristic symptoms being impaired learning capabilities and memory loss [2]. The hippocampus is the primary region affected by BBB damage in type 2 diabetes mellitus (T2DM) and is also a crucial area for regulating cognitive functions in the brain [3-5]. A prominent feature of diabetic encephalopathy is the structural and functional abnormalities of the BBB resulting from the downregulation of tight junction proteins, which in turn lead to variations in the BBB’s transport function for essential substances such as glucose, amino acids, choline, sodium, potassium, ketone bodies,
and insulin [6]. The BBB is a unique structure composed of vascular endothelial cells, pericytes, astrocytes, and others. It plays a critical role in restricting the efflux of plasma components, maintaining cerebral homeostasis, regulating the transport of materials, and participating in immune responses [7]. A pivotal element concerning the BBB’s permeability is the tight junction, which not only underpins the integrity of the BBB structure but is also essential for controlling the passage of hydrophilic molecules [8]. Tight junctions consist of unbroken transmembrane proteins such as occludins, zona occludens-1 (zo-1), claudins, endothelial cell-selective adhesion molecules, and junctional adhesion molecules [8]. Although various central lesions may trigger BBB damage through numerous mechanisms, research suggests that the downregulation of tight junction proteins expression can lead to increased BBB permeability in a range of central nervous system lesions [9]. BBB integrity is significantly compromised in various diabetes models, including in T2DM patients [10], rhesus monkeys [11], and mice [12]. Diabetic hyperglycemia can cause pathological changes such as the pathological proliferation of endothelial cells, a reduction in tight junction proteins [13], swelling of astrocyte pseudopodia [14], and decreased coverage by pericytes [15]. Studies on human diabetes have shown that the downregulation of the tight junction proteins occludin and zo-1 correlates with increased expression of matrix metalloproteinase-9 (MMP-9) [16]. Inhibiting MMP-9 in a diabetic mouse model prevented the increase in BBB permeability triggered by streptozotocin in type 1 diabetes mellitus (T1DM) mice [17].

Neuron-glia antigen 2 (NG2)-glia, also known as oligodendrocyte precursor cells, are capable of differentiating into oligodendrocytes, thereby forming myelin structures that envelop axons and are crucial for supporting bioelectrical signaling and preserving the normal function of nerve cells [18]. Emerging evidence suggests that NG2-glia are significantly associated with BBB component cells (e.g., endothelial cells, pericytes, and astrocytes), playing a vital role in maintaining the BBB’s structural and functional integrity [5,19-24]. NG2-glia can proliferate and migrate to adjacent injured areas after a nerve injury and respond to a diverse array of pathological injuries [25-27]. In the adult brain, an increase in NG2-glia following injury and disease is often thought to preserve BBB homeostasis and mediate the long-term repair of white matter [28]. However, in white matter lesions caused by cerebral underperfusion, NG2-glia can disrupt the integrity of the BBB by secreting MMP-9 during the early stages of BBB breakdown [29]. NG2-glia can accumulate around blood vessels in acute lesions of multiple sclerosis, thus contributing to BBB damage by disrupting the tight junctional integrity of astrocyte pseudopods and endothelial cells [30]. In summary, NG2-glia are critically important in maintaining the structural and functional integrity of the BBB under normal conditions, yet they also play a role in BBB disruption in acute lesions (e.g., cerebral hypoperfusion and multiple sclerosis), indicating that NG2-glia have varied roles in BBB injury and white matter lesions associated with different diseases.

Existing research has suggested that MMPs play a role in BBB destruction in a variety of diseases, including diabetes, cerebral ischemia, and multiple sclerosis [27]. Studies have shown that MMP-9 degrades components of both tight junction proteins and the extracellular matrix, including laminin, collagen, and fibronectin [31]. It has been reported that activation of the Wnt/β-catenin signaling pathway in target cells is significantly correlated with diabetic nephropathy and diabetic cardiomyopathy in peripheral lesions induced by diabetic mice. Activation of the Wnt/β-catenin signaling pathway triggers symptoms of diabetic nephropathy (e.g., proteinuria, renal insufficiency, and dyslipidemia) in glomerular foot cells of T1DM and T2DM mice [32], and this mechanism is likely correlated with increased secretion of MMPs, the downstream targets of the Wnt/β-catenin signaling pathway [33]. Diabetes-induced myocardial fibrosis is correlated with the activation of the Wnt/β-catenin signaling pathway, and treatment with the Wnt/β-catenin signaling pathway inhibitor XAV-939 notably reduces myocardial hypertrophy and fibrosis and improves cardiac function [34,35]. MMP-9 is a clear target of the Wnt/β-catenin signaling pathway [36]. It is found that the Wnt/β-catenin signaling pathway is activated in target cells in various diseases such as tumors, inflammation, and diabetic nephropathy [32,37,38]. Upon activation of the Wnt/β-catenin signaling pathway, β-catenin protein accumulates in the cytoplasm and then translocates to the nucleus. Once in the nucleus, the β-catenin protein can form the β-catenin/T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) transcription complex, which can directly bind to the MMP-9 promoter. This process can promote the transcription, translation, and expression of MMP-9, which is involved in disease onset and progression [39,40].

The aim of this study was to investigate whether NG2-glia play a destructive role in BBB injury in db/db mice and to explore the related molecular mechanisms. The findings revealed that the Wnt/β-catenin signaling pathway was activated in hip-
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pocampal NG2-glia in db/db mice, which upregulated the expression of MMP-9 and ultimately led to the degradation of tight junction proteins, causing structural and functional disruption of the diabetic BBB. Overall, these findings contribute new insights into the mechanism of diabetes-induced BBB damage and provide new targets for drug development to combat diabetes-induced cognitive impairment.

METHODS

Experimental animals
The experimental animals were derived from db/m mice of a C57BL/6J background, sourced from the Jackson Laboratory (Bar Harbor, ME, USA). Both db/db and db/m mice were bred and maintained at the Experimental Animal Center of the Second Affiliated Hospital of the Army Medical University. For the experimental T2DM mouse model, a fasting blood glucose level of >16.7 mmol/L was used as a criterion. All mice were housed in specific pathogen free conditions with a 12-hour light/dark cycle and ad libitum access to food and water. Laboratory conditions were kept constant at a temperature of 22°C±2°C and a relative humidity of 60%±5%. The experimental protocols and procedures complied with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and were initiated after receiving approval from the Experimental Animal Welfare Ethics Review Committee of the Army Medical University (Approval No. AMUWEC2019359).

Experimental grouping and drug administration
The mice were randomly selected for all experimental groups. To assess the trends in blood glucose and body weight, fasting blood glucose levels and body weights were measured in db/db mice and compared with db/m mice at corresponding ages. For BBB structure and function assessments: four db/m mice and four db/db mice were randomly chosen at ages of 4th, 8th, 12th, 14th, 15th, and 16th week. The Evans-Blue assay was used to examine BBB permeability in db/db mice. Based on the experimental outcomes, four db/m mice and four db/db mice at the age of 16th week were randomly chosen to examine the BBB ultrastructure in the hippocampal region using transmission electron microscopy. To investigate changes in NG2-glia numbers: four db/m mice and four db/db mice at ages of 4th, 8th, 12th, and 16th week were utilized to examine NG2-glia number and distribution in the hippocampus using immunofluorescence. For the study of NG2-glia involvement in BBB injury, eight db/m mice and 16 db/db mice at 12 weeks of age were randomly divided into three groups (eight mice each): a physiological saline+db/m group, a saline+db/db group, and an XAV-939+db/db group. Saline and XAV-939 (10 mg/kg) were administered intraperitoneally in proportion to body weight daily starting from the 12th week for 4 weeks until the 16th week. The selection of the XAV-939 dose and dosing regimen was based on published studies [41]. After 4 weeks of saline and XAV-939 treatment, the experimental animals were further divided into two subgroups of four for BBB permeability assays and immunofluorescence staining experiments.

Blood glucose testing
Fasting blood glucose levels were measured at a fixed time from blood samples taken from the tail vein. Fasting commenced at 8:00 PM the day before the test, with bedding changed beforehand. Testing began at 8:00 AM the following morning. Once 12 hours of fasting were completed, blood was collected by tail-tip clipping. The first drop of blood was discarded, and the fasting blood glucose level was determined with the second drop of blood using an steady blood glucose meter-1 (BGMS-1) (with a measuring range of 2.2 to 27.8 mmol/L) made by Sano Bio (Changsha, China).

Daily diet and water intake monitoring
The mass of the mice’s water bottles was weighed at regular intervals throughout the day to calculate each animal’s daily water intake. Similarly, the mass of the mouse feed was weighed at regular intervals each day to determine the daily diet per animal. Water intake was calculated as the difference in the water bottle mass from the previous day to the current day, divided by the number of mice (n=5). The amount of feed was calculated as the difference in the mass of feed from the previous day to the current day, divided by the number of mice (n=5).

Evens-blue BBB permeability test
BBB permeability was investigated through the Evans-Blue dye leakage assay (G1810, Solarbio, Beijing, China). Mice were injected with 0.5% Evans-Blue dye iv in the tail at 4 mg/kg. After 1-hour Evans-Blue injection was completed, the mice were anesthetized with 1% pentobarbital sodium (40 mg/kg) and then perfused with pre-cooled phosphate buffered saline (PBS). Next, the brain tissues were quickly separated, and hippocampus was peeled off under the colorless discharged liquid. After
weighing, the tissues were homogenized into 1 mL PBS and then centrifuged (15,000 rpm, 20 minutes). When the centrifugation process was completed, the supernatant was acquired, and an equal volume of trichloroacetic acid (50%) was introduced and incubated throughout the night at 4°C and then centrifuged again (15,000 rpm, 20 minutes). The absorbance of Evens-Blue dye at 620 nm was examined using a spectrophotometer (Multiskan FC microplate reader, Thermo Scientific, Waltham, MA, USA) and quantified in accordance with the standard curve. The content of Evens-Blue dye in the brain was expressed as μg/g wet weight.

Transmission electron microscopy examination
After anesthetizing mice with 1% sodium pentobarbital (40 mg/kg), the heart was perfused with pre-cooled PBS buffer. When the outflowing liquid became colorless, 0.1% glutaraldehyde-4% paraformaldehyde (PFA) fixing solution was perfused. After perfusion fixation, the hippocampal tissue was quickly dissected, and samples measuring 1 mm in length, 1 mm in width, and 1 mm in thickness were cut with a surgical knife and fixed with 2.5% glutaraldehyde. All samples were sent to the Electron Microscopy Laboratory of the Second Affiliated Hospital of the Army Medical University to prepare ultra-thin sections. The BBB ultrastructure was identified using a transmission electron microscope (TEM system, Hitachi, Tokyo, Japan).

Immunofluorescence experiment
The mice were anesthetized with 1% sodium pentobarbital (40 mg/kg). Before the immunofluorescence sections were made, blood was collected from the cardiac of db/m mice and db/db mice at 4th, 8th, 12th, and 16th week of age and immediately subjected to heart perfusion, fixation, and dehydration. First, the mice were perfused with pre-cooled PBS for 5 minutes and then with 4% PFA for 20 minutes. After the perfusion fixation was completed, brain tissue was dissected and then fixed with 4% PFA (4°C) for 12 hours. After the fixation process, the brain tissue was placed in 10%, 20%, and 30% sucrose (4°C) for dehydration for 12 hours respectively. Next, the tissue was frozen and then cut into 25 μm sections on a cryostat to perform immunofluorescence experiments. The sections were cleaned three times with PBS for 5 minutes each time. Afterward, the sections were incubated in the blocking solution (supplemented with 0.3% Triton X-100 and 10% bovine serum albumin) at ambient temperature (25°C) for 30 minutes. After removing the serum, the sections were incubated with primary antibody throughout the night at 4°C. The sections were then rinsed three times with PBS for 5 minutes each time, and incubated with fluorescent secondary antibody (diluted 1:400) under dark conditions in a humidified chamber for 1 hour at ambient temperature. Subsequently, the sections were rinsed three times with PBS for 5 minutes each time. Lastly, the sections were mounted with anti-fluorescence quenching sealing solution containing 4’,6-diamidino-2-phenylindole (DAPI). The immunofluorescence results were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) by randomly selecting images. The sections were identified using a fluorescence microscope (TSC STED, Olympus, Tokyo, Japan).

The antibodies employed are presented as follows: platelet-derived growth factor receptor α (PDGFRα; 1:400, rabbit, ab203491, Abcam, Cambridge, UK), CD31 (1:200, mouse, MA1-26196, Invitrogen, Carlsbad, CA, USA), CD31 (1:200, mouse, 3528S, Cell Signaling Technology, Danvers, MA, USA), β-catenin (1:100, mouse, 13-8400, Invitrogen), zo-1 (1:100, rabbit, 40-2200, Invitrogen), as well as MMP-9 (1:200, mouse, sc-13520, Santa Cruz, Santa Cruz, CA, USA).

ELISA kit detection
Blood was collected from mice at the ages of 4th, 8th, 12th, and 16th week based on a cardiac puncture method prior to the immunofluorescence cryosection experiment. After blood collection, the samples were rapidly centrifuged (20 minutes, 1,500 rpm), and the supernatant was acquired, and incubated with fluorescent secondary antibody (diluted 1:400) under dark conditions in a humidified chamber for 1 hour at ambient temperature. The supernatant was acquired, and incubated with fluorescent secondary antibody (diluted 1:400) under dark conditions in a humidified chamber for 1 hour at ambient temperature. The significance among multiple groups was determined through one-way analysis of variance (ANOVA). The probability of \( P < 0.05 \) indicated a difference with statistical significance. The analyzed charts conventionally represent the statistical results, where \( P < 0.05 \) and \( P < 0.01 \) indicated levels of significance. Error bars had the expression of mean ± standard error of the mean. Sample sizes for all experiments were obtained in accordance with standard practices and expertise extensively employed in the field: seven mice per group for fasting blood glucose and weight detection, and four exper-
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RESULTS

Biological characterization of db/db mice and db/m mice

The blood glucose, body weight, and dietary water intake metabolism variations in db/db mice and db/m mice were examined starting from the 4th week of age, with measurements taken every 2 weeks till the 18th week of age. Results showed that in contrast to identically aged db/m mice, db/db mice had notably higher fasting blood glucose levels starting from the 6th week of age ($P<0.01$). Obvious symptoms of polydipsia and polyphagia appeared at the 8th week of age (Fig. 1C and D), and high blood glucose levels persisted starting from the 10th week of age (Fig. 1A). Starting from the 8th week of age, db/db mice showed a significant increase in body weight ($P<0.01$), with body weights exceeding 40 g for db/db mice above 10th week of age (Fig. 1B).

Abnormal BBB function and structure in the hippocampus of db/db mice

After 0.5% Evens-Blue dye (4 mL/kg) was intravenously injected via the tail vein, the absorbance values of Evens-Blue were examined at 620 nm with a spectrophotometer after 1 hour. The permeability of the hippocampal BBB in db/db mice and db/m mice at several ages (i.e., 4th, 8th, 12th, and 16th week) was examined through Evens-Blue BBB permeability detection. In contrast to identically aged db/m mice, the permeability of the hippocampal BBB in db/db mice aged the 16th week was notably increased, about three times higher than that in db/m mice at the identical age ($P<0.01$) (Fig. 2A). To determine the onset time of increased BBB permeability in db/db mice, Evens-Blue BBB permeability detection was performed on db/db mice and db/m mice of 14 and 15th week of age, respectively. No remarkable difference was reported when in contrast to identically aged db/m mice ($P>0.05$) (Fig. 2A).

The research method of Hayden and Banks [42] was adopted

Fig. 1. Fasting blood glucose levels and weight change trends of db/db mice and db/m mice. (A) The trend of variations in fasting blood glucose levels of db/db mice and db/m mice, as well as the comparison of fasting blood glucose levels between db/db mice and db/m mice at the same age. (B) The trend of variations in body weight of db/db mice and db/m mice, as well as the comparison of body weight between db/db mice and db/m mice at the same age. (C, D) The trend of variations in daily diet and water intake of db/db mice and db/m mice, as well as the comparison of daily diet and water intake between db/db mice and db/m mice at the same age. $^aP<0.01$. 
Fig. 2. Blood-brain barrier (BBB) structural and functional abnormalities in db/db mice. (A) Trend of variations in db/m and db/db mice’s BBB permeability. In contrast to db/m mice of the identical age, db/db mice aged the 16th week achieved notably improved BBB permeability. (B) Structural remodeling of perivascular cells around the microvasculature (BBB) in db/db mice. (a, b) Panels present the distribution of astrocytes around the microvasculature. (a) Panel presents considerable electron density-lower astrocytes (yellow pseudocolor labeling) surrounding the microvasculature in db/m mice. (b) Panel presents activated microglia cells (aMGC) extending “pseudopodia” invading into the gap between the astrocytes and the microvessels (blue pseudocolor labeling), such that the astrocytes shrink and detach from the microvascular basement membrane in db/db mice. (c, d) Panels present astrocyte-microvessel relationships under a magnification of 8,000 times. (c) Panel shows that the pseudopodia of astrocytes surrounding the microvasculature of db/m mice are tightly adhered to the outer basal membrane of the microvasculature (note the structure within the white dashed border). (d) Panel shows that the astrocytes around the microvasculature of db/db mice have retracted and separated from the microvascular basement membrane: ×2,000, scale bar=5 μm (a, b); ×8,000, scale bar=1 μm (c, d). (C) Structural remodeling of endothelial cells and tight junctions around the microvasculature in db/db mice. (a, b) Panels indicate the distribution of astrocytes surrounding the microvasculature. (c, d) Panels indicate magnified images of panels (a, b), suggesting the tight junctions (yellow arrows) and abnormal mitochondria (red pseudocolor labeling) between endothelial cells under a magnification of 8,000 times. The gap between the tight junctions of endothelial cells was widened, with a notable decrease in electron density and even discontinuity or absence in some areas (red arrows). Notably, the number of abnormally shaped mitochondria (aMt) increased notably in endothelial cells and perivascular cells that make up the BBB in db/db mice. Abnormal mitochondria are characterized by a significant decrease in electron density of mitochondrial matrices and varying degrees of loss of mitochondrial cristae. (e, f) Panels show endothelial cell remodeling. The endothelial cells making up the BBB were swollen in db/db mice, and some endothelial cells exhibited low-density shadowy vesicles with “phagocytic bubbles” (note the structure in the white dashed border). As indicated by the result, large nucleus endothelial cells were abnormal (marked in red font): ×2,000, scale bar=5 μm (a, b); ×8,000, scale bar=1 μm (c, d); ×4,000, scale bar=2 μm (e, f). (D) Comparison of the number of aMt in BBB constitutive cells of db/db mice and db/m mice. (E) Comparison of astrocyte-basement membrane contact area around the BBB in db/db mice and db/m mice. NVU, neurovascular unit; CL, capillary; N, nucleus; AC, astrocyte; PCN, pericellular nucleus; TJ, tight junction; PC, pericellular; EC, endothelial cell. *P<0.01.
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to observe the ultrastructure of the hippocampal BBB in db/db mice aged the 16th week through transmission electron microscopy to observe whether BBB structure was damaged when BBB permeability was improved in db/db mice. In contrast to identically aged db/m mice, abnormal hippocampal BBB structures were identified in db/db mice, where activated microglia appeared around the BBB and infiltrated the microblood vessels, thus destroying the tight structure formed by the pseudopodia of astrocytes and the microvascular basement membrane (Fig. 2Ba and b). Under high magnification, the astrocytes around the microblood vessels of db/db mice had retracted and moved away from the microvascular basement membrane (Fig. 2Bd, Cb, and E), which may be one of the reasons for the increased BBB permeability. The tight junction gaps be-

Fig. 3. Elevated number of neuron-glia antigen 2 (NG2)-glia within db/db mice’s hippocampus and their aggregation around blood-brain barrier (BBB). (A) Immunofluorescence staining of NG2-glia. In contrast to db/m mice at the identical age, NG2-glia’s number within db/db mice’s hippocampus increased notably from 12th week of age. Images were obtained at 40× magnification; scale bar, 20 µm. Images were obtained at 20× magnification; scale bar, 50 µm. Green platelet-derived growth factor receptor α (PDGFRα) labels NG2-glia, and blue 4’,6-diamidino-2-phenylindole (DAPI) labels cell nuclei. (B) Trend of variations in NG2-glia’s number within db/m mice’s and db/db mice’s hippocampus, as well as the comparison of NG2-glia’s number within db/m mice’s and db/db mice’s hippocampus at the same age. At 12th week of age, NG2-glia’s number within db/db mice’s hippocampus was notably increased. (C) Immunofluorescence double-staining of NG2-glia with endothelial cells (CD31). Images (c, d) are enlarged views of (a, b), respectively. Images (a, c, e) show the distribution of NG2-glia around the hippocampal BBB in db/m mice aged the 16th week. Images (d, f, g) show the elevated number of NG2-glia around the hippocampal BBB in db/db mice aged the 16th week, and the formation of cell clusters locally around the microvasculature. (g) Microvessels and NG2-glia were clearly labeled using double arrows and textual descriptions. The boxed area is the BBB. Images were obtained at 40× magnification; scale bar, 20 µm. Images were obtained at 20× magnification; scale bar, 50 µm. Green PDGFRα-labeled NG2-glia, blue DAPI-labeled nuclei, red represents CD31-labeled BBB endothelial cells. *P<0.01.
between endothelial cells widened, with a significant decrease in electron density at the tight junctions, and some areas had continuous interruption or even absence (Fig. 2Cd), as indicated by the observation of brain microvessels. Moreover, swollen endothelial cells were reported in several microblood vessels with “bubble-like” low-density shadows inside, as well as endothelial cells with giant nuclei (Fig. 2Cf). The above-mentioned abnormal remodeling of endothelial cells and tight junction proteins within db/db mice’s hippocampus are important causes of increased BBB permeability. Notably, a significant increase in the number of “vacuolated” abnormal mitochondria was found in endothelial cells and pericytes, which are important constituent cells of the BBB in db/db mice (Fig. 2Cd and D). As indicated by the above experimental results, db/db mice showed increased BBB permeability at 16th week of age, while astrocytes and microglia around the microvasculature were remodeled morphologically. Endothelial cells and tight junctions, i.e., important and vital structures of the BBB, showed different degrees of damage and remodeling.

Variations in hippocampal NG2-glia in db/db mice

Immunofluorescence experiments were performed to observe the variations in NG2-glia’s number (PDGFRα markers) within db/m mice’s and db/db mice’s hippocampus at the ages of 4th, 8th, 12th, and 16th week. As indicated by the result, NG2-glia’s number within db/m mice’s and db/db mice’s hippocampus varied dynamically with age. In contrast to db/db mice and db/m mice at 4th week of age, NG2-glia’s number within db/m mice’s and db/db mice’s hippocampus at 8th week of age slightly decreased. At 12th week of age, NG2-glia’s number was not notably increased in db/m mice’s hippocampus, while NG2-glia’s number within db/db mice’s hippocampus notably increased (P<0.01) (Fig. 3A and B). As BBB permeability was notably increased in db/db mice aged the 16th week (Fig. 4A and B). In the double immunofluorescence staining experiment of NG2-glia and β-catenin protein, the proportion of NG2-glia expressing β-catenin protein in the hippocampus of db/db mice aged the 16th week was notably higher than that in the same age db/m mice. After XAV-939 was employed, a Wnt/β-catenin signaling pathway inhibitor able to degrade β-catenin protein, the proportion of NG2-glia expressing β-catenin protein in the hippocampus of db/db mice aged the 16th week decreased notably (Fig. 4C and D). In conclusion, in the hippocampus of db/db mice aged the 16th week, the expression of β-catenin protein in NG2-glia was upregulated, and β-catenin protein was translocated from the cytoplasm to the nucleus. After treatment with XAV-939, db/db mice aged the 16th week achieved the notably alleviated upregulation of β-catenin protein in NG2-glia in the hippocampus. The above results indicate that the signaling pathway of Wnt/β-catenin was activated in NG2-glia within db/db mice’s hippocampus.

XAV-939 ameliorates structural and functional variations in db/db mice’s hippocampal BBB

We assessed whether BBB function was abnormal by Evans-Blue BBB permeability assay. Zo-1, an important component of tight junction proteins [43], is normally expressed on blood vessels. We found that zo-1 and CD31-labeled microvessels were morphologically similar by zo-1 and CD31 immunofluorescence co-localization experiments. We assessed changes in tight junction proteins expression by the percentage of zo-1 area co-localized with CD31 as an indicator of whether the
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BBB structure was damaged. The physiological saline+db/m mice group, physiological saline+db/db mice group, and XAV-939+db/db mice group were subjected to Evens-Blue BBB permeability test to determine the variations in BBB permeability. The results showed that at 16th week of age, db/db mice’s BBB permeability was notably higher than that of identically aged db/m mice (P<0.01). Compared with db/db mice without XAV-939 treatment, db/db mice aged the 16th week had significantly lower BBB permeability after completion of XAV-939 treatment (P<0.01) (Fig. 5A). In addition, zo-1 and CD31 immunofluorescence co-localization semi-quantitative analysis revealed that at 16th week of age, the expression level of zo-1 within db/db mice’s hippocampus was notably reduced in contrast to identically aged db/m mice (Fig. 5B and C). In contrast to identically aged db/db mice, treatment with XAV-939 increased the expression level of zo-1 in the hippocampus of db/db mice aged 16th week.

Fig. 4. Co-localization of neuron-glia antigen 2 (NG2)-glia and β-catenin protein immunofluorescence. (A, B) Immunofluorescence staining images of β-catenin protein show that in contrast to age-matched db/m mice, the overlap between β-catenin protein and nuclei in the hippocampus of db/db mice aged the 16th week was notably increased (P<0.01), suggesting that the β-catenin protein was translocated into the nucleus. Red indicates β-catenin, blue indicates 4’,6-diamidino-2-phenylindole (DAPI)-labeled nuclei. Images were obtained at 40× magnification; scale bar, 20 µm. (C) Double-stained image of NG2-glia with β-catenin protein immunofluorescence. Green platelet-derived growth factor receptor α (PDGFRα) labels NG2-glia, red β-catenin labels β-catenin protein, and blue DAPI labels the nucleus. Images were obtained at 40× magnification; scale bar, 20 µm. (D) Immunofluorescence co-localization analysis of NG2-glia and β-catenin protein in saline+db/m mice group, saline+db/db mice group, and XAV-939+db/db mice group. The results showed increased expression of β-catenin protein in NG2-glia in the hippocampus of db/db mice aged the 16th week, and the use of XAV-939 inhibitor notably alleviated the upregulation of β-catenin protein in NG2-glia in the hippocampus of db/db mice aged the 16th week. One-way analysis of variance (ANOVA) was employed for the analysis of data. DG, dentate gyrus; CA1, hippocampal subregion. In contrast to db/m, *P<0.01; in contrast to db/db, †P<0.01, n=4.
XAV-939 reduces MMP-9 secretion from NG2-glia in the hippocampus of db/db mice

MMP-9 is a direct target protein of the signaling pathway of Wnt/β-catenin, and its secretion can be promoted after pathway activation [44]. Through co-localization experiments of NG2-glia and MMP-9 using immunofluorescence, the proportion of NG2-glia co-localizing with MMP-9 was analyzed to determine whether NG2-glia that secreted MMP-9 showed an elevated number. The area of MMP-9 co-localizing with NG2-glia was also analyzed to determine whether the expression of MMP-9 increased. In contrast to identically aged db/m mice, at 16th week of age, the area of MMP-9 co-localizing with NG2-glia within db/db mice's hippocampus was notably increased (P<0.01) (Fig. 6B), and NG2-glia's number under the co-localization with MMP-9 was also notably increased (P<0.01) (Fig. 6C). After treatment with XAV-939, the area of MMP-9 co-localizing with NG2-glia and NG2-glia's number under the co-localization with MMP-9 notably decreased in the hippocampus of db/db mice aged the 16th week. As revealed by the correlation analysis, NG2-glia's number under the co-localization with MMP-9 was linearly correlated with the area of MMP-9 co-localizing with NG2-glia (Fig. 6D). The MMP-9 content in the blood of db/db and db/m mice were examined at weeks 4, 8, 12, and 16, and it was found that there was no significant increase in MMP-9 content in the blood of db/db mice at week 16 (Fig. 6E). The above experimental results suggest that upregulated MMP-9 expression in the hippocampus of db/db mice aged the 16th week is highly correlated with elevat-
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Fig. 6. Co-localization of neuron-glia antigen 2 (NG2)-glia and matrix metalloproteinase-9 (MMP-9) immunofluorescence. (A) Double-staining image of NG2-glia and MMP-9 immunofluorescence shows that in contrast to age-matched db/m mice, db/db mice aged the 16th week had more NG2-glia secreting MMP-9, and the area co-localized with NG2 cells was increased. The area of MMP-9 co-localized with NG2-glia was notably reduced in XAV-939-administrated db/db mice in contrast to db/db mice without any treatment. Images were obtained at 40× magnification; scale bar, 20 μm. Green denotes platelet-derived growth factor receptor α (PDGFRα)-labeled NG2-glia, red denotes MMP-9, blue expresses 4′,6-diamidino-2-phenylindole (DAPI)-labeled nuclei. (B, C) Double-staining analysis of NG2-glia and MMP-9 shows that the percentage of MMP-9 co-localized with NG2-glia in the hippocampus of db/db mice aged the 16th week was notably increased in contrast to age-matched db/m mice. NG2-glia’s number under the co-localization with MMP-9 in the hippocampus of db/db mice aged the 16th week was also notably increased in contrast to age-matched db/m mice. (D) The area of MMP-9 co-localized with NG2-glia in the hippocampus of db/db mice aged the 16th week showed a notable correlation with NG2-glia’s number under the co-localization with MMP-9 ($R^2=0.6232$). (E) Detection of MMP-9 content in the blood using an enzyme-linked immunosorbent assay (ELISA) kit. The MMP-9 content in the blood of db/db mice was not notably increased at 16th week of age in contrast to that of age-matched db/m mice, suggesting that the increase in MMP-9 expression within db/db mice’s hippocampus was not related to the amount of MMP-9 in the blood. DG, dentate gyrus; CA1, hippocampal subregion. *vs. db/m: $P<0.01$, †vs. db/db: $P<0.01$, $n=4$, one-way analysis of variance (ANOVA) analysis.
ed numbers of NG2-glia.

DISCUSSION

Diabetes has been established as a prevalent chronic metabolic disease in people's daily lives. For pathological research on diabetes, the selection of appropriate animal models is critical for exploring the pathogenesis of diabetes-related complications. The db/db mice, a spontaneous T2DM animal model, present a defect in the leptin receptor gene, akin to T2DM in humans. Consequently, they are extensively utilized in research on the pathological mechanisms of T2DM. BBB structural and functional damage has been documented in vitro and in vivo under diabetic conditions [1]. Diabetes leads to BBB damage associated with hyperglycemia, chronic inflammation, and oxidative stress [45], while diabetes-associated hypertension, hyperlipidemia, and insulin resistance all trigger BBB structural and functional impairment [46]. As indicated by the comparison of various diabetes animal models, the location and timing of BBB damage differ across these models [1,47-50]. The hippocampus, a central region for memory processing, has been confirmed as a common site of BBB injury in various T2DM animals, and its structural and functional abnormalities are capable of triggering memory loss and cognitive dysfunction associated with diabetic encephalopathy [4]. Accordingly, we focused on the hippocampus of db/db mice, a T2DM model, to observe the structural and functional changes in the hippocampal BBB and the variations of NG2-glia in diabetic pathological injury. As indicated by the results in different diabetic model animals, the timing of BBB injury varied. In streptozotocin-induced T1DM, BBB injury usually occurs 4 weeks after drug induction [6]. However, the timing of BBB injury in T2DM db/db mice is controversial. Several studies have shown that BBB damage in db/db mice typically occurs around 16th week [51,52], consistent with our research findings. Nevertheless, there are also reports of BBB damage in db/db mice occurring before 16th week [12,53]. The discrepancies in research on the timing of BBB damage in db/db mice in the literature may be attributed to a variety of factors, such as experimental animal background, feeding conditions, tracer species and dose differences, as well as the use of different instruments and detection methods for assessing BBB structure and function.

Extensive research indicates that activated NG2-glia play a role in the pathological repair of various injuries, and that proliferation or migration of NG2-glia is a protective response to pathological injury. In contrast, in acute lesions (e.g., cerebral hypoperfusion and multiple sclerosis) [27,29], NG2-glia contribute to BBB destruction, suggesting that NG2-glia play different roles in BBB damage and white matter lesions caused by various diseases. Our research supports this view. Our findings demonstrate that BBB structural and functional disorders in db/db mice generally occur around the 16th week of age (Fig. 2). Immunofluorescence double-staining of NG2-glia and endothelial cells showed that significant clusters of NG2-glia accumulated around the BBB in db/db mice at 16 weeks of age (Fig. 3), accompanied by an increase in MMP-9 expression levels in the hippocampus, while plasma MMP-9 levels remained normal (Fig. 6). To verify whether the upregulated MMP-9 in the hippocampus of db/db mice is secreted by NG2-glia, we performed immunofluorescence co-localization analysis of NG2-glia with MMP-9 and the MMP-9 co-localization area. Our correlation analysis revealed a strong association between the two (Fig. 6D), suggesting that the heightened expression of MMP-9 in the hippocampus of db/db mice is related to the increased number of NG2-glia. This rise in NG2-glia and MMP-9 within the hippocampus of db/db mice may contribute to the impairment of BBB structure and function.

To further investigate the mechanism behind MMP-9 secretion by NG2-glia in diabetes, we examined the closely associated Wnt/β-catenin signaling pathway in NG2-glia. It is well known that the Wnt/β-catenin signaling pathway is critically important for the survival and proliferation of human embryonic stem cells. In the central nervous system, this signaling pathway influences the proliferation and differentiation of various progenitor cells, including NG2-glia. The Wnt/β-catenin pathway also serves as an important regulatory factor for NG2-glia differentiation and myelin formation [54]. Our study revealed that both the number of NG2-glia and MMP-9 expression increased following the onset of BBB injury in db/db mice (Figs. 3 and 6), with the increase in NG2-glia within the hippocampus of db/db mice occurring prior to the BBB injury. Elevated MMP-9 levels were found to correlate strongly with NG2-glia in NG2-glia and MMP-9 immunofluorescence co-localization experiments (Fig. 6D). We also observed increased hippocampal BBB permeability, downregulated expression of the tight junction protein zo-1, and activation of the Wnt/β-catenin signaling pathway in NG2-glia in 16-week-old db/db mice through Evans-Blue BBB permeability assay and immunofluorescence co-localization analysis. After treatment with the Wnt/β-catenin signaling pathway inhibitor XAV939, MMP-9 levels in the hippocampus of db/db mice decreased, indicating that the Wnt/β-catenin signaling pathway is involved in the upregulation of MMP-9 in diabetes.
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9 protein secretion by NG2-glia in the hippocampus of 16-week-old db/db mice decreased, and the structural and functional impairment of the BBB improved (Figs. 5 and 6). This suggests that in diabetic central neuropathy, NG2-glia induce MMP-9 expression through the activation of the Wnt/β-catenin signaling pathway and contribute to diabetes-induced BBB damage.

In summary, hippocampal NG2-glia in db/db mice may cause structural and functional disruption of the diabetic BBB by activating the Wnt/β-catenin signaling pathway in NG2-glia, upregulating MMP-9 expression, and ultimately degrading tight junction proteins. Therefore, future studies should aim to target NG2-glia that secrete MMP-9 in order to mitigate the onset and progression of diabetic BBB injury and diabetic encephalopathy.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTIONS

Conception or design: X.L., Y.C., J.Z.
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