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Glycogen synthase kinase-3β mediates toll-like receptors 4/nuclear factor kappa-B-activated cerebral ischemia-reperfusion injury through regulation of fat mass and obesity-associated protein

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Abstract:

BACKGROUND: Glycogen synthase kinase-3β (GSK3β), fat mass and obesity-associated protein (FTO), and toll-like receptors 4 (TLR4) take on critical significance in different biological processes, whereas their interactions remain unclear. The objective was the investigation of the interaction effect in cerebral ischemia-reperfusion (I/R) injury.

METHODS: The function of the cerebral cortex in the mouse middle cerebral artery occlusion **(**MCAO) model (each group $n = 6$) and P12 cells oxygen-glucose deprivation/reoxygenation (OGD/R) model was analyzed using short hairpin GSK3β lentivirus and overexpression of FTO lentivirus (*in vitro*), TLR4 inhibitor (TAK242), and LiCl to regulate GSK3β, FTO, TLR4 expression, and GSK3β activity, respectively.

RESULTS: After GSK3β knockdown in the OGD/R model of PC12 cells, the levels of TLR4 and p-p65 were lower than in the control, and the level of FTO was higher than in the control. Knockdown GSK3β reversed the OGD/R-induced nuclear factor kappa-B transfer to the intranuclear nuclei. As indicated by the result, TLR4 expression was down-regulated by overexpressed FTO, and TLR4 expression was up-regulated notably after inhibition of FTO with the use of R-2HG. After the inhibition of the activity of GSK3β *in vivo*, the reduction of FTO in mice suffering from MCAO was reversed.

CONCLUSIONS: Our research shows that GSK3β/FTO/TLR4 pathway contributes to cerebral I/R injury.

Keywords:

Cerebral ischemia-reperfusion injury, fat mass and obesity-associated protein, glucose-oxygen deprivation/reoxygenation, glycogen synthase kinase-3β, middle cerebral artery occlusion, N6-methyladenosine, nuclear factor kappa-B, stroke, toll-like receptors 4

Introduction

Stroke has been confirmed as the main reason for death and adult disability around the world.[1,2] An epidemiological survey concerning stroke in China has confirmed that the stroke mortality rate is 127.2/100,000[3] and that 75% suffer from varying degrees of disability due to stroke.[4] Following an ischemic stroke, neuronal damage is the result of a wide variety of biochemical and

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molecular processes (e.g., excitatory toxicity, apoptosis, endoplasmic reticulum stress, oxidative stress, ion imbalance, and inflammation[5]). However, due to poor methods and understanding, stroke remains a massive problem worldwide.[6] Accordingly, the fundamental pathophysiological changes and potential treatments after stroke should be studied in depth.

Glycogen synthase kinase-3 (GSK3) refers to a ubiquitous serine/threonine kinase, existing in two isoforms in mammals: a and b.[7] GSK3β shows a correlation with the regulation of multiple molecular and cellular functions (e.g., cytoskeletal regulation,[8] intracellular vesicle transport,[9] cell cycle progression,[10] and apoptosis[11]). A considerable amount of research has highlighted the vital effect of GSK3β in mediating ischemic neuronal death.[12] GSK3β inhibition is the primary pattern involved in pre-treatment and post-treatment. 24 h prior to ischemia or shortly prior to ischemia, or prior to reperfusion, administration of GSK3β inhibitors can narrow the infarct area.[13,14]

Fat mass and obesity-associated protein (FTO) belongs to the Alkb protein family linked to obesity, i.e., the alkB homolog9. It also serves as an effective mediator of nuclear messenger RNA (mRNA) selective splicing and mRNA 3′UTR procession.[15] As indicated by existing research, FTO is highly expressed in multiple brain regions in mice.[16] FTO knockdown results in altered dopaminergic neurotransmission, weight loss, and metabolic rate changes in mice.[17,18] Furthermore, the loss of FTO function suppresses neural axon extension.[19] FTO also takes on a critical significance to memory and learning. Besides, a lack of FTO may prevent adult neural stem cells from proliferating and differentiating into neurons *in vivo*, such that memory and learning are impaired.[20] Faulds *et al*. confirmed that GSK3β regulates FTO expression through phosphorylation and ubiquitination of FTO.[21] Recent reports have also suggested FTO-mediated m6A modification in hemorrhagic thalamic pain by toll-like receptors 4 (TLR4).[22] Moreover, previous results indicated that FTO affects cerebral ischemic-reperfusion injury to a certain extent.[23] However, the exact mechanism of FTO in stroke should be studied in depth.

The TLR, i.e., innate immune receptors found on the cell surface or in the intracellular space, become active in response to inflammation.[24] Within the core and penumbra of ischemic brain tissue, a wide variety of cytokines (interleukins, interferons, and chemokines) were produced for the regulation of TLR expression.[25] Within neurons after cerebral ischemia, TLR4 gene expression showed a notable increase, followed by the enhancement of multiple inflammatory cytokines. TLR4 knockdown in neurons is conducive to boosting survival under glucose-deficient conditions.[26] TLR2 and TLR4 more remarkably affect the pathogenesis of ischemic brain injury than other TLRs.[27] Nalamolu *et al*. have suggested that simultaneous inhibition of the neuroprotective effect of TLR2/TLR4 in ischemic stroke may receive the mediation through proinflammatory cytokines' alleviated induction (tumor necrosis factor [TNF], interleukin-1 [IL-1], and IL-6).[28] A study has indicated that the regulation of GSK3β phosphorylation is capable of inhibiting TLR4/MyD88/NF-κB signalling pathway-mediated inflammatory responses for protecting against renal ischemia-reperfusion (I/R).[29]

The aim of this study was to investigate the interaction of GSK3β, FTO, and TLR4 on cerebral I/R injury.

Methods

Animals

The study conformed to the principles outlined in the Declaration of Helsinki. The Animal Experiment Guidelines of the First Affiliated Hospital of Wenzhou Medical University was followed in this study, with the number of 2021-0184, and the protocol of the experiment in this study gained approval from the The First Affiliated Hospital of Wenzhou Medical University's Animal Experiment Ethics Committee. Beijing Vital River Laboratory Animal Center, China provided male C57BL/6 mice (20–25 g, 4–6 weeks, total number: 24). They were maintained within a controlled environment (12 h light/dark cycle; humidity 60%–70%; 21 ± 2°C) for 7 days prior to surgery. Animals were given water and food that were not restricted in the laboratory.

Antibodies and drugs

Several antibodies were employed, which are presented as follows: rabbit anti-TLR4 antibody (western blotting assay), Goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP), rabbit anti-GAPDH antibody, Goat anti-mouse IgG-HRP, rabbit anti-TLR4 antibody (immunofluorescence), rabbit anti-GSK3β antibody, rabbit anti-p-GSK3β (Ser9) antibody, rabbit anti-GSK3β antibody (1:50 for immunoprecipitation), β-Actin polyclonal antibody, mouse monoclonal to FTO, rabbit anti-BCL-2 antibody, rabbit anti-Bax antibody, rabbit anti-TRIF antibody (western blotting assay), NF-κB p65 Antibody (immunofluorescence), Phospho-NF-κB p65 (Ser536) Antibody (western blotting assay), Alexa488-Donkey anti-rabbit IgG, Alexa594-Donkey anti-rabbit IgG. Several drugs were used, which comprised TAK242, R-2HG, LiCl, Actinomycin D (ActD). For detailed information, please refer to Supplementary Table 1.

Cell culture

We carried out the culture of PC12 cells (EY-X0109, ATCC) and HEK293T (CRL-3216, ATCC) within Dulbecco's modified eagle medium (DMEM) containing 1% v/v penicillin/streptomycin and 10% v/v fetal bovine serum (complete medium). The culture was kept at 37°C within a 5% v/v CO_2 incubator and the fresh medium was substituted within cell passage of 2–3 days and 3–4 days.

Oxygen-glucose deprivation/reoxygenation

First, serum-free/glucose-free DMEM media replaced the complete medium within the plate. Subsequently, the plate was placed in an anaerobic chamber with 95% N_2 and 5% CO_2 and cultured at a temperature of 37°C for 6 h. Next, the cells were placed in the oxygenated incubator in the reperfusion process and cultured continuously in a complete medium for 12 h.

Middle cerebral artery occlusion

Middle cerebral artery occlusion (MCAO) model followed the above description.[30] Anesthesia on the animals with 2% isoflurane. Along with the external carotid artery, we inserted the nylon thread into the internal carotid artery, thus resulting in middle cerebral artery occlusion. The removal of nylon thread was achieved in terms of reperfusion after 1.5 h of ischemia. LiCl (i. p.) injected with 1.0 mEq/(kg. 12 h) 7 days before I/R and divided into four groups: Sham, LiCl+Sham, MCAO, and LiCl+MCAO each group of 6. The ischemic penumbra was selected to perform tissue sampling. The protocols are elucidated in Supplementary Method.

Immunohistochemistry

Immunohistochemistry followed the above description.[23] The brain was taken out and collected after 24 h of reperfusion. 4% paraformaldehyde was injected into the mice via the left ventricle. Paraffin sections were deparaffinized and submerged within hydrogen peroxide for 10 min and were boiled within citrate buffer. The sections were cultured with primary antibodies and secondary antibodies. Fluorescence images were taken with the use of the Leica DM6B fluorescence microscope. The figure's IOD was quantified using Image-Pro Plus. The protocols are elucidated in Supplementary Method.

Immunocytochemistry

Immunocytochemistry followed the above description.[23] The treated PC12 cells were placed in paraformaldehyde and permeabilized with 0.3% Triton-100. The primary antibody and secondary antibody were added, followed by washing with PBS and DAPI sealing. The Leica DM6B fluorescence microscope was adopted to take images of fluorescence. The figure's IOD was quantified using Image-Pro Plus. The protocols are elucidated in Supplementary Method.

Quantitative real-time polymerase chain reaction

Trizol reagent was adopted for total RNA. cDNA was generated using the cDNA Synthesis Kit. With the use of the SYBR Kit, QuantStudio 5 was adopted to perform real-time PCR. The amplified products were quantified using the $2^{-\Delta\Delta CT}$ method. The target gene expression was normalized to β-actin. TLR4 Forward: TTGCTGCCAACATCATCCAGGAAG, TLR4 Reverse: CAGAGCGGCTACTCAGAAACTGC. The protocols are elucidated in Supplementary Method.

Coimmunoprecipitation

Immunoprecipitation was performed following the producer's guideline. The protocols are elucidated in Supplementary Method.

Western blotting assay

For the extraction of protein, we employed RIPA lysis buffer, and obtain protein concentration was determined using the BCA kit. We divided the protein with the use of 10% SDS-PAGE and placed it in a PVDF membrane. 5% skimmed milk was employed for blocking. An appropriate primary antibody and HRP conjugated secondary antibody was added. We identified the reaction using an ECL chemiluminescence reagent. The blot's intensity was quantified using Image Lab. The protocols are elucidated in Supplementary Method.

Lentivirus production and infection

The lentiviral vector construction and lentiviral production followed the above description.[23,31] The protocols are elucidated in Supplementary Method.

Cell counting Kit-8 assay

1×10^4 PC12 cells in the respective well were seeded into 96-well plates. The culture of the cells was achieved based on normal culture conditions. After culturing for 1 day, using the oxygen-glucose deprivation/reoxygenation (OGD/R) model, 10 µl of CCK8 solution was introduced to each well and we continued the incubation for 2 h. We selected the wavelength of 450 nm for examining the absorbance and recording the result.

Enzyme-linked immunosorbent assay

We followed the manufacturer's instructions for the enzyme-linked immunosorbent assay. The protocols are elucidated in Supplementary Method.

Statistical analysis

GraphPad Prism was utilized for the data analysis and statistical analysis. The sample size was not predetermined. The data have an expression of mean ± standard error of the mean (SEM). Through ANOVA and Student's *t*-test, the difference between two or more groups was studied. $P < 0.05$ indicated a significant difference at a statistical level.

Results

Glycogen synthase kinase-3β affects cerebral ischemia/reperfusion injury

The result indicated that p-GSK3β expression increased within the ischemic penumbra region after exposure to MCAO [Figure 1a and b]. Then, we used the GSK3β inhibitor LiCl to inhibit the activity of GSK3β. The result indicated that compared with the MCAO group, the apoptotic protein BCL2 increased, and bax declined [Figure 1c]. LiCl treatment reverses the increased levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α in the ischemic penumbra following cerebral I/R [Figure 1d]. Next, the *in vitro* results on PC12 cells were confirmed, and the result indicated that p-GSK3β expression was suppressed after OGD/R [Figure 1e]. Notably, immunofluorescence indicated that GSK3β protein clustered toward the perinuclear following OGD/R [Figure 1f]. Furthermore, GSK3β knockdown was achieved, and a reduction in OGD/R induced cell damage and increased cell viability were indicated [Figure 1g and h].

Glycogen synthase kinase-3β affects fat mass and obesity-associated protein expression

The result indicated that inhibit the activity of GSK3β in the MCAO model, the N^6-methyladenosine demethylase FTO increased [Figure 2a]. We further observed that GSK3β knockdown and followed by OGD/R treatment, the decline of FTO expression in PC12 cells was reversed [Figure 2b and c].

Glycogen synthase kinase-3β affects toll-like receptors 4 and nuclear factor kappa-B expression

Given that the TLR4 inflammatory signaling pathway also significantly affects cerebral I/R injury, we inhibit the activity of GSK3β in the MCAO model and observed the TLR4 protein decreased [Figure 3a]. We performed GSK3β knockdown followed by OGD/R treatment to detect the expression of cellular TLR4, and we observed a significant reduction in TLR4 expression [Figure 3b and c]. Furthermore, the result indicated that a decrease in p-nuclear factor kappa-B (NFκB) expression after GSK3β knockdown [Figure 3d] and immunofluorescence showed that the transfer of NFκB to intranuclear nuclei by OGD/R treatment was reversed after GSK3β knockdown [Figure 3e].

Glycogen synthase kinase-3β knockdown regulates toll-like receptors 4 expression by fat mass and obesity-associated protein

We performed protein immunoprecipitation for in-depth research on the interaction between GSK3β, TLR4, and FTO. We did not find the binding of GSK3β and TLR4 or GSK3β to FTO [Figure 4f]. We further overexpressed FTO and examined the level of TLR4. The result indicated that overexpression of FTO notably reduced TLR4 protein expression [Figure 4a]. In addition, we performed GSK3β knockdown and adopted the FTO inhibitor R-2HG to inhibit FTO expression. The result indicated that inhibiting FTO up-regulated TLR4 expression [Figure 4b] while increasing cell damage and the production of inflammatory factors [Figure 4c and d].

Given that FTO mainly acts by regulating mRNA modification, we used ActD to inhibit RNA extension. The result indicated that the FTO overexpression group's TLR4 mRNA level was higher than the control after the ActD treatment [Figure 4e]. However, in this study, the level of TLR4 rose markedly both at the mRNA level [Figure 4e] and protein level while overexpression of FTO [Figure 4a]. Thus, we speculate that FTO not only regulates TLR4 degradation but may also regulate TLR4 expression by affecting TLR4 mRNA production.

Toll-like receptors 4 mediates cerebral ischemia/reperfusion injury

We employed the TLR4 inhibitor TAK242 to inhibit TLR4 expression for studying TLR4's effect on cerebral I/R injury. The result indicated that in the PC12 cells treated with OGD/R, TLR4 downstream protein TRIF was notably reduced in the TLR4 inhibitor group [Figure 5a], and cell damage and inflammatory factors were notably reduced [Figure 5b-d].

Discussion

This article reveals that the GSK3β/FTO/TLR4 pathway is critical to cerebral I/R injury. We proved that the damage attributed to OGD/R treatment was notably reduced after neurons knocked down GSK3β. Next, we demonstrated that PC12 cells knocked down GSK3β had lower levels of TLR4 and p-p65 after suffering from OGD/R in contrast to that in the control and reversed the OGD/R-induced NFκB transfer to the intranuclear nuclei. After using the TLR4 inhibitor TAK242, we observed notably reduced damage attributed to OGD/R treatment, demonstrating that TLR4 participates in cerebral I/R injury. In addition, the result indicated that GSK3β did not directly bind to TLR4 but regulated FTO and indirectly affected TLR4 expression. Given the lack of clinical treatment methods for stroke, discovering novel targets and mechanisms contributes to the development of novel treatment options.

A considerable number of articles have reported inconsistent or even contradictory findings though there have been several investigations on GSK3β over the past few years. GSK3β inhibition or deletion is capable of modulating cerebral I/R injury, whereas GSK3β's role is difficult to determine. Several aspects of GSK3β

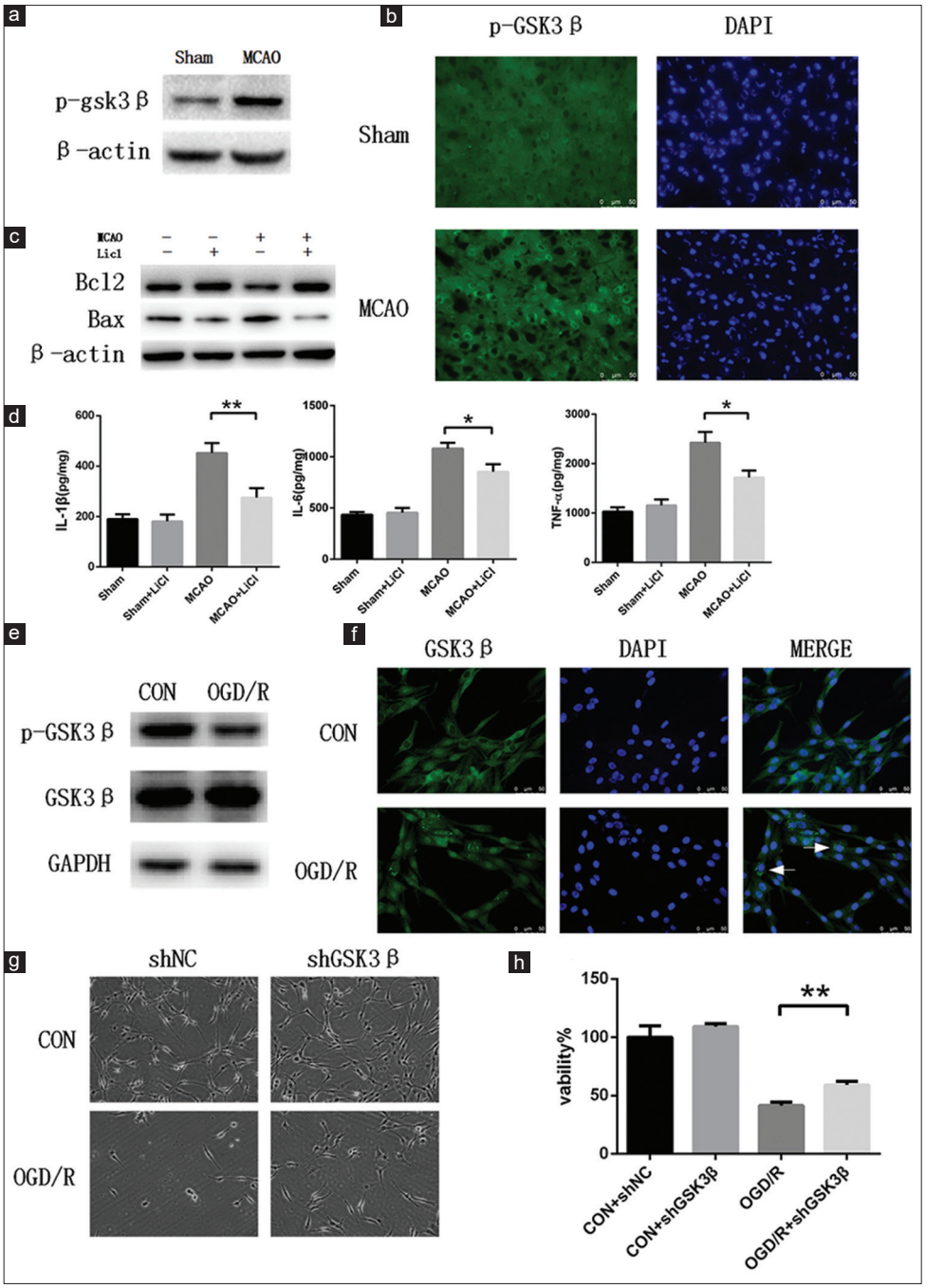

Figure 1: The influence exerted by GSK3β on cerebral I/R injury. (a) The levels of MCAO-administrated mouse cerebral cortex p-GSK3β. Mean ± SEM; $n = 6$; ***$P < 0.001$, (b) Immunofluorescence results of the cerebral cortex of mice treated with MCAO: green staining denotes a p-GSK3β protein, and blue staining corresponds to DAPI. Mean ± SEM; *$n = 3$; $P < 0.05$, (c) mouse cerebral cortex treated with LiCl intraperitoneally and subjected to MCAO. Bcl2 and Bax levels were obtained using the Western blotting assay. Mean ± SEM; $n = 6$; **$P < 0.01$, MCAO vs. MCAO + LiCl, (d) The ischemic penumbra tissue was examined for inflammatory factors, showing the quantification of TNF-α, IL-6, and IL-1β. Mean ± SEM; **$P < 0.01$; *$P < 0.05$, $n = 6$, (e) The levels of OGD/R-administrated PC12 cells p-GSK3β and GSK3β. Mean ± SEM; $n = 3$; ** $P < 0.01$, (f) immunofluorescence of PC12 cells based on OGD/R treatment: Green staining indicates GSK3β protein, and blue staining indicates DAPI. Bar = 50 μm, (g) Knockdown GSK3β protein and OGD/R-administrated PC12 cells, the change in PC12 cell morphology was indicated in the image.(h) Knockdown GSK3β protein and OGD/R-administrated PC12 cells. The PC12 cell viability percentage was quantified. Mean ± SEM; $n = 8$; ** $P < 0.01$. GSK3β: Glycogen synthase kinase-3β, MCAO: Middle cerebral artery occlusion, OGD/R: Oxygen-glucose deprivation/reoxygenation, I/R: Ischemia-reperfusion, SEM: Standard error of mean, DAPI: 4',6-diamidino-2-phenylindole, TNF: Tumor necrosis factor, IL: interleukin

function have been studied, whereas the phosphorylation of GSK3β and its corresponding signal transduction pathway mediating cerebral I/R injury have aroused wide interest.[14,32] The result indicated that p-GSK3β expression decreased in neurons undergoing OGD/R treatment, consistent with previous studies. In addition, the result indicated that GSK3β protein aggregated around the nucleus after suffering from OGD/R. From a traditional

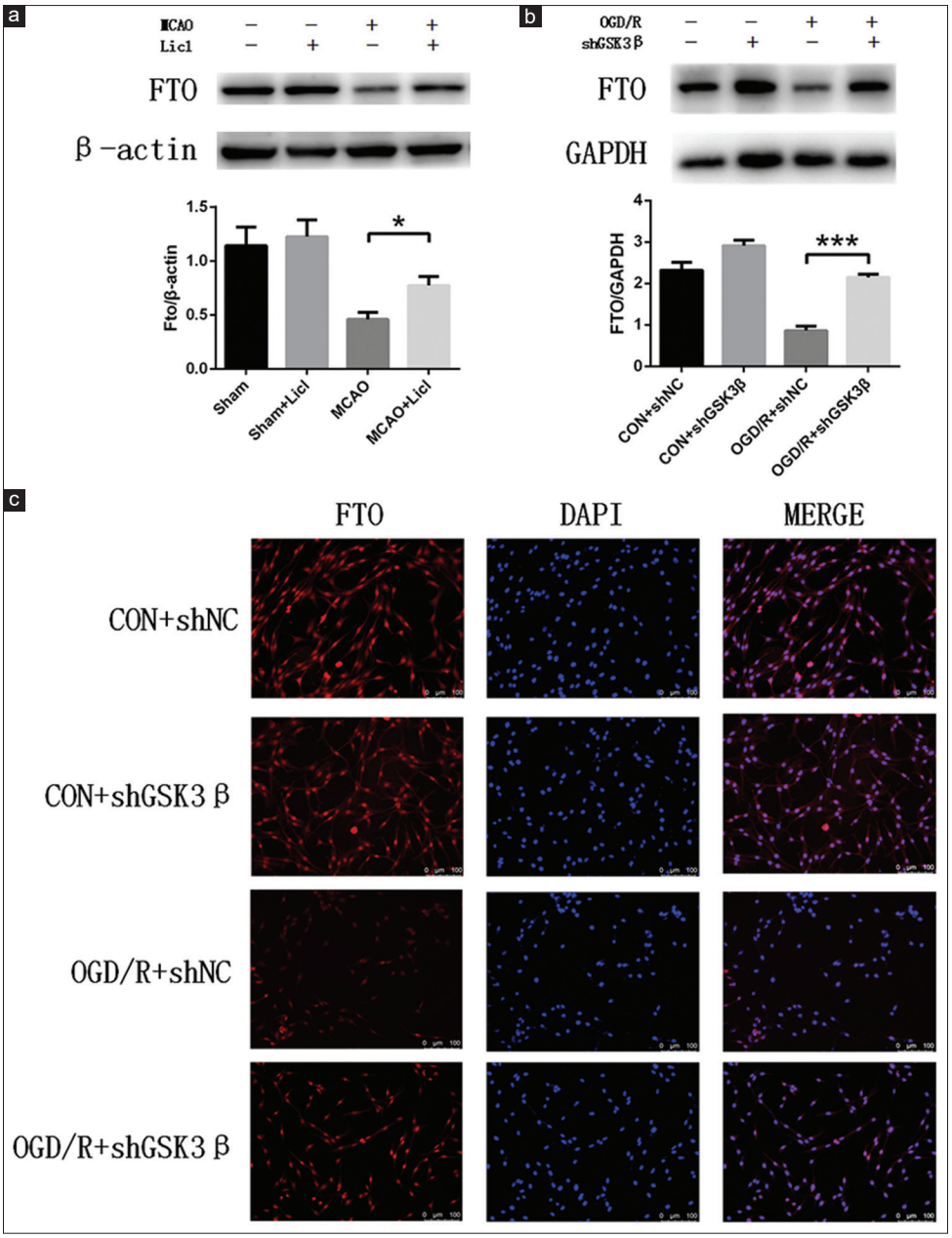

Figure 2: GSK3β knockdown increase the expression of FTO. (a) The levels of MCAO-administrated mouse cerebral cortex FTO. Mean ± SEM; *n* = 6; * *P* < 0.05, (b) Western blotting assay was performed to obtain knockdown GSK3β protein and OGD/R-administrated PC12 cells, as well as the levels of FTO. Mean ± SEM; *n* = 3; ****P* < 0.001, (c) PC12 cells knockdown GSK3β and OGD/R treatment immunofluorescence results: red staining corresponds to FTO protein, and blue staining corresponds to DAPI. Bar = 100 μm. Mean ± SEM; *n* = 6; ****P* < 0.001 OGD/R + shNC vs. OGD/R + shGSK3β. GSK3β: Glycogen synthase kinase-3β, FTO: Fat mass and obesity-associated protein, MCAO: Middle cerebral artery occlusion, OGD/R: Oxygen-glucose deprivation/reoxygenation, shGSK3β: Short hairpin GSK3β lentivirus, DAPI: 4',6-diamidino-2-phenylindole, SEM: Standard error of mean

perspective, GSK3β has been largely considered a cytoplasmic protein. Nevertheless, GSK3β is also present in mitochondria, nuclei, and other subcellular compartments, where local signaling activity is capable of regulating its level and activation state. Mitochondrial GSK3β is particularly significant with the presence of oxidative stress and certain apoptotic conditions.[33] It has been reported that various stimuli that cause cell damage can induce an inherent apoptotic signal transduction cascade (e. g., endoplasmic reticulum stress, oxidative stress, DNA damage, and other damages). The above conditions activating intrinsic apoptotic signal transduction can induce mitochondrial disruption and cellular disruption, and this process is promoted by GSK3β.[7] Accordingly, it is speculated that the GSK3β-medicated intrinsic apoptotic signal cascade that destroys mitochondria affects cerebral I/R injury to a certain extent.

The m6A modification has been considered a posttranscriptional regulatory marker of several RNA

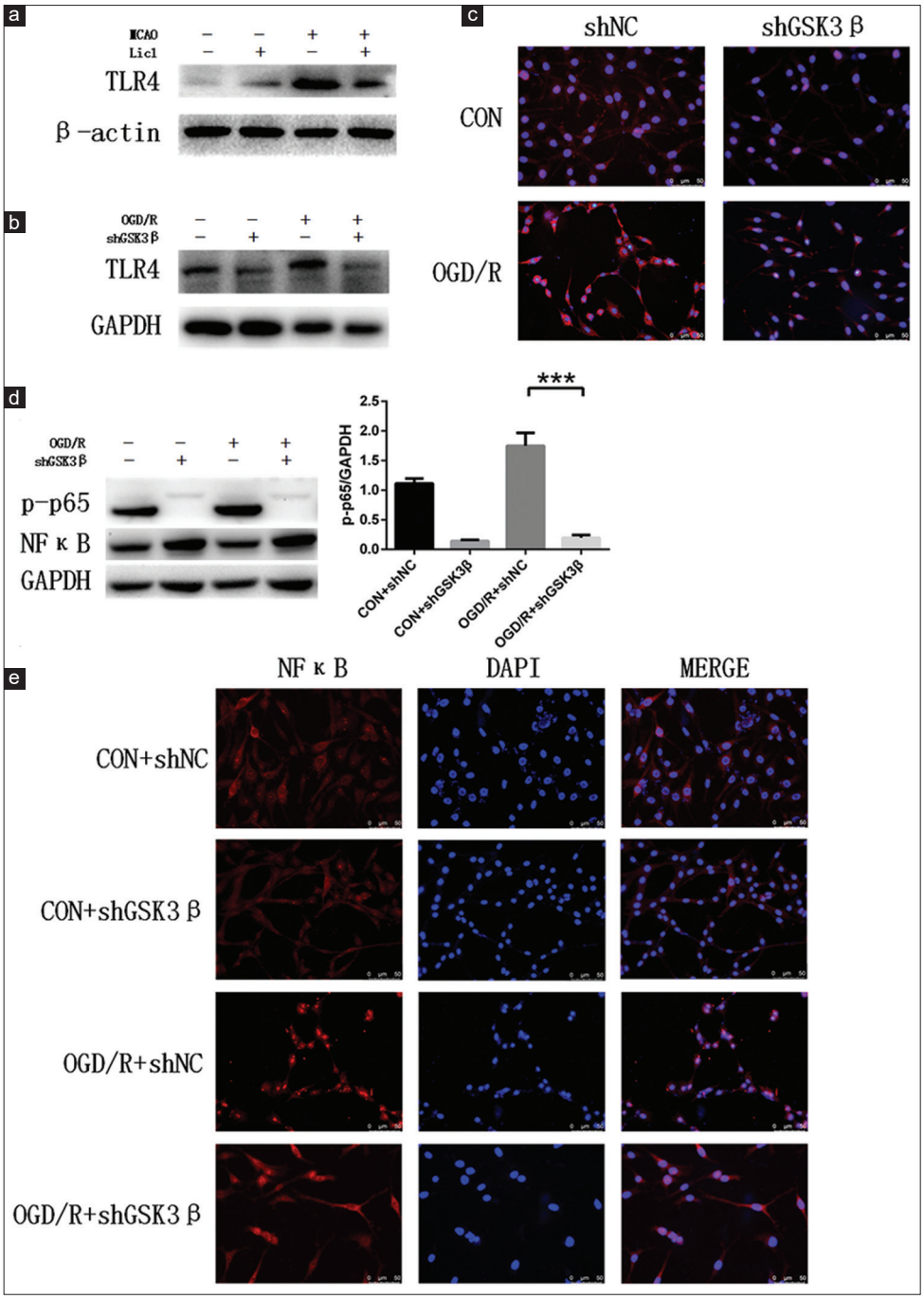

Figure 3: GSK3β knockdown down-regulated the expression of TLR4 and p-p65 within PC12 cells and reversed OGD/R-induced NFκB transfer to the nucleus. (a) The levels of MCAO-administrated mouse cerebral cortex TLR4. Mean ± SEM; $n = 6$; ***$P < 0.001$ MCAO vs. MCAO + LiCl, (b) PC12 cells knockdown GSK3β and OGD/R treatment. The level of TLR4 was obtained using the Western blotting assay. Mean ± SEM; $n = 6$; ***$P < 0.001$ OGD/R + shNC vs. OGD/R + shGSK3β, (c) PC12 cells knockdown GSK3β and OGD/R treatment immunofluorescence results: red staining corresponds to TLR4 protein, and blue staining corresponds to DAPI. Mean ± SEM; $n = 3$; ***$P < 0.001$ OGD/R + shNC vs. OGD/R + shGSK3β, (d) PC12 cells knockdown GSK3β and OGD/R treatment. The p-p65 level was obtained using the Western blotting assay. Mean ± SEM; *** $P < 0.001$; $n = 6$, (e) PC12 cells knockdown GSK3β and OGD/R treatment immunofluorescence results: red staining corresponds to NFκB protein. Blue staining corresponds to DAPI. Bar = 50 μm. GSK3β: Glycogen synthase kinase-3β, TLR4: Toll-like receptors 4, MCAO: Middle cerebral artery occlusion, OGD/R: Oxygen-glucose deprivation/reoxygenation, NFκB: Nuclear factor kappa-B, DAPI: 4',6-diamidino-2-phenylindole, SEM: Standard error of mean

types (long non-coding RNA, microRNA, circular RNA, ribosomal RNA, transfer RNA, and messenger RNA[34]). RNA m6A modification notably affects RNA splicing, translation, stability, translocation, and higher structure.[35] Previous studies, including our own, have shown that FTO facilitates cerebral I/R injury through m6A modification.[23,36] In this study, the result indicated that GSK3β regulates cerebral I/R injury through FTO, and GSK3β knockdown increases the expression of FTO. Similarly, existing research has suggested that GSK3β mediates mRNA methylation within embryonic stem cells of mice through phosphorylation and ubiquitination

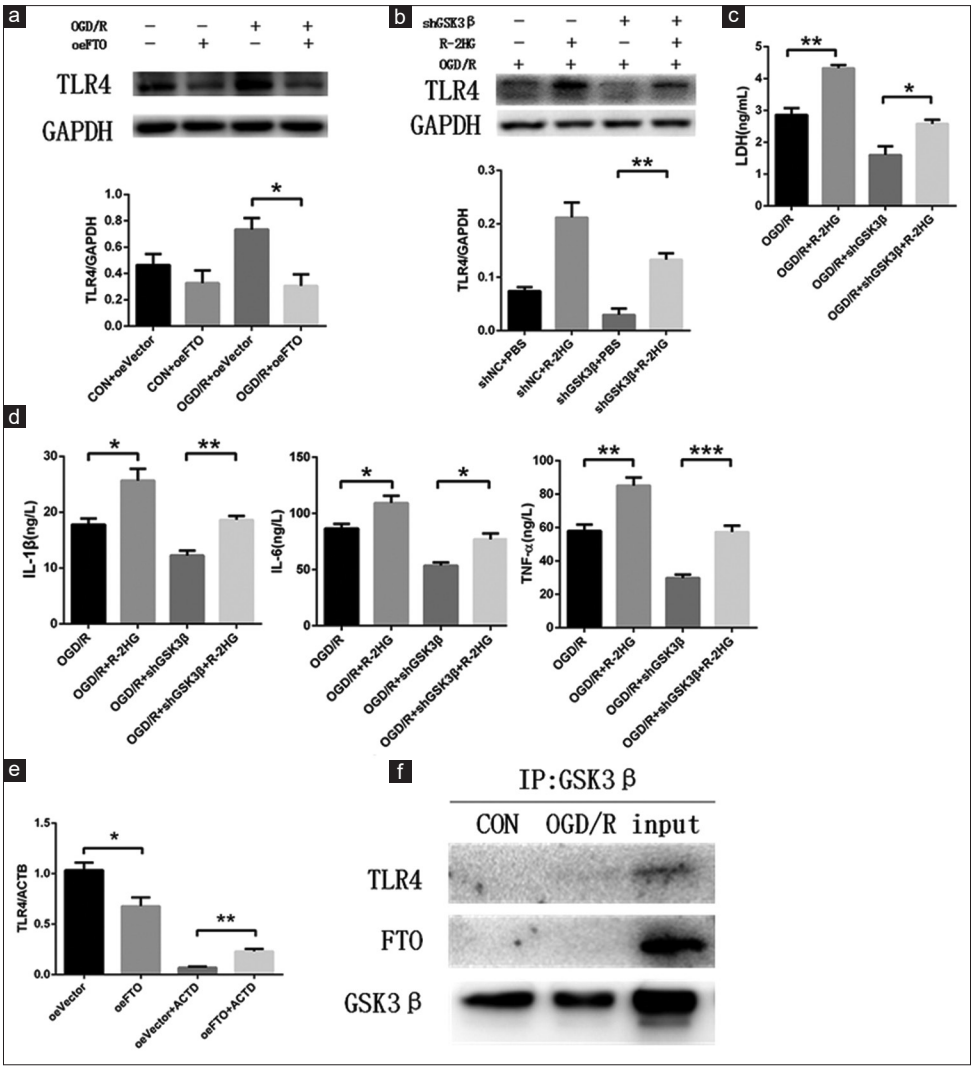

Figure 4: FTO overexpression suppressed the increase in TLR4 within PC12 cells due to OGD/R treatment. (a) PC12 cells overexpress FTO and OGD/R treatment. The level of TLR4 was obtained using the Western blotting assay. Mean ± SEM; **P* < 0.05; *n* ≥ 3, (b) PC12 cells led to GSK3β knockdown and underwent FTO inhibitor R-2HG and OGD/R treatment. The level of TLR4 was obtained using the Western blotting assay. Mean ± SEM; *n* = 3; ***P* < 0.01, (c) PC12 cells knockdown GSK3β and are given FTO inhibitor R-2HG and OGD/R treatment. The cell supernatant was examined for lactate dehydrogenase. Mean ± SEM; *n* ≥ 8; ***P* < 0.01, (d) PC12 cells knockdown GSK3β and are given FTO inhibitor R-2HG and OGD/R treatment. The cell supernatant was examined for inflammatory factors. Mean ± SEM; *n* ≥ 8; **P* < 0.05. (e) PC12 overexpressed FTO and cultured with 5 μg/ml Act D for 24 h, the relative mRNA level of TLR4 by qRT-PCR. Mean ± SEM; ***P* < 0.01; **P* < 0.05, *n* = 3).(f) Endogenous protein was collected from OGD/R. TLR4, FTO, and GSK3β in PC12 cells by co-IP. GSK3β: Glycogen synthase kinase-3β, FTO: Fat mass and obesity-associated protein, TLR4: Toll-like receptors 4, OGD/R: Oxygen-glucose deprivation/reoxygenation, co-IP: Coimmunoprecipitation, SEM: Standard error of mean

of FTO and ultimately regulates stem cell pluripotency through this mechanism.[21,37]

Furthermore, the result indicated that FTO mediates cerebral I/R injury through TLR4. FTO overexpression and the use of inhibitors to inhibit FTO notably affected the content of TLR4. Our previous studies have demonstrated a brain-protective role for FTO through regulating mRNA stability in cerebral I/R injury. The result indicated the improved stability of TLR4 after FTO overexpression. However, under FTO overexpression, TLR4 levels at both mRNA and protein levels notably decreased. Thus, other mechanisms can more probably affect TLR4 mRNA production. Studies have suggested that FTO can affect several steps of gene expression, from mRNA pre-processing steps (e. g., alternative splicing and alternative polyadenylation) to translation.[19] However, in cerebral I/R injury, whether FTO regulates TLR4 mRNA production through an indirect or direct pathway should be investigated in depth.

Toll-like receptors refer to the receptors of transmembrane pattern recognition, critical to producing and controlling immune/inflammatory responses. Cerebral I/R injury is accompanied by inflammation. TLR4 expression level affects the production of a variety of cytokines involved in inflammatory signalling pathways while determining the outcome of cerebral ischemia and reperfusion.[27] TAK242 intraventricular injection has been indicated to dramatically reduce infarct volume after occlusion and reperfusion of the middle cerebral artery and improve neurological score.[38] A

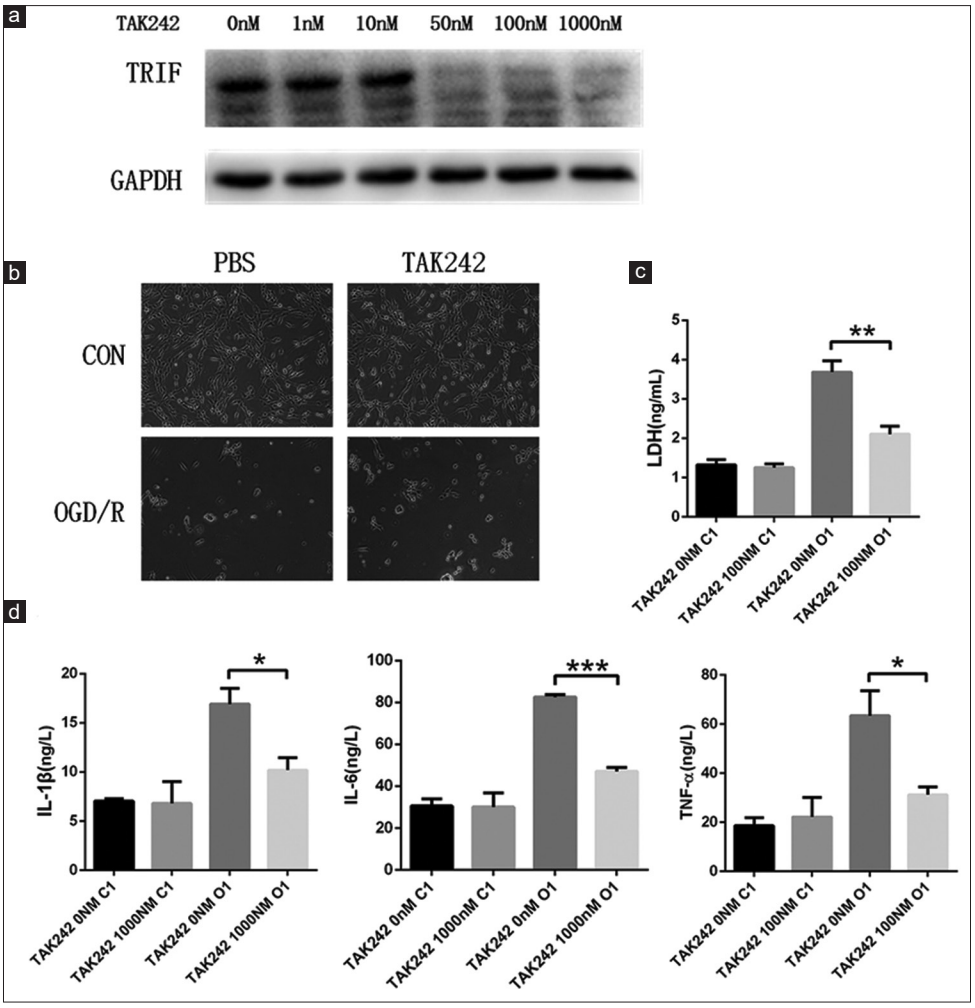

Figure 5: TAK242, a TLR4 inhibitor, alleviates the damage attributed to OGD/R. (a) PC12 cells were given TAK242. The level of TRIF was obtained using the Western blotting assay, (b) PC12 cells were given TAKA242 and OGD/R treatment. A representative image shows the morphology of PC12 cells, (c) PC12 cells were given TAKA242 and OGD/R treatment. The cell supernatant was examined for lactate dehydrogenase. Mean ± SEM; ** $P < 0.01$; $n \geq 8$, (d) PC12 cells were given TAKA242 and OGD/R treatment. The cell supernatant was examined for inflammatory factors. Mean ± SEM; ***$P < 0.001$; *$P < 0.05$, $n \geq 8$. TLR4: Toll-like receptors 4, OGD/R: Oxygen-glucose deprivation/reoxygenation, OGD/R: Oxygen-glucose deprivation/reoxygenation, SEM: Standard error of mean

notably increased neuronal activity and notably decreased inflammatory factor expression were identified after the administration of the TLR4 inhibitor. Some research has reported that TLR4 takes on a vital significance in ischemic stroke by activating NF-κB and inducing the overproduction of inflammatory factors.[39] Moreover, previous studies reported that GSK3β affects TNFα-induced NF-κB activity.[40] Furthermore, the result indicated that the notably reduced expression of TLR4 and phosphorylated NF-κB after GSK3β knockdown, and GSK3β knockdown reversed the OGD/R-induced NFκB transfer to intranuclear nuclei.

Conclusions

This study confirmed that the GSK3/FTO/TLR4 pathway has a certain effect on cerebral I/R injury.

Data availability statement

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

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Conflicts of interest

There are no conflicts of interest.

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Middle Cerebral Artery Occlusion

Anaesthesia on the animals with 2% isoflurane. Along with the external carotid artery, we inserted the nylon thread (RWD Life Science, China). The nylon thread was adjusted to enter the common carotid artery to the internal carotid artery, thus leading to the occlusion of middle cerebral artery. At this point, the blood flow was blocked. The removal of nylon thread was achieved in terms of reperfusion after 1.5 h of ischemia, and the wound was sutured.

Immunohistochemistry

The brain was taken out and collected after 24 h of reperfusion. 4% paraformaldehyde was injected into the mice through the left ventricle. 3.5 mm thick paraffin sections were deparaffinized and submerged in 3% hydrogen peroxide for 10 min to block the endogenous peroxidase activity. The sections were boiled in 10 mmol/L citrate buffer for antigen retrieval before being allowed to cool naturally to ambient temperature. 5% BSA diluted in PBS was adopted to block the sections for 1 h at ambient temperature, and the sections were cultured throughout the night with corresponding antibodies diluted in 1% BSA. After the sections were washed 3 times with PBS, we added the fluorescent secondary antibody for 1 h. We sealed the plate with 4'6-diamidino-2-phenylindole (DAPI). Fluorescence images were taken with the use of the Leica DM6B fluorescence microscope. The figure's IOD was quantified using Image-Pro Plus.

Immunocytochemistry

The treated PC12 cells were placed in PBS containing 4% paraformaldehyde for 15 min before being permeabilized with 0.3% Triton-100 for 15 min. 5% BSA in PBS was adopted to block cells for 1 h at ambient temperature. Next, the primary antibody was adopted to culture the cells in 1% BSA in PBS throughout the night. After three times of washing with PBS, fluorescent secondary antibody was added for 1 h, followed by three times of washing with PBS and DAPI sealing. The Leica DM6B fluorescence microscope was adopted to take images of fluorescence. The figure's IOD was quantified using Image-Pro Plus.

Quantitative Real-time Polymerase Chain Reaction

The extraction of total RNA was achieved with the use of Trizol reagent. With the use of the RevertAid First Strand cDNA Synthesis Kit, cDNA was generated using 1 μg of RNA per sample. With the use of SYBR® Premix Ex TaqII Kit, Applied Biosystems QuantStudio 5 Real-Time PCR Systems were adopted to perform real-time PCR. The 2-ΔΔCT method was used to quantify the amplified products. The expression of the target protein was normalized to the relative expression of β-actin (B661202-0001, Sangon Biotech). The FTO primers used in this study were chemically synthesized by Sangong Biotechnology (Shanghai) Co., Ltd., TLR4 Forward: TTGCTGCCAACATCATCCAGGAAG, TLR4 Reverse: CAGAGCGGCTACTCAGAAACTGC.

Coimmunoprecipitation (co-IP)

The cells were cultured to 70-80% confluence in a 100 mm cell culture dish, then OGD/R treatment was conducted. 3 ml of ice-cold NP-40 buffer (AR0107, BOSTER) was added to the cell culture dish, and cultured at 4°C for 10 min. 1.0 g rabbit IgG and 20 μl Protein A/G PLUS-Agarose were added. Cultured at 4°C for 30 min before being centrifuged at 2,500 rpm for 5 min at 4°C. The supernatant was transferred on ice to a new tube. 1 μg of the primary antibody was added and cultured at 4°C for 1 h. Then, 20 μl of Protein A/G PLUS-Agarose was added and cultured throughout the night at 4°C on a rotating device. The immunoprecipitates were collected by centrifugation. The pellet was washed with 1.0 ml NP-40 buffer. The supernatant was aspirated and discarded and the pellet was resuspended in 40 μl 1x electrophoresis sample buffer. The sample was boiled for 2-3 min and then was detected by Western blotting assay.

Western Blotting Assay

RIPA lysis buffer was adopted for the extraction of protein, and the BCA Protein Assay Kit was adopted to obtain protein concentration. Equal quantities of protein were separated using 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked for 1 h at ambient temperature with 5% skimmed milk dissolved in TBST before being cultured with the primary antibody throughout the night at 4°C. After three washes with TBST, an appropriate horseradish peroxidase (HRP) conjugated secondary antibody was added and cultured at

ambient temperature for 1 h. After another three times of washing with TBST, we identified the reaction using ECL chemiluminescence reagent. The blot's intensity was quantified using Image Lab.

Lentivirus Production and Infection

293T cells were co-transfected with pVSVG, pMDL, pRev, polyethyleneimine, and pCDH-EF1-FHC-FTO expression vector. After 16 h of transfection, fresh medium was used and we continued to culture the sample for another 24/48 h, before harvesting the virus-containing medium. We filtered it through a 0.45 μm syringe filter and stored it at -80°C until the time we used it. PC12 was infected with a lentivirus carrying GSK3β shRNA (shGSK3β) (Neuron biotech, Shanghai, China) to knockdown GSK3β. A lentivirus expressing scramble-shRNA (shNC) served as a control. The target sequences of GSK3β and scramble siRNA are shGSK3β: GCTAGATCACTGTAACATAGT and shNC: TTCTCCGAACGTGTCACGT. PC12 cells were infected with a lentivirus carrying pCDH-EF-FHC-FTO (oeFTO) for FTO overexpression. A lentivirus expressing pCDH-EF1-FHC (oeVector) served as a control. After 48 h of infection, a complete medium containing 2ug/mL puromycin was cultured for 48 h to select stable transgenic strains.

Enzyme-linked Immunosorbent Assay

The enzyme-linked immunosorbent kits applied are elucidated as follows: TNF-α (BP-E30635, Boyun), IL-6 (BP-E30646, Boyun), IL-1β (BP-E30419, Boyun), LDH (BP- E30652, Boyun). In short, the standards, the samples, and the reagents were produced. The produced samples and standards, as well as biotin antigen were introduced and reacted at 37°C for 30 min. After washing the plate, we added avidin-HRP, and react at 37°C for 30 min. The plate was washed, and the colour developing solutions A and B were added. We let the colour to develop at 37°C for 10 min before the introduction of the stop solution. The OD value within 10 min was read, and finally the results were obtained.

Supplementary Table 1: The drugs used in the study

Reagents	Company	Catalogue number
Goat anti-mouse IgG-HRP	Biosharp Life Sciences	BL001A
Goat anti-rabbit IgG-HRP	Biosharp Life Sciences	BL003A
Rabbit anti-GAPDH antibody	Bioworld Technology	AP0063
Rabbit anti-TLR4 antibody	Solarbio	K106584P
Rabbit anti-TLR4 antibody	Abcam	ab13556
Rabbit anti-GSK3β antibody	Cell Signalling Technology	12456S
NF-κB p65 Antibody	Cell Signalling Technology	8242S
β-Actin polyclonal antibody	Bioworld Technology	AP0060
Mouse monoclonal to FTO	Abcam	ab92821
Phospho-NF-κB p65 (Ser536) Antibody	Affinity	AF2006
Rabbit anti-BCL-2 antibody	Affinity	AF6139
Rabbit anti-Bax antibody	Abcam	ab32503
Rabbit anti-TRIF antibody	Abcam	ab13810
Rabbit anti-GSK3β antibody	Affinity	AF5016
Rabbit anti-p-GSK3β (Ser9) antibody	Affinity	AF2016
Alexa488-Donkey anti-rabbit IgG	abcam	ab150073
Alexa594-Donkey anti-rabbit IgG	abcam	ab150076
TAK242	MedChemExpress	CLI-095
R-2HG	Sigma-Aldrich	SML2200
LiCl	Sigma-Aldrich	L9650
Actinomycin D	MedChemExpress	HY-17559
DMEM	Gibco	11966-025
Penicillin/streptomycin	Gibco	15140-122
Fetal bovine serum	Gibco	10099-141
Serum-free/glucose-free DMEM	Gibco	C11995500BT
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific	K1622
SYBR® Premix Ex TaqII Kit	Takara	RR820A
NP-40 buffer	BOSTER	AR0107
Protein A/G PLUS-Agarose	Santa	sc-2003
BCA Protein Assay Kit	Thermo Scientific	23227
PVDF membrane	Bio-Rad	1620177
ECL chemiluminescence reagent	Thermo Scientific	34094

HRP: Horseradish peroxidase, IgG: Immunoglobulin G, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, TLR4: Toll-like receptor 4, GSK3: Glycogen synthase kinase-3, NF-kB: Nuclear factor kappa-B, FTO: Fat mass and obesity-associated protein, ECL: Enhanced chemiluminescence, PVDF: polyvinylidene fluoride, BCA: Bicinchoninic Acid, NP-40: Nonidet P-40, DMEM: Dulbecco's modified eagle medium, LiCl: Lithium chloride, TRIF: TIR-domain-containing adaptor inducing interferon-□, R-2HG: Octyl-D-2HG, TAK242: Resatorvid