Lipin2 ameliorates diabetic encephalopathy via suppressing JNK/ERK-mediated NLRP3 inflammasome overactivation

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Research Article

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Abstract

OBJECTIVES: Diabetic encephalopathy (DE) is a common complication of diabetes in the central nervous system, which can cause cognitive dysfunction in patients. However, its pathophysiological mechanism has not been elucidated, and thus effective prevention and treatment methods are still lacking. Previous studies reported that neuroinflammation involved in the central neuropathy, while lipin2 plays an important role in inflammatory response. Therefore, we aimed to investigate the effects of lipin2 on regulating inflammatory response in the pathogenesis of DE.

METHODS: BV2 cells were treated with high glucose and infected with lipin2 overexpression or knockdown virus to observe the cell viability. Then, we constructed a mouse model of DE, and constructed a lipin2 knockdown or overexpression model by injecting lentivirus into the brain with stereotaxis. The expression of lipin2 in inflammatory bodies and related inflammatory factor signaling pathway-related proteins were examined by western blot and quantitative real-time PCR. Morris water maze was used to evaluate the spatial learning and memory of mice.

RESULTS: High glucose decreased the expression of lipin2 in BV2 cells, while overexpression of lipin2 in BV2 cells significantly suppressed the inflammatory response and apoptosis induced by high glucose. Meanwhile, the expression of lipin2 was down-regulated in the hippocampus in a DE mice model. Up-regulation of lipin2 in the hippocampus of DE mice inhibited JNK/ERK signaling pathway, reduced NLRP3 inflammasome-mediated inflammatory response, down-regulated IL-1/TNF-α expression, and improved synaptic plasticity and cognitive dysfunction in mice. Conversely, knockdown of lipin2 increased NLRP3 inflammasome overactivation, caused neuronal abnormalities and cognitive impairment in mice.

CONCLUSIONS: Lipin2 may play a neuroprotective role in DE by inhibiting JNK/ERK-mediated NLRP3 inflammasome overactivation and subsequent inflammatory responses. It may be a potential therapeutic target for DE therapy.

1. Introduction

Diabetic encephalopathy (DE) is due to abnormal brain function caused by long-term hyperglycemia. The main pathological manifestations are neuronal loss and degenerative changes, and the clinical characteristics are acquired cognitive dysfunction and behavioral defects, which seriously affect the quality of life of diabetic patients[1]. With the extension of life expectancy of diabetic patients, the DE prevalence increases rapidly. However, the pathogenesis of DE is currently unclear, and thus resulted in the lack of specific treatment for DE. Increasing evidence showed that the overactivation of NLRP3 inflammatory plays an important role in the development of diabetes mellitus (DM)[2][3], which suggested that the chronic inflammation maybe a critical risk factor of DE[4][5],

The mammalian lipin proteins (lipin1, lipin2, and lipin3) are phosphatidic acid phosphatase (PAP) enzymes that regulate levels of cellular triacylglycerols and phospholipids[6]. They play an important role
in maintaining normal nerve conduction function. Lipin proteins act as lipid intermediates in cellular signaling pathways and have transcriptional activity\[^7\]. In addition, cognitive impairment is present in Lpin1\(^{\text{fid}}\)/J mice\[^8\]. Lipin1 expression in hippocampal CA1 region decreased in diabetic rats, and the up-regulation of Lipin1 expression could improve synaptic function in rats\[^9\]. In addition, lipin2 was observed closely associated with inflammation. \(\text{LPIN2}\) mutations in humans caused Majeed syndrome, a disease whose clinical manifestations can be reduced by blocking IL-1\(\beta\) or its receptor\[^10\],[^11],[^12],[^13]\]. This finding highlights the key role of lipin2 in inflammatory signal transduction. In human and mouse macrophages, lipin2 inhibits inflammation by affecting NLRP3 inflammasome activation, caspase-1 processing, and IL-1\(\beta\) mRNA transcription\[^14\],[^15\]. However, it is unclear whether lipin2 plays a role in inflammatory responses associated with cognitive dysfunction in DE mice model. Therefore, the study aimed to clarify the mechanisms of lipin2 underlying the neuroinflammation and whether characteristics changes of lipin2 involved in the pathogenesis of DE.

2. Materials And Methods

2.1 Animals

Six-week-old male C57/BL6 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (License No. SCXK (Beijing) 2016-0006). The mice were fed standard animal feed under pathogen-free conditions (SPF grade) and at constant room temperature (22 ± 2°C), relative humidity of 60 ± 4%, and with a light/dark cycle of 12 h/12 h (the lamp was turned on from 8 am to 20 pm). All animal experiments were approved by the Research Ethics Committee of the Second Hospital of Shandong University (KYLL-2021 (KJ) A-0429) and carried out in accordance with the International Guiding Principles for Animal Research provided by the World Health Organization.

2.2 Diabetes animal Model

After 1 week of adaptive feeding and 8 h of fasting, the mice were randomly divided into diabetic model and control groups. Streptozocin (STZ, Solarbio, China) was prepared and dissolved in 0.1 M (mol/L) sodium citrate buffer (pH 4.2, Solarbio, China), and the mice in the diabetic group were given a one-time intraperitoneal injection of 130 mg/kg body weight STZ. The mice in the control group were intraperitoneally injected with the same amount of sodium citrate buffer. In this study, blood glucose was measured from the tail vein of mice. Mice with fasting blood glucose higher than 11.1 mM at 3 days after injection of STZ were considered to be diabetic, and feeding was continued for 12 weeks. Tail vein blood glucose levels and body weight were measured 3 days, 1 week, 2 weeks, 4 weeks, 8 weeks, and 12 weeks after STZ injection. All animal experiments were conducted at the Animal Center of the Second Hospital of Shandong University.

2.3 Virus Packaging

Lipin2 overexpression (LV-Lipin2) and knock-down (LV-Lipin2-RNAi) lentivirus and the blank vectors (LV-Lipin2-Ctrl and LV-Lipin2-RNAi-Ctrl) were purchased from GeneChem Corporation (Shanghai, China).
of the following were encoded with GFP: LV-Lipin2 (1×10^9 TU/mL), LV-Lipin2-Ctrl (2×10^9 TU/mL), LV-Lipin2-RNAi (7.5×10^8 TU/mL), and LV-Lipin2-RNAi-Ctrl (1×10^9 TU/mL). The sequence of Lipin2-RNAi was gcGGCTCTCTATTTCCCTAAA. The sequence of Lipin2-RNAi-Ctrl was TTCTCCGAACGTGTCACGT. Transfection and expression efficiency of overexpressed and knockout lentiviruses (determined by GFP expression) were over 80%.

### 2.4 Stereotactic viral injection

At 12 weeks after STZ injection, mice were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneal injection) and fixed on the brain stereo-positioning instrument (Stoelting, USA). The hair at the top of the head was shaved to expose the skin. The skin was swabbed with iodine. The skull was exposed by making a 1 cm incision on the scalp. The stereotactic coordinates of the hippocampus were located according to the mouse brain atlas (coordinates relative to Bregma: +2.06 mm A/P, ± 1.74 mm L/M and − 2.0 mm D/V). A small burr hole was drilled at the anchor point using an electric miniature animal cranial drill. Lipin2-knockdown lentivirus and blank vector were injected into the wild-type (WT) mice (WT + LV-Lipin2-RNAi and WT + LV-Ctrl groups). Lipin2-overexpressing lentivirus and blank control were injected into DE mice (DE + LV-Lipin2 and DE + LV-Ctrl group). Lentivirus at 4 µL (2 µL per side of hippocampus) was injected into the CA1 region of hippocampus at a flow rate of 0.15 µL/min with a special 5 µL (Gaoge, Shanghai, China). The needle was left at the injection site for 10 min and then gently and slowly withdrawn. The wound was disinfected and closed. Behavioral tests were performed 2 weeks after intracerebral injection.

### 2.5 Morris Water Maze (MWM)

The MWM was used to evaluate spatial learning and memory of mice 2 weeks after stereoscopic brain injection. The experiment was conducted in a warm and quiet environment, with a shading cloth (with a diameter of 150 cm) around the pool to eliminate reflection on the water surface. The first day of adaptation period was used to exclude motor and visual impairments and non-swimming mice. Days 2 to 6 were training time. The water surface was divided into four quadrants. A platform with a diameter of 5 cm was placed in the middle of the first quadrant under 1 cm of water. Thrice a day, the mice were gently placed into the water from the other three quadrants (except the first quadrant) with their heads toward the pool wall. The mice were allowed to find the platform within 60 s. If the mice did not find the platform within 60 s, they were gently guided to the platform and kept there for 20 s. The exercise time, trajectory, and speed of the mice in the pool were recorded for each training session. The time it took to find the platform (called escape latency) was used as an indicator of spatial learning and memory (maximum 60 s). On the 7th day, for the space exploration period, the hidden platform was removed, the mice swam freely for 60 s, and the distance and the number of crossings at the platform area were recorded. All experimental data and tracks were recorded using Any-maze software (Stoelting Co., Wood Dale, IL, USA).

### 2.6 In Vitro Diabetes Model

The microglia cell line BV2 (CL-0493) was purchased from the Procell Cell Bank (Wuhan, China). Cells were cultured in Minimal Essential Medium (MEM) (PM150410, Procell, China), supplemented with 10%
fetal bovine serum (FBS) (Lonsera, China) and 1% penicillin/streptomycin (HyClone, USA) at 37°C in a humidified atmosphere of 5% CO₂ and were passaged once every 3–4 days.

### 2.7 Cell Transfection

BV2 cells were seeded in 12-well plates at a density of $5.0 \times 10^4$ cells/well and allowed to adhere overnight. Then, the instructions for viral infection were followed. According to the recommended Multiplicity of Infection value (MOI = 100), the corresponding amount of virus was added to the culture medium, and a transfection enhancer, HitransG A (GeneChem, Shanghai, China) was added. After 12 h of culture, the solution was changed. Cells were subcultured 3 days later to confirm the infection effect.

### 2.8 Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation was determined by CCK-8 assay (Dojindo, Japan). Cell suspension were seeded at 4000–5000 cells per well in 96-well plates in triplicate and then treated under the indicated conditions the following day. After processing, 10 µL of CCK-8 solution was added and incubated for another 2–4 h at 37°C. The proliferation rate was determined by measuring the absorbance at 450 nm with the microplate reader.

### 2.9 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated and extracted from BV2 cells and mouse hippocampal tissues using Trizol reagent (Invitrogen, USA). One microgram of total RNA was reverse transcribed into cDNA using the primeScript RT reagent Kit (TaKaRa, Otsu, Japan) following the manufacturer’s protocol. Then, the mRNA levels were quantified with a SYBR Green Select Master Mix (ABclonal, RK21203) on a CFX96 real-time system (Bio-Rad). Relative mRNAs levels were calculated using the $2^{-\Delta \Delta Ct}$ method; β-actin was used as the internal control. The primer sequence is as follows: lipin2-forward AGTTGACCCCATCACCGTAG, reverse CCCAAAGCATCAGACTTGGT; β-actin-forward GGGAATCGTGCGTGAC, reverse AGGGCTGAAAAAGAGGCTT; IL-1β-forward GAAATGCCACCTTTTGACAGTG, reverse TGGATGCTCTCATCAGGACAG; TNF-α-forward CTGAACCTCGGGTGATCGG, reverse GGCTTGTCACTGAATTTTGAGA; IL-4-forward GGTCTCAACCCCCAGCTAGT, reverse GCCGATGATCTCTCAAGTGAGT (Generay, Shanghai China).

### 2.10 Western blot (WB)

Mouse hippocampal tissues and BV2 cells were extracted with precooled RIPA lysis buffer (Beyotime, Shanghai, China) containing protease inhibitors and phosphatase inhibitors (Solarbio, Beijing, China) and ice lysed for 30 min after homogenization. The samples were ultrasonically treated 3 times at 10 s each, and centrifuged at 12,000 RPM for 20 min at 4 °C. Total protein concentration was determined using BCA kit (Beyotime, Shanghai, China). The proteins were isolated in equal quantities using either 8% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes. Then, the PVDF membrane was sealed in 5% skim milk for 1 h and then incubated overnight with primary antibody at 4 °C. The primary antibody treatment was as follows: anti-lipin2 (1:300,
ab176347), anti-NLRP3 (1:1000, CST15101), anti-cleaved caspase-1 (1:1000, CST24232), anti-SAPK/JNK (1:1000, CST9252), anti-Phospho-SAPK/JNK (1:1000, CST4668), anti-p44/42 MAPK (ERK1/2) (1:1000, CST4695), anti-Phospho-p44/42 MAPK (ERK1/2) (1:1000, CST4370), anti-PSD95 Rabbit (1:1000, A7889, ABclonal), anti-Synaptophysin (1:1000, A6344, ABclonal), anti-BDNF (1:1000, A1307, ABclonal), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5000, proteintech), anti-β-actin (1:5000, Proteintech), and anti-tubulin (1:5000, Proteintech). The membrane was washed in TBST thrice for 10 min each and incubated in TBST with secondary antibody (1:5000, Abcam Co., UK) at room temperature for 1 h. After washing with TBST, the enhanced chemiluminescence detection kit (Merck Millipore, Billerica, MA, USA) was used. Blots were developed using an automatic image analysis system (Tanon 4800, China), and the relative protein expression level was quantified using ImageJ software.

2.11 Electron Microscopy (EM) Analysis

Mice were anesthetized by pentobarbital sodium, and the brains were rapidly removed. The hippocampus was separated and cut into 1 mm3 pieces, and these were quickly placed in pre-cooled glutaraldehyde for four nights, fixed with PBS, fixed with osmium tetroxide for 2 h, dehydrated by ethanol gradient, fixed with propylene oxide for 2 h, and embedded with resin. The tissue was cut into ultra-thin sections (Leica) with a thickness of 70 nm and stained with 2% uranium acetate and lead citrate. The ultrastructure of mouse hippocampal neurons was observed and photographed by transmission electron microscopy (Philips Tecnai 20, Holland).

2.12 Immunofluorescence

After anesthesia, the mouse heart was perfused with 0.9% normal saline, and the brain was perfused with 4% paraformaldehyde (PFA). Then, they were fixed in 4% PFA for 24 h and conventionally dehydrated and paraffin embedded. They were cut into 5 µm-thick coronal sections. Brain slices and 4% PFA fixed cell slides were washed with PBS, sealed with 5% BSA at room temperature for 30 min, and incubated overnight with primary antibody at 4 °C. The primary antibodies were as follows: anti-iNOS (1:500, ab178945, Abcam) and anti-MAP2 (1:50, CST4542). Samples were washed with PBS thrice and FITC-labeled secondary antibody (Alexa 488 conjugate anti-rabbit IgG, 1:400) was added. Then, they were incubated at room temperature, dark for 1 h and nucleated with DAPI (Beyotime). Tablets were sealed with a sealing solution containing anti-fluorescence quenching agent, and a laser scanning confocal microscope (LSM 800, ZEISS, Germany) was used to collect images.

2.13 Statistical Analysis

All experimental data were expressed as mean ± SEM and analyzed using the IBM SPSS Statistics 26.0 program. Comparison between groups was performed by two-tailed Student’s T test or one-way ANOVA. A statistical difference of \( P<0.05 \) was considered significant.

3. Results
3.1 Overexpression of lipin2 enhanced the viability of BV2 cells exposed to high glucose.

To clarify the role and mechanism of lipin2 in DE, microglia cells (BV2) were cultured in vitro, and normal MEM medium (0 mM/48 h) and high glucose medium were added. Under high glucose conditions, the expression of lipin2 was down-regulated, and cell viability decreased in BV2 cells (Fig. 1A). The same concentration of mannitol was used as control to exclude the influence of osmotic pressure (Fig. 1B), and 100 mM/48 h was finally selected as the subsequent high glucose treatment condition.

Lipin2 level decreased under high glucose condition and so did the activity of BV2 cells (Figs. 1A and 1B). Therefore, lentiviruses with overexpression and knockdown of lipin2 were constructed and used to infect BV2 cells. The relationship between lipin2 and the above changes was clarified. Fluorescence microscopy was used to observe the infection efficiency, and the infection rate in each group was more than 80% (Fig. 1C), and the cell growth was good enough for subsequent tests. Cells infected with LV-Lipin2-RNAi and LV-Lipin2-RNAi-Ctrl were were given normal medium (NG + LV-Lipin2-RNAi and NG + LV-Ctrl groups). Cells infected with LV-Lipin2 and LV-Lipin2-Ctrl were subjected to 100 mM glucose (HG + LV-Lipin2 and HG + LV-Ctrl). PCR and Western blot were used to verify the infection efficiency. Compared with the control group, mRNA and protein expressions of the HG + LV-Lipin2 group significantly increased ($P < 0.01$), and the lipin2 mRNA and protein expressions of the NG + LV-Lipin2-RNAi group decreased ($P < 0.05$) (Fig. 1D).

CCK-8 assay was used to detect the cell viability of each group. Under high glucose conditions, the cell viability of the HG + LV-Lipin2 group increased compared with the HG + LV-Ctrl group ($P < 0.05$), and the cell viability of the NG + LV-Lipin2-RNAi group decreased significantly compared with the NG + LV-Ctrl group ($P < 0.01$) (Fig. 1E). The above results showed that low expression of lipin2 led to decreased cell viability, whereas the overexpression of lipin2 can ameliorate cell damage caused by high glucose, suggesting that the expression of lipin2 level may affect BV2 cell activity.

Further PCR was used to detect IL-1$\beta$ and TNF-\(\alpha\) mRNA levels in each group. Compared with the control group, IL-1$\beta$ and TNF-\(\alpha\) mRNA levels in the lipin2 overexpression group increased, whereas the lipin2 knockdown group showed an opposite trend (Fig. 1F). Lipin2 may affect cell viability by mediating inflammatory response.

3.2 The expression of lipin2 was decreased in the hippocampal area of diabetic mice.

To further clarify whether lipin2 expression is changed in the DE model, we constructed a DE mouse model. Three days after intraperitoneal injection of STZ, the blood glucose and body weight of the mice were significantly higher than those of the control group (Fig. 2A), indicating the successful modeling of diabetic mice. Compared with the control group, the expression of lipin2 protein in the hippocampus of diabetic mice was significantly decreased ($P < 0.01$) (Fig. 2B), suggesting that lipin2 expression level in the hippocampal region of mice could be related to cognitive dysfunction in DE mice.
3.3 Lipin2 in the hippocampus of mice regulate the cognitive function and neuronal synaptic plasticity

To determine the effect of lipin2 on the cognitive function of mice 12 weeks after the STZ injection, LV-Lipin2 was injected into the hippocampus of DM mice stereoscopically to construct the DE mouse model with a high expression of lipin2. LV-Lipin2-RNAi was injected into the WT mice, and the lipin2 knockout model was constructed. (Fig. 3A). Corresponding blank vectors were injected as control.

Western blot was used to detect the expression of lipin2 protein in hippocampus of each group of mice (Fig. 3B). Compared with WT + LV-Ctrl group, lipin2 protein level was significantly decreased in WT + LV-Lipin2-RNAi group and DE + LV-Ctrl group (P<0.01). Compared with the DE + LV-Ctrl group, DE + LV-Lipin2 group had increased levels of lipin2 proteins (P<0.05).

Two weeks after stereotactic injection, the MWM test was performed to observe the cognitive and memory abilities of the mice (Fig. 3C). As the training period progressed, the latency of escape in DE + LV-Ctrl group was higher than that in WT + LV-Ctrl (P<0.05). On day 5, the latency of escape in WT + LV-Lipin2-RNAi group was significantly higher than that in WT + LV-Ctrl group (P<0.05), whereas the escape latency in DE + LV-Lipin2 group was shorter than that in DE + LV-Ctrl group (P<0.05). During the space exploration period, the number of mice crossing the platform in the WT + LV-Lipin2-RNAi group and the DE + LV-Ctrl group was significantly less than that in the WT + LV-Ctrl group (P<0.05). Compared with the DE + LV-Ctrl group, the number of times of mice crossing the platform in the DE + LV-Lipin2 group significantly increased (Fig. 3C). Reduced lipin2 level can lead to cognitive decline, whereas the overexpression of lipin2 can improve the cognitive impairment caused by diabetes.

The structure of mouse hippocampal neurons was observed by electron microscopy, as shown in Fig. 3D. The synaptic number per visual field in the WT + LV-Lipin2-RNAi group and the DE + LV-Ctrl group was significantly lower than that in the WT + LV-Ctrl group (P<0.01). Compared with that in the DE + LV-Ctrl group, the number of synapses in the DE + LV-Lipin2 group significantly increased (P<0.01). Lipin2 levels affect the number of synapses in hippocampal neurons, leading to changes in the learning and memory ability of mice.

3.4 Lipin2 regulate synaptic plasticity via NLRP3/JNK/ERK inflammatory signaling pathways

To further clarify the role of lipin2 in DE, immunofluorescence staining was performed on mouse brain tissue sections (Fig. 4A). Compared with the WT + LV-Ctrl group, the expression of microtubule associated protein 2 (MAP2) was down-regulated and inducible nitric oxide synthase (iNOS) increased in WT + LV-Lipin2-RNAi group (P<0.01). In the DE + LV-Lipin2 group, compared with DE + LV-Ctrl group, MAP2 expression was up-regulated, whereas iNOS decreased. iNOS is released by microglia in the M1 state, and the change of microglia M1 may reflect the inflammatory response (P<0.01). Lipin2 may affect mouse synapses by regulating inflammatory response.
To further clarify the relationship between lipin2 and inflammatory pathway, we tested relevant inflammatory indicators (Figs. 4B and 4C). Western blot analysis results of mouse hippocampal tissue showed that compared with WT + LV-Ctrl, the expression of nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), caspase-1, phosphorylation of c-Jun amino terminal kinase (p-JNK), and phosphorylation of extracellular signal-regulated protein kinases 1/2 (p-ERK1/2) of the WT + LV-Lipin2-RNAi and the DE + LV-Lipin2 groups was up-regulated (Figs. 4B). In the DE + LV-Lipin2 group, compared with the DE + LV-Ctrl group, the expression levels of the above indicators decreased ($P < 0.05$). The expression levels of synaptic plasticity-related indicators postsynaptic density protein 95 (PSD-95), synaptophysin (SYP, or SYN), and brain derived neurotrophic factor (BDNF) in the WT + LV-Lipin2-RNAi and the DE + LV-Lipin2 groups decreased compared with those in the WT + LV-Ctrl group (Figs. 4B). However, compared with the DE + LV-Ctrl group, the expression of these proteins in the DE + LV-Lipin2 group increased ($P < 0.05$). PCR was used to detect the mRNA levels of IL-1$\beta$, TNF-$\alpha$, and IL-4 in the hippocampus of mice in each group (Figs. 4C). Compared with the WT + LV-Ctrl, the mRNA expressions of IL-1$\beta$ and TNF-$\alpha$ in the WT + LV-Lipin2-RNAi and DE + LV-Lipin2 groups were up-regulated, whereas the level of IL-4 was decreased ($P < 0.05$). Compared with the DE + LV-Ctrl group, the mRNA expressions of IL-1$\beta$ and TNF-$\alpha$ decreased in the DE + LV-Lipin2 group, whereas the level of IL-4 increased (Fig. 4C) ($P < 0.05$). Lipin2 affected synaptic plasticity through regulation of NLRP3 inflammasome associated proteins.

4. Discussion

The cognitive dysfunction caused by DE seriously affects the quality of life of patients, and the research on related mechanisms has attracted considerable attention which focuses on the neuroinflammation\textsuperscript{[16]}, autophagy changes\textsuperscript{[17]}, mitochondrial dysfunction\textsuperscript{[18]}, advanced glycation end products (AGEs)\textsuperscript{[19]}, $\beta$ amyloid peptides (A$\beta$), synaptic plasticity\textsuperscript{[20]}, and other aspects. Inflammation was therein considered play a important role in the pathogenesis of diabetes and its central nervous system complications. Sustained hyperglycemia can trigger the activation of multiple inflammatory pathways, such as NLRP3 inflammasome related pathways and the release of pro-inflammatory factors, which resulting in the imbalance between pro-inflammatory and anti-inflammatory networks, the increase of reactive oxygen species, and the formation of inflammatory mediators without any control. Thus, the dysfunction of synaptic transmition may subsguently leads to the neuron damage and behavioral disorders\textsuperscript{[4],[5]}.

Regulation of lipid homeostasis is an important cellular function that involves a variety of biological processes, including the regulation of transport and structural maintenance. Lipin2 is a member of the Lipin protein family which highly expressed in liver, small intestine, macrophages, and central nervous system, especially hippocampus and cerebellum\textsuperscript{[21],[22]}. Lipin family contains PAP and transcriptional co-regulators that regulate cellular lipid metabolism, innate immunity, cognitive function, cerebellar function, and intestinal lipoprotein regulation\textsuperscript{[7],[9],[23]}. Our previous studies showed that increasing Lipin1 content can reduce the sciatic nerve autophagy disorder and improve the peripheral nerve conduction velocity in DM rats\textsuperscript{[24]}, and thus relieve painful neuropathy\textsuperscript{[25]}. Meanwhile, we found the cognitive impairment in
Lpin1^flld/J mice[^8^], and the decreased expression of lipin1 in the hippocampal CA1 region of diabetic rats. Moreover, up-regulation of lipin1 expression could improve DE in rats[^9^]. These results indicated that the decreased Lipin1 levels are closely associated with diabetic peripheral and central neuropathy.

As a member of the lipin family, lipin2 is more closely associated with inflammation. Mutations in the human LPIN2 gene are associated with the autoinflammatory disease Majeed syndrome, which is characterized by spontaneous inflammation of the bone and skin[^4^][^10^][^11^][^13^]. The application of IL-1β or its receptor blockers significantly improved clinical manifestations and radiographic changes[^13^]. Defects in the LPIN2 gene are linked to autoinflammatory disease, highlighting its key role in innate immune signaling. Lipin2 can regulate excessive IL-1β formation in human and mouse macrophages through multiple mechanisms, including activation of NLRP3 and caspase-1 treatment, as well as inhibition of purinergic receptor P2X7 and K^+^ efflux[^14^]. Lipin2 deficiency can lead to the elevation of NF-κB signaling pathway in mouse macrophage lines[^26^], the activation of MAPK signaling pathway in human and mouse macrophages, and the induction of pro-inflammatory factors, such as IL-1β and TNF-α[^15^][^27^]. Increased lipin2 expression limits the hyperphosphorylation of ERK, P38, and JNK and reduces IL-1β synthesis and release[^14^][^27^]. In addition, Lipin2^-/-^ mice showed increased sensitivity to LPS-induced inflammation, highlighting the role of lipin2 as a key regulator of inflammation in vivo[^14^]. Coincidentally, in a study involving Dutch people, researchers found a single nucleotide polymorphism (SNP 9-rs3745012) in the 3'-untranslated region of LPIN2, which was significantly associated with type 2 diabetes; these people's higher body mass index affected fat distribution[^28^]. Human LPIN2 mutations may influence diabetes development. This study was conducted in vivo and in vitro to elucidate the effects and mechanisms of lipin2 involving the neuroinflammation and cognitive dysfunction in DE mice.

BV2 is a mouse microglial cell line with activated inflammatory and phagocytic properties. It is widely used in the study of inflammatory diseases in the central nervous system. Microglia, the dominant immune cells in the central nervous system, act as neuroprotectants in a variety of ways, including clearing abnormal neurons, tissue debris, and synapses[^29^][^30^]. In this study, we treated BV2 cells with high glucose and found that the expression of lipin2 decreased, whereas the activity of BV2 cells infected with LV-Lipin2 increased. These findings indicated that the up-regulation of lipin2 level can improve the cytotoxicity induced by high glucose. INOS and pro-inflammatory cytokines such as TNF-α, IL 1-β, and IL-6 released by activated M1-type microglia are the main mediators of neuroinflammatory deterioration[^31^]. TNF-α is the center of neuroinflammatory reaction under pathological condition. IL-1β, a biomarker of early neuroinflammation and brain tissue damage, promotes the increase of other pro-inflammatory cytokines and chemokines and synergistically leads to neurotoxicity with TNF-α[^32^]. IL-1β expression is upregulated in rat retinal nerve cell cultures exposed to high glucose[^33^]. In the present study, we treated BV2 cells with high glucose and found that the expressions of IL-1β and TNF-α mRNA increased. On the contrary, the expressions of IL-1β and TNF-α decreased in BV2 cells infected with LV-Lipin2. This finding is consistent with observations in human and mouse macrophages[^14^][^15^]. Also, in vivo experiments, lipin2 can reduce the expression of pro-inflammatory factors TNF-α and IL 1-β in the hippocampus of DE
mice and increase the expression of anti-inflammatory factor IL-4. Lipin2 can improve high glucose-induced inflammatory response in vivo and in vitro.

In vivo, lipin2 significantly affected cognitive function in DE mice. In the MWM test, compared with the mice in the WT + LV-Ctrl group, the mice in the DE + LV-Ctrl group had longer escape latency and lower number of crossing platforms, indicating that the DM mice exhibited cognitive impairment. This finding is consistent with those of previous research works\[^{34,35}\]. However, compared with the DE + LV-Ctrl group, the DE + LV-Lipin2 group had shorter escape latency, and the number of crossings on the platform during the positioning cruise period significantly increased. Lipin2 improved the learning and memory ability of DE mice. After knocking down the expression of lipin2 in the hippocampus of WT mice, we were surprised to find that the animals' behavior was similar to that of DE mice. This results indicated that the reduction of hippocampal lipin2 content impaired the cognitive function of mice.

The changes of synaptic number and function are related to cognitive dysfunction. Then, we observed the changes of synaptic number in hippocampus of mice by transmission electron microscopy, and detected MAP2 by immunofluorescence method. We found that the above indicators were consistent with the changes of lipin2 content and cognitive function. MAP2 is widely distributed in dendrites and is directly involved in dendritic growth, bifurcation, remodeling, and synaptic plasticity\[^{36,37}\]. Next, we detected the levels of synaptic plasticity-related proteins PSD95, synaptophysin, and BDNF in the hippocampus of mice by Western blot and found that the above indexes of the mice in the DE + LV-Lipin2 group were significantly higher than those in the control group. The decrease of lipin2 level in the WT group could inhibit the production of the above proteins, which was consistent with changes in MAP2.

PSD95 is a homogeneous layer of dense material on the cytoplasmic surface of the medial postsynaptic membrane that contains neurotransmitter receptor cytoskeleton and signaling molecules. Changes in PSD95 indicate synaptic remodeling, which is the basis of learning and memory\[^{38}\]. SYP is a calcium-binding acid glycoprotein that is widely distributed on the membrane of presynaptic vesicles; it is directly involved in the formation of synapses and regulates the expression of other synaptic related proteins and is closely related to the transport and release of synaptic vesicles and synaptic plasticity\[^{39}\]. BDNF plays an important role in the survival, differentiation, growth, and development of neurons\[^{40}\]; the inhibition of BDNF leads to reduced synaptic plasticity and neurogenesis in the hippocampus, leading to impaired learning and memory\[^{41}\]. Damage to synaptic structure and information transmission is a key risk factor for cognitive dysfunction. In this study, lipin2 increased the expressions of PSD95, SYP and BDNF in DE mice and affected the number of synapses, thereby indicating improved synaptic plasticity.

NLRP3 inflammasome is a multi-protein complex that is assembled in macrophages in response to pathogen exposure; it coordinates the innate immune response by activating caspase-1, leading to the maturation of pro-inflammatory cytokines to produce IL-1β. In previous studies, lipin2 inhibits NLRP3 overactivation and caspase-1 processing of inflammasome and regulates excessive IL-1β formation in human and mouse macrophages\[^{14}\]. In the present study, the same phenomenon was observed in animals. In DE mice, increasing the level of lipin2 inhibited the activation of the NLRP3 inflammasome,
decreased the production of caspase-1, and decreased the release of IL-1β and TNF-α, while the p-JNK and p-ERK pathways were inhibited. The opposite phenomenon was observed in WT mice when lipin2 level was reduced.

Mitogen-activated protein kinase (MAPK) cascade, which includes ERK1/2, P38, JNK, and ERK5, is an important intracellular signal transduction system involved in cell growth and differentiation, apoptosis, inflammatory stress response, and other physiological and pathological processes. Among them, ERK\(^{[42],[43]}\) and JNK\(^{[44],[45]}\) signaling pathways are implicated in the regulation of cognitive function and synaptic plasticity. Lipin1 knockout mice mediated cognitive dysfunction through the ERK pathway\(^{[8]}\). In the present study, lipin2 improved synaptic plasticity and cognitive function by inhibiting the JNK and ERK signaling pathways, the overactivation of NLRP3 inflammasome, and the release of IL-1β and TNF-α. Specific down-regulation of lipin2 in WT mice activates the JNK/ERK pathway and NLRP3 inflammasome, promoting inflammatory responses and exacerbating cognitive dysfunction. Therefore, lipin2 can be a potential target for DE treatment by improving NLRP3 inflammasome-mediated inflammatory responses.

It should be noted out that this study also has some limitations as follows. First of all, the lipin2 expression was specifically regulated only in the hippocampus, and lipin2 gene knockout was not performed in animals. Second, the relationship between members of the lipid family needs further study.

5. Conclusions

In the present study, we confirmed the important role of lipin2 in amelioration of cognitive dysfunction in DE mice. Hyperglycemia can reduce lipin2 expression, resulting in neuroinflammation and cognitive dysfunction. In contrast, overexpression of lipin2 inhibits JNK/ERK pathway and NLRP3 inflammasome-mediated inflammatory response, thereby improving synaptic plasticity and cognitive function.

Abbreviations

BDNF
brain derived neurotrophic factor
CCK-8
Cell Counting Kit-8
DE
Diabetic encephalopathy
DM
Diabetes mellitus
EM
Electron Microscopy
GAPDH
Glyceraldehyde-3-phosphate dehydrogenase
GFP
Green fluorescent protein
MAPK
Mitogen-activated protein kinase
MAP2
Microtubule associated protein 2
MWM
Morris Water Maze
NLRP3
Nucleotide-binding oligomerization domain-like receptor protein 3
PAP
Phosphatidic acid phosphatase
PFA
Paraformaldehyde
p-ERK
Phosphorylation of extracellular signal-regulated protein kinases 1/2
p-JNK
Phosphorylation of c-Jun amino terminal kinase
PSD-95
Postsynaptic density protein 95
qRT-PCR
Quantitative Real-Time Polymerase Chain Reaction
SDS-PAGE
Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STZ
Streptozocin
WB
Synaptophysin (SYP, or SYN) Western blot
WT
Wild-type

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Research Ethics Committee of the Second Hospital of Shandong University (KYLL-2021 (KJ) A-0429) and carried out in accordance with the International Guiding Principles for Animal Research provided by the World Health Organization.

Consent for publication
Availability of data and materials

All data generated or analyzed during this study are included in this published article and are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors’ contributions

Shihong Chen, Xianghua Zhuang, and Shuyan Yu designed the experiments and made valuable suggestions during the revision of this manuscript. Xiaochen Zhang and Shan Huang performed most of the experiments; Ziyun Zhuang, Xiaolin Han, Mengyu Hua, Zhonghao Liang, Chao Meng and Ling Yin helped with experimentation. Xiaocheng Zhang, Xiaolin Han, and Shan Huang analyzed the data. Xianghua Zhuang and Shuyan Yu provided technical assistance with the experiments. Xiaochen Zhang wrote the manuscript. All authors critically reviewed, revised, and approved the final version of the manuscript.

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Conflict of interest statement

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References


Figures

Figure 1

Overexpression of lipin2 enhanced the viability of BV2 cells exposed to high glucose. (A) The lipin2 protein expressions in BV2 cells incubated with glucose was determined using Western blot. *P < 0.05, **P < 0.01, ***P < 0.001 versus 0 mM/48 h. (B) After treating BV2 cells with different concentrations of glucose and mannitol for 48 h, cell viability was standardized with that of the control group. n=3/group. *P < 0.05, **P < 0.01, ***P < 0.001 versus 0 mM/48 h. (C) BV2 cells were infected with lentiviruses that were up-regulated or down-regulated by lipin2. After 5 days of stable culture, the infection efficiency was detected by fluorescence microscope (infection rate > 80%). (D) The mRNA and protein expression of lipin2 in BV2 cells after viral infection, as detected by qPCR and Western blot. n = 3/group. (*P < 0.05, **P < 0.01, ***P < 0.001 versus NG+LV-Ctrl, #P < 0.05, ###P < 0.001 versus HG+LV-Ctrl). (E) Determination of BV2 cell viability under different treatment conditions. The activity of LV-Lipin2-RNAi infected BV2 cells in normal medium was significantly decreased compared with that in the control group. Under high glucose conditions, the activity of LV-Lipin2 infected BV2 cells was enhanced compared with the blank vector control group. n=3/group. (*P < 0.05, **P < 0.01, ***P < 0.001 versus NG+LV-Ctrl, #P < 0.05, ###P < 0.001 versus HG+LV-Ctrl). (F) Effect of lipin2 on the mRNA levels of IL-1β and TNF-α in BV2 cells. Data represent the means ± SEMs, n = 3/group. (*P < 0.005, **P < 0.01 versus NG+LV-Ctrl, #P < 0.05 versus HG+LV-Ctrl).

Figure 2

Expression of Lipin2 in hippocampus of diabetic mice. (A) Changes in body weight and blood glucose levels of mice after STZ injection, indicating the occurrence of diabetes (n = 30 animals/group). (***(P < 0.001 versus wile-type group). (B) The lipin2 protein expression in hippocampus of wile-type group (WT) and diabetic group (DM) mice. Data are presented as the means ± SEMs. n = 3 animals/group, **P < 0.01 versus wild-type group.

Figure 3

Lipin2 in the hippocampus of mice regulate the cognitive function and neuronal synaptic plasticity. (A) Spontaneous fluorescence of mouse hippocampus after LV-Lipin2 injection. (B) Expression of lipin2 protein in hippocampus of each group after infection detected by Western blot. Data represent the means ± SEMs, n = 3/group. (**P < 0.01, ***P < 0.001 versus WT+LV-Ctrl, #P < 0.05, ###P < 0.01, versus DE+LV-Ctrl). (C) Results of Morris Water Maze test. The number of escape incubation period in 5-day training period and crossing platform in space exploration period were determined. The trajectory of mouse space
exploration was recorded by camera. Data were expressed as mean ± SEM, n = 8-9 animals/group. (*P < 0.05, **P < 0.01, ***P < 0.001 versus WT+LV-Ctrl, #P < 0.05 versus DE+LV-Ctrl). (D) Effects of lipin2 on synaptic number of mouse hippocampus. Arrow indicates synaptic location (scale bar = 0.5 µm). n=3 animals/group. (**P < 0.01 versus WT+LV-Ctrl, ##P < 0.01 versus DE+LV-Ctrl).

Figure 4

Effect of Lipin2 on synaptic plasticity and NLRP3/JNK/ERK inflammatory signaling pathways. (A) The relative expression levels of MAP2 (Magnification: 200) and iNOS (Magnification: 100) in hippocampal region of mice after viral infection (**P < 0.01 versus WT+LV-Ctrl, ###P < 0.001 versus DE+LV-Ctrl). (B) Western blot was used to detect the effects of lipin2 on the expression levels of NLRP3, Caspase-1, PSD95, SYP, BDNF, p-JNK, p-ERK, and other proteins in the hippocampus of mice. Data were expressed as mean ± SEM, n = 3 animals/group. (*P < 0.05, **P < 0.01, ***P < 0.001 versus WT+LV-Ctrl, #P < 0.05, ##P < 0.01, ###P < 0.001 versus DE+LV-Ctrl). (C) The effects of lipin2 on the relative expression levels of IL-1β, TNF-α, and IL-4 mRNA were detected by PCR. Data were expressed as mean ± SEM, n = 3 animals/group. (*P < 0.05, **P < 0.01 versus WT+LV-Ctrl, #P < 0.05, ##P < 0.01, ###P < 0.001 versus DE+LV-Ctrl).

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