**N^6^-methyladenosine demethylases Alkbh5/Fto regulate cerebral ischemia-reperfusion injury**

**Kaiwei Xu*, Yunchang Mo*, Dan Li*, Qimin Yu, Lu Wang, Feihong Lin, Chang Kong, Meita Felicia Balelang, Anqi Zhang, Sijia Chen, Qinxue Dai and Junlu Wang**

**Abstract**

**Background:** Although N^6^-methyladenosine (m^6^A) plays a very important role in different biological processes, its function in the brain has not been fully explored. Thus, we investigated the roles of the RNA demethylases Alkbh5/Fto in cerebral ischemia-reperfusion injury.

**Methods:** We used a rat model and primary neuronal cell culture to study the role of m^6^A and Alkbh5/Fto in the cerebral cortex ischemic penumbra after cerebral ischemia-reperfusion injury. We used Alkbh5-shRNA and Lv-Fto (in vitro) to regulate the expression of Alkbh5/Fto to study their regulation of m^6^A in the cerebral cortex and to study brain function after ischemia-reperfusion injury.

**Results:** We found that RNA m^6^A levels increased consecutive to the increase of Alkbh5 expression in both the cerebral cortex of rats after middle cerebral artery occlusion, and in primary neurons after oxygen deprivation/reoxygenation. In contrast, Fto expression decreased after these perturbations. Our results suggest that knocking down Alkbh5 can aggravate neuronal damage. This is due to the demethylation of Alkbh5 and Fto, which selectively demethylate the Bcl2 transcript, preventing Bcl2 transcript degradation and enhancing Bcl2 protein expression.

**Conclusion:** Collectively, our results demonstrate that the demethylases Alkbh5/Fto co-regulate m^6^A demethylation, which plays a crucial role in cerebral ischemia-reperfusion injury. The results provide novel insights into potential therapeutic mechanisms for stroke.

**Keywords:** Alkbh5, Bcl2, cerebral ischemia-reperfusion injury, Fto, m^6^A, MCAO, OGD/R, RNA methylation

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**Introduction**

Stroke is the leading cause of death and disability in humans. With limited treatment options and the undesirable outcomes of current therapies, stroke remains a huge problem worldwide. Therefore, further study of basic pathophysiological changes and possible interventions after stroke are necessary. Similar to DNA and histones, mRNAs and long non-coding RNAs can be chemically modified. Post-transcriptional regulation of mRNAs can affect the expression of key proteins and brain functioning. However, our understanding of this process is incomplete and thus requires further exploration. To that end, we studied the regulation of epitranscriptomics after cerebral ischemia-reperfusion. In our study, we found a major, but previously unidentified, mechanism that contributes to cerebral ischemia-reperfusion injury through regulated RNA modification.

Although the mechanisms of epitranscriptomics have been studied extensively, our knowledge of their biological functions in physiological and pathological conditions remains limited. Recent studies have shown that the most abundant internal chemical modification of RNA, N^6^-methyladenosine (m^6^A), is a key regulator of mRNA stability, protein

*These authors contributed equally to this study.
expression, and several other cellular processes.\textsuperscript{3,4,7,8} With advances in Me-RIP-seq technology, m\^6A has been the subject of extensive study.\textsuperscript{9} According to studies, m\(^6\)A exists mainly in the 5\(^\prime\)-RRACH-3\(^\prime\) (R=A or G; H = A, C or U) consensus sequence, in gene-coding regions, and in 3\(^\prime\)UTRs.\textsuperscript{9,10} Recent findings indicate that the m\(^6\)A modification of mRNA is reversible and dynamically regulated by writers (methyltransferases) that catalyze the addition of m\(^6\)A (such as METTL3, METTL4, METTL14, and WTAP) and erasers (demethylases) that catalyze removal of m\(^6\)A (such as Fto and ALKBH5) from mRNA.\textsuperscript{11} Differential expression of m\(^6\)A levels regulates a variety of biological functions in mammals, such as transcript alternative splicing, nuclear RNA export, protein translation, heat shock response, cell fate determination, meiotic progression, and myocardial function.\textsuperscript{3,5,12} Thus, variations in the regulatory mechanisms of m\(^6\)A modification may be associated with human disease. Although dysregulated m\(^6\)A is associated with various types of cancer and brain diseases,\textsuperscript{4,13} the role of m\(^6\)A in cerebral ischemia-reperfusion injury has not been studied.

The aim of this study is to investigate the role of m\(^6\)A modification in glucose oxygen deprivation/reoxygenation (OGD/R)-treated neurons and to explore the mechanism in which m\(^6\)A is involved in cerebral ischemia-reperfusion injury.

Materials and methods

**Animals**

The experimental protocol used in this study was approved by the Animal Experimental Ethics Committee of Wenzhou Medical University and was conducted according to the Animal Experiment Guide of Wenzhou Medical University, number: wydw2019-0539. Male Sprague-Dawley rats (250–300 g) were obtained from the Beijing Vital River Laboratory Animal Center, China. and were housed in a controlled environment (12h light/dark cycle; 21 ± 2°C; humidity 60–70%) for 1 week before surgery. Animals had free access to standard laboratory food and water.

**Antibodies and drugs**

The following antibodies were used: anti-m\(^6\)A antibody (202003, Synaptic Systems, 1:1000 for Dot blot, 1:100 for Immunofluorescence), Mouse Anti-NeuN antibody (ab104224, Abcam, 1:500) rabbit anti-ALKBH5 antibody (ab195377, Abcam, 1:1000), rabbit anti-METTL3 antibody (ab195352, Abcam, 1:1000), mouse monoclonal to FTO (ab92821, Abcam, 1:1000), BCL-2 antibody (ab32503, Abcam, 1:5000), Akt (4691T, Cell Signaling Technology, 1:1000), Phospho-Akt-Ser473 (4060S, Cell Signaling Technology, 1:1000), mTOR (2983T, Cell Signaling Technology, 1:1000), PARP (9542T, Cell Signaling Technology, 1:1000), β-Actin polyclonal antibody (AP0060, Bioworld Technology, 1:1000), Goat anti-rabbit IgG-HEK293T were obtained (CRL-3216, ATCC) and cultured in DMEM containing 10% v/v fetal bovine serum (FBS), 1% v/v penicillin/streptomycin. Replace fresh medium in 2–3 days, 3–4 days cell passage.

**Cell culture**

Primary cortical neurons were cultured from embryonic day 16–18 (E16-E18) rats. Briefly, embryos were removed from maternal rats anesthetized with isoflurane and euthanized by an excess of isoflurane anesthesia. The cortex was dissected in Hank’s balanced salt solution and then digested with 0.125% w/v trypsin. Neurons were centrifuged (1000 × rpm, 5 min, 4°C) and resuspended in neurobasal medium containing 2% B27 serum-free supplement, 0.5% v/v penicillin/streptomycin, 0.5 mM glutamine (Neuronal standard medium). The dissociated cells were then plated at a density of 5 × 10\(^4\) cells per cm\(^2\) in plates pre-coated with poly-L-lysine. The culture was maintained at 37°C in a 5% v/v CO\(_2\) humidified incubator. Thereafter, one-half of the medium was replaced every 3 days.

HEK293T were obtained (CRL-3216, ATCC) and cultured in DMEM containing 10% v/v fetal bovine serum (FBS), 1% v/v penicillin/streptomycin. Replace fresh medium in 2–3 days, 3–4 days cell passage.

**Glucose oxygen deprivation/reoxygenation**

The neuron standard medium in the plates was initially replaced with serum-free/glucose-free DMEM medium and then transferred to an
anaerobic chamber containing a mixture of 5% CO₂ and 95% N₂ at 37°C for 3h. During reperfusion, the cells were then returned to an oxygen-containing incubator (95% air and 5% CO₂) for 24h with normal neuron standard medium.

**Middle cerebral artery occlusion**

Intraluminal filament technique was used to induce focal cerebral ischemia, as previously described. Briefly, animals were anesthetized with 10% chloral hydrate (350 mg/kg) intraperitoneally. The nylon thread (2636A4/2838A4, Bei Jing Ciontech Co. Ltd) was inserted through the external carotid artery, and the blood flow was blocked by the adjustment of the nylon thread into the common carotid artery to the internal carotid artery and finally occluded the middle cerebral artery. Reperfusion was achieved by withdrawing the nylon thread after 1.5h of ischemia, and the wound was stitched up. Regional cerebral blood flow was monitored using a transcranial laser Doppler flowmeter (PeriFlux 5000; Perimed AB, Sweden). MCAO was considered sufficient if local cerebral blood flow decreased to 20% of pre-ischemia; if not, animals were excluded.

**RNA m⁶A dot blot**

The dot blot was performed as described above and some minor changes were made. Total RNA was isolated using TRIzol (Invitrogen, 15596018) according to the manufacturer’s instructions, and RNA quality was analyzed using DeNovix. RNA (100ng) was spotted onto a nylon membrane (RPN303B, GE Healthcare Life Sciences). The membrane was then UV-crosslinked and blocked in TBST [0.1% Tween 20 plus TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl)] containing 5% milk for 1h. Rabbit anti-m⁶A antibody was incubated overnight with the membrane (4°C). After washing three times with PBS, Goat anti-rabbit IgG-HRP was incubated with the membrane for 1h at room temperature. The ‘TBST’ was washed three times and the reaction was observed using an ECL chemiluminescent reagent. The same amount of RNA was spotted on the membrane, stained with 0.02% methylene blue in 0.3M sodium acetate (pH 5.2) for 2h, and washed with ribonuclease-free water for 5h.

**Immunohistochemistry**

The brain was removed 24h post-reperfusion. Rats were perfused with 4% paraformaldehyde through the left ventricle. Paraffin sections (3.5 mm thick) were deparaffinized and immersed in 3% hydrogen peroxide for 10min to block endogenous peroxidase activity. The sections were boiled with 10mmol/L citrate buffer for antigen retrieval and cooled to room temperature by natural cooling. Sections were blocked with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1h at room temperature, and sections were incubated overnight with 1% BSA diluted Anti-ALKBH5 antibody. The next day, sections were washed three times with PBS, and horseradish peroxidase (HRP) -conjugated secondary antibody (PV-6001, ZSGB-BIO) was added, incubated for 30min at 37°C, then washed 3 times with PBS. DAB coloring solution (ZLI-9018, ZSGB-BIO) was then added, and stop color reaction with double distilled water in time. Hematoxylin was counter-dyed for 3–5min, washed for 5s and sealed with a neutral resin. We analyzed the results using Image-Pro Plus.

**Immunofluorescence**

The brain was removed 72h post-reperfusion. Rats were perfused with 4% paraformaldehyde through the left ventricle. Paraffin sections (3.5 mm thick) were deparaffinized and immersed in 3% hydrogen peroxide for 10min to block endogenous peroxidase activity. The sections were boiled with 10mmol/L citrate buffer for antigen retrieval and cooled to room temperature by natural cooling. Sections were blocked with 5% BSA in PBS for 1h at room temperature and sections were incubated overnight with 1% BSA diluted rabbit anti-m⁶A antibody and mouse Anti-NeuN antibody. After washing three times with PBS, a fluorescent secondary antibody was added for 1h, and, after washing 3 times with PBS, the plate was sealed with 4’6-diamidino-2-phenylindole (DAPI). Fluorescence images were obtained using an OLYMPUS BX51 fluorescence microscope. Gain, threshold, and black levels did not change during the respective experiments. All image analyses were performed under experimental conditions.

**Immunocytochemistry**

Immunofluorescence staining was performed in cultured neurons of DIV14. Briefly, neurons were fixed by PBS containing 4% paraformaldehyde for 15min and permeabilized with 0.2% Triton-100 for 10min. Neurons were blocked with 5% BSA
in PBS for 1 h at room temperature and then incubated overnight with primary antibody in PBS containing 1% BSA. After washing three times with PBS, a fluorescent secondary antibody was added for 1 h, and, after washing 3 times with PBS, the plate was sealed with DAPI. Fluorescence images were obtained using an OLYMPUS BX51 fluorescence microscope. Gain, threshold, and black levels did not change during the respective experiments. All image analyses were performed under experimental conditions.

**Quantitative real-time polymerase chain reaction**

Total RNA was extracted using Trizol reagent. cDNA was generated using 1 μg of RNA from each sample using a RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Scientific) according to the manufacturer’s protocol. Real-time PCR was performed using SYBR® Premix Ex TaqII Kit (RR820A, Takara) of Applied Biosystems QuantStudio 5 Real-Time PCR Systems. Amplification products were quantified using the 2−ΔΔCT method. The relative expression of the protein of interest was normalized to the expression of β-actin (B661202-0001, Sangon Biotech). All primers used in this study were synthesized chemically by Sangon Biotech (Shanghai) Co., Ltd, and are detailed in Supplementary Materials.

**Western blotting**

Protein was extracted using RIPA lysis buffer and protein concentration was determined using the BCA Protein Assay Kit. Equal amounts of protein were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (1620177, Bio-Rad). The membrane was blocked with 5% skim milk in TBST for 1 h at room temperature and incubated with primary antibody overnight at 4°C. After washing three times with TBST, it was then incubated with a suitable horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature and washed three times with TBST. The reaction was observed using an ECL chemiluminescent reagent. The intensity of the blot was quantified using Image lab.

**Generation of Fto expression constructs**

The Fto cDNA was cloned from previously obtained Rat cDNA using PrimeSTAR Max DNA Polymerase (R045Q, Takara) using the following primers and conditions: (forward 5′ C G T C T A G A T G A A G C G C G T C C A G ACCG3′, reverse 5′ CGGAAATCCCTAGGAT C T T G C T T C C A A G C T G G G3′); 25 cycles at 98°C (10 s), 60°C (15 s), 72°C (30 s). PCR products were subsequently digested with EcoRI and XbaI and purified by AxyPrep DNA Gel Extraction Kit (AP-GX-50, Axygen) and ligated into similarly digested pCDH-EF-FHC vector. pCDH-EF-FHC-Fto was propagated and sequenced for inserting verification.

**Lentivirus production**

To generate lentiviruses for transduction, 6 × 10⁶ 293T cells were seeded into 10 cm² culture dishes with 10 mL complete medium and grown to 60–80% confluence. 293T cells were co-transfected with pRev (0.5 μg), pMDL (1 μg), pVSVG (2 μg) (the lentiviral envelope and packaging plasmids were kindly provided by Jianjing Lin) and pCDH-EF-FHC-Fto (4 μg) expression vectors using polyethylenimine. Fresh medium was replaced after 16 h of transfection, and the culture continued for 24/48 h to harvest the virus-containing medium, which was filtered through a 0.45 μm syringe filter and stored at −80°C until use.

**Lentivirus infection**

DIV7 cultured cortical neurons were infected with a lentivirus carrying GV112-Alkbh5-shRNA (Alkbh5-shRNA) (Shanghai Genechem Co., Ltd) according to the vendor’s protocol to knock down Alkbh5. Lentivirus expressing GV112-scramble-shRNA (Ctrl-shRNA) was used as a control. Target sequences for Alkbh5 and Ctrl siRNAs are siRNA1: GCGCAGTCATCAACGACTA, siRNA2: GCC TCAGGACATCAAAGAA, and Ctrl: TTCT CCGAACGTGTCACGT. DIV10 cultured cortical neurons were infected with lentivirus carrying pCDH-EF-FHC-Fto (Lv-Fto) to overexpress Fto. A lentivirus expressing pCDH-EF-FHC (Lv-Ctrl) (Addgene plasmid # 6487426) was used as a control. These were subsequently used for Western blotting or flow cytometry.

**Cycloleucine or betaine treatment**

Neurons were cultured in vitro for neuron standard medium supplemented with cycloleucine (CL) [A1063, TCI (Shanghai) Chemical Industry Development Co., Ltd.] (10 mM, 20 mM, 40 mM)
and betaine (61962, Sigma) (4 mM, 8 mM, 16 mM) from 24 h before OGD to the end of the experiment. According to a previously published article, the optimal concentration was determined and then used in subsequent experiments.

Apoptosis analysis
The neurons were processed according to the experimental requirements, and the supernatant and PBS washing solution were collected, trypsinized for 5 min, 10% v/v FBS was used to terminate the digestion, and the cells were harvested. Cell death assays were performed using FITC Annexin V Apoptosis Detection Kit I (556419, BD). Briefly, the cells were resuspended in 300 μl of 1X Binding Buffer, 5 μl of FITC Annexin V was added and mixed, then incubated at room temperature for 15 min in the dark. Then, 5 μl of propidium iodide (PI) was added, mixed, and incubated at room temperature in the dark for 5 min. Finally, 200 μl of 1X binding buffer was added to each tube. Samples were analyzed on CytoFLEX LX flow cytometers (Beckman Coulter, USA) and all flow cytometers operations were performed by laboratory professionals. CytExpert V2.3 software was used to calculate the percentage of cells positive for FITC Annexin V and PI.

Cell counting Kit-8 assay
Primary cortical neurons were extracted as described above, and a single cell suspension was prepared using neuron standard medium; 2 × 10^4 cells per well were seeded into 96-well plates at a volume of 100 μl per well. The cells were cultured under normal culture conditions. After 14 days of culture, the OGD/R model was used, and 10 μl of CCK8 solution was added per well, and incubation was continued for 2 h. The wavelength of 450 nm was selected, and the light absorption value of each well was measured on a microplate reader, and the results were recorded.

Hoechst 33342/PI double stain
Neurons were processed according to experimental requirements and stained according to the manufacturer’s instructions. Briefly, the supernatant was removed, washed once with PBS, 1 ml of cell staining buffer was added, 5 μl of Hoechst staining solution was added, incubated at 4°C for 15 min, 5 μl of PI staining solution was added, and incubated at 4°C for 5 min. The cells were washed once with PBS, and the results were observed and recorded under a fluorescence microscope.

Statistical analysis
GraphPad Prism (version 6.01, Graph-Pad Software Inc.) was used for data display and statistical analysis. We did not predetermine the sample size. Data were showed as mean ± SEM. Differences between two or more groups were analyzed by Student’s t test and ANOVA, respectively. P values < 0.05 were considered statistically significant.

Results

Increased m^6A expression after OGD/R and MCAO
To investigate whether m^6A modification is involved in ischemia/reperfusion (I/R)-induced brain tissue injury, middle cerebral artery occlusion (MCAO) was performed in Sprague-Dawley rats. The level of m^6A modification was measured by m^6A dot blot. The level of m^6A modification was significantly increased in the brain after MCAO (Figure 1A). To further confirm the change in the m^6A modification level, immunofluorescence staining was performed on MCAO-treated rat brain tissue. We observed a similar significant increase in the m^6A modification level after MCAO treatment that was localized mainly in neurons (Figure 1B). We therefore measured m^6A modification levels within in vitro primary cortical neurons after OGD/R-exposure. Consistent with the brain tissue of rats subjected to MCAO treatment, the levels of neuronal m^6A modification were significantly increased after OGD/R (Figure 1C). We also performed immunofluorescence staining on primary neurons. A significant increase in m^6A levels after OGD/R treatment was observed (Figure 1D). These results indicate that m^6A levels are dynamically regulated in both primary neurons after OGD/R treatment and rat brain tissue after MCAO treatment.

OGD/R and MCAO induce Fto upregulation in neurons and cerebral cortex
To determine the regulators responsible factors for the increase in m^6A levels after I/R, we examined previously identified proteins that affect m^6A methylation (writers such as Mettl3 and Mettl14) and demethylation (erasers such as Fto and
Alkbh5) levels in primary neurons and rat brain tissue. Our Western blot and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) results show that Fto mRNA and protein levels were decreased significantly both in rat brain tissue after MCAO and in primary neurons following OGD/R (Figure 2A–C). In addition, our histochmical results show that MCAO treatment significantly reduced the number of Fto-positive cells in the ischemic penumbra of rat brain tissue.

**Figure 1.** MCAO and OGD/R induces increased levels of m6A RNA modification in rat cerebral cortex and primary cerebral cortical neurons, respectively. (A) RNA dot blot analysis of m6A levels in MCAO-treated rat cerebral cortex (top left panel). Methylene blue staining was used as a loading control (bottom left panel). Quantification of RNA dots is shown (right panel) (mean ± SEM; n = 3; *p < 0.05 versus sham). (B) Immunofluorescence results of MCAO-treated rat cerebral cortex: red staining represents m6A modification, green staining represents NeuN protein, and blue staining represents DAPI. The m6A modification were quantified by IOD values (mean ± SEM; n = 6; ***p < 0.001 versus sham). (C) RNA dot blot analysis of m6A levels in primary cerebral cortical neurons treated with OGD/R (top left panel). Methylene blue staining was used as a loading control (bottom left panel). Quantification of RNA dots was shown (right panel) (mean ± SEM; n = 9; *p < 0.05 versus Ctrl). (D) Immunofluorescence results of primary cerebral cortical neurons treated with OGD/R: red staining represents m6A modification and blue staining represents DAPI, and quantification of m6A modification was shown (mean ± SEM; n = 4; **p < 0.01 versus Ctrl).

Ctrl, control; DAPI, 4',6'-diamidino-2-phenylindole; m6A, N6-methyladenosine; IOD, integrated optical density; MCAO, middle cerebral artery occlusion; OGD/R, glucose oxygen deprivation/reoxygenation; SEM, standard error of the mean.
Interestingly, Western blot and qRT-PCR data shows that Alkbh5 expression levels were significantly increased in rat brain tissue after MCAO and in primary neurons following OGD/R (Figure 3A and Figure S1A–C). Similarly, we observed a significantly increased number of
Alkbh5-positive cells after MCAO in rat brain tissue (Figure 3B). Alkbh5 is a demethylase that acts to erase m^6^A. However, we observed a seemingly paradoxical increase in m^6^A modification after I/R as shown in Figure 1. Therefore, we hypothesized that the upregulation of Alkbh5 is compensatory. Furthermore, we did not observe a significant change in Mettl3 mRNA and protein levels (Figure 3C and Figure S1D) or the level of other demethylases and methyltransferases such as Mettl3, WTAP, RBM15, and VIRMA (Figure 3D).

Knockdown of Alkbh5 enhances apoptosis in OGD/R-treated neurons, and overexpression of Fto plays a protective role

To investigate the role of Alkbh5 in I/R, we used Alkbh5-shRNA to knock down Alkbh5 expression in primary neurons. As shown in Figure S2A, shRNA1 and shRNA2 achieved knockdown efficiencies of more than 70%. We next performed OGD/R treatment on primary cortical neurons with or without Alkbh5 knockdown, and detected apoptosis using flow cytometry. Consistent with...
the expected results, Alkbh5 knockdown aggravated neuronal death after OGD/R treatment (Figure 4A). We then found that Alkbh5 knockdown significantly increased cleaved-caspase3 levels after OGD/R treatment (Figure S2B). Furthermore, we tested the activity of primary cortical neurons in the same treatment using the Cell Counting Kit-8 (CCK8) assay. The results were consistent with those from our flow cytometry experiments: Alkbh5 knockdown aggravated the damage caused by OGD/R (Figure 4B). Finally, we performed Hoechst 33342/PI double staining on the same treated cells and found that neuronal death was increased in the Alkbh5-shRNA transduction + OGD/R group relative to the OGD/R group (Figure 4C).

To investigate the role of Fto in I/R, we used Lv-Fto to overexpress Fto in primary neurons. As shown in Figure S2C, Lv-Fto significantly increased the levels of Fto in primary neurons. We next treated primary cortical neurons overexpressing Fto with OGD/R and detected apoptosis using flow cytometry. Consistent with the expected results, overexpression of Fto attenuated neuronal death after OGD/R treatment (Figure 4D). We then found that primary neurons overexpressing Fto had significantly reduced cleaved-caspase3 levels compared with the OGD/R group (Figure S2D). Furthermore, we tested the activity of primary cortical neurons in the same treatment using CCK8. Our findings were consistent with our flow cytometry results: overexpression of Fto alleviated the damage caused by OGD/R (Figure 4E). Bcl2 mRNA and protein levels were then examined using qRT-PCR. It was observed that, after treatment with Act D, Bcl2 mRNA was more unstable in primary cortical neurons undergoing Alkbh5 knockdown than in corresponding control cells (Figure 4F). Bcl2 mRNA was more stable in primary cortical neurons overexpressing Fto than in corresponding control cells (Figure 4G).

m^6^A demethylation works through Bcl2
To investigate the specific mechanism of Alkbh5 in I/R, we determined the expression levels of related proteins in primary cortical neurons with or without Alkbh5 knockdown using Western blot. We found that Alkbh5 knockdown significantly reduced the expression of the apoptosis-related protein Bcl2 in primary neurons, but that the effect of Alkbh5 knockdown on Bax was insignificant (Figure 5A). Previous studies have reported that m^6^A is involved in regulation of the Akt/mTOR signaling pathway and PARP protein expression. Considering that these proteins play an important role in cerebral ischemia-reperfusion injury, we therefore examined expression of these proteins in primary cortical neurons after Alkbh5 knockdown. Surprisingly, we found that these proteins were not affected by Alkbh5 knockdown in primary cortical neurons (Figure 5B). To further confirm that Bcl2 mRNA is a direct target of m^6^A modification, we obtained the Bcl2 mRNA sequence. Sequence analysis indeed revealed multiple matches of the 5'-RRACH-3'-m^6^A consensus sequence in Bcl2 mRNA (Figure 5C). The overexpression of Fto in primary neurons increased the Bcl2 mRNA and protein levels (Figure 5D–E). We examined whether Alkbh5 and Fto affect the stability of Bcl2 mRNA. To this end, primary cortical neurons undergoing Alkbh5 knockdown or Fto overexpression were treated with actinomycin D (Act D) to inhibit RNA elongation. Bcl2 mRNA levels were then examined using qRT-PCR. It was observed that, after treatment with Act D, Bcl2 mRNA was more unstable in primary cortical neurons undergoing Alkbh5 knockdown than in corresponding control cells (Figure 4F). Bcl2 mRNA was more stable in primary cortical neurons overexpressing Fto than in corresponding control cells (Figure 4G).

m^4^A methylation and demethylation drugs play a role in aggravating and reducing cerebral ischemia-reperfusion injury
In order to investigate the role of m^4^A in I/R, we used Betaine, which is used as a methyl donor to increase global m^4^A levels, and CL, which inhibits the activity of methionine adenosyltransferase, thereby reducing S-adenosylmethionine concentration and decreasing global m^4^A levels, during OGD/R treatment in primary cortical neurons. CCK8 data show that, compared with the OGD/R group, neuronal activity was decreased after adding Betaine (Figure 6C) and increased after adding CL (Figure 6C) following OGD/R treatment in primary cortical neurons. Similarly, we performed Hoechst 33342/PI double staining on the same treated cells. Consistent with CCK8 results, neuronal death was increased in the Betaine group (Figure 6A) and reduced after the addition of CL (Figure 6B) relative to the OGD/R group. Finally, we performed Western blot to quantify the levels of cleaved-caspase3 on the same treated cells. Cleaved-caspase3 was increased in the Betaine group (Figure 6D) and reduced after the
Figure 4. Alkbh5 knockdown enhanced OGD/R-treated primary neuronal apoptosis and Fto overexpression attenuated OGD/R-treated primary neuronal apoptosis. (A) Primary neurons were infected with Alkbh5-shRNA and then cultured under OGD/R conditions or normoxia. Apoptosis analysis was performed and representative flow cytometry images are shown. Quantifications of the percentage of apoptotic cells are shown (mean ± SEM; n = 3; ***p < 0.001 versus Ctrl, **p < 0.01 OGD/R + Alkbh5-shRNA versus OGD/R + Ctrl-shRNA). (B) Primary neurons were infected with Alkbh5-shRNA and then cultured under OGD/R conditions or normoxia prior to performing cell activity assays. Quantifications of the percentage of primary neuronal activity are shown (mean ± SEM; n ≥ 8; **p < 0.01 OGD/R + Alkbh5-shRNA versus OGD/R). (C) Primary neurons were infected with Alkbh5-shRNA and then cultured under OGD/R conditions or normoxia. Cell death was quantified by microscopy for PI-positive cells. Quantifications of the percentage of dead cells are shown (mean ± SEM; n = 8; *p < 0.05 versus OGD/R). (D) Primary neurons were infected with Lv-Fto and then cultured under OGD/R (Continued)
addition of CL (Figure 6E) relative to the OGD/R group.

Discussion
In this article, we reveal that Alkbh5 and Fto play important roles in cerebral ischemia-reperfusion injury. We first demonstrated that m6A modification levels increased after both neuronal OGD/R treatment and rat MCAO treatment, and that the demethylases Alkbh5 and Fto (but not the methylases Mettl3 and Mettl14) were mainly responsible for abnormal m6A modification. We also demonstrated that Fto reduction may result in apoptosis in OGD/R-treated neurons, and that compensatory increases in Alkbh5 protect against the development of ischemia-reperfusion injury. Interestingly, the overexpression of Fto alleviated neuronal damage in OGD/R-treated neurons. These findings demonstrate the treatment potential of the demethylases Alkbh5 and Fto in stroke patients. In addition, we found that Fto mRNA and protein levels were significantly reduced in neurons treated with OGD/R. This is therefore reason to believe that cerebral ischemia-reperfusion injury causes corresponding changes in Fto expression. Because Fto is a dioxygenase that oxidatively demethylates mRNA containing m6A,15 we hypothesized that ischemia or hypoxia would cause a decrease in Fto expression and function in cerebral ischemia-reperfusion injury. Because we observed that overexpression of Fto significantly reduced neuronal apoptosis after OGD/R treatment, this speculation seems reasonable. Since demethylating m6A in single-stranded nuclear RNA is a major function of Fto, we attributed the effect of Fto alteration directly to altered m6A levels in the target transcript. However, we do not rule out Fto-dependent N6,2'-O-methyladenosine (m6Am) demethylation,16 long non-coding RNA demethylation,13 or other indirect effects that regulate miRNA expression.

Furthermore, the demethylase Alkbh5 is a dioxygenase that uses α-ketoglutarate and O2 as substrates in the m6A demethylation reaction.17 The scientific fields examining Alkbh5 are mainly oncology,18 fertility,3 cerebellum development,5 and so on. Recent studies have shown that Alkbh5 and Mettl3 act together to regulate hypoxia/reoxygenation of cardiomyocytes by autophagy in an m6A-dependent manner.8 Our investigation also showed that Alkbh5 mRNA and protein levels were elevated in both OGD/R-treated neurons and MCAO-treated rat cerebral cortex. Consistent with previous studies, hypoxia induced Alkbh5 expression,19 but the previous article did not specifically report the function of Alkbh5. Considering that Alkbh5 is a demethylase, however, we
observed a paradoxical increase in m^6^A levels in Figure 1. We thought that the increase in Alkbh5 expression likely serves to compensate for stress responses in the brain or neuronal ischemia/hypoxia. Supporting our view, Mathiyalagan et al.\(^1\)\(^2\) found a compensatory increase in Alkbh5 expression around the infarct after heart failure. Knocking down Alkbh5 in OGD/R-treated
Betaine treatment enhanced OGD/R treated primary neuronal death and CL treatment attenuated OGD/R-treated primary neuronal death. (A–C) Primary neurons were treated with 8 mM Betaine or 20 mM CL and then cultured under OGD/R conditions or normoxia. Cell death was quantified by CCK8 assay or by microscopy for PI-positive cells. Quantifications of the percentage of dead cells were shown. (mean ± SEM; n = 6–20; *p < 0.05, ***p < 0.001 versus OGD/R). (D–E) Representative images of Western blots of primary neuron treated with 8 mM Betaine or 20 mM CL. Cleaved-caspase3 levels were determined by Western blot (mean ± SEM; n = 3; *p < 0.05 versus OGD/R). (F) A proposed model for Alkbh5 and Fto in cerebral ischemia-reperfusion injury.

CCK8, Cell Counting Kit-8; CL, cycloleucine; OGD/R, glucose oxygen deprivation/reoxygenation; PI, propidium iodide; SEM, standard error of the mean.

Figure 6.

neurons aggravated neuronal damage, indicating that the compensatory rise of Alkbh5 is likely neuroprotective. Interestingly, we performed immunohistochemical staining of MCAO-treated rat brain tissue, we found that Alkbh5 is differentially expressed between the MCAO group and the sham group. Alkbh5 is located mainly in the cg1 area and cg2 area on the caudal of anterior cingulate cortex. These areas are related to attention/perception in the rat, and dysfunction in these areas has also been implicated in attention deficit hyperactivity disorder and mental illnesses.20 In addition, stress regulates m6A/m in specific brain regions and impaired blood m6A/m modification observed in depressed patients.4 We must think about whether the specific spatial expression of Alkbh5 affects the pathophysiological process of post-stroke depression by affecting local brain methylation. A systematic review and another meta-analysis have concluded that the prevalence...
of depression between 1 and 5 years after stroke is estimated to be 30%. Therefore, the hypothesis that Alkbh5-dependent methylation is involved in post-stroke depression deserves further study.

Our results show that Alkbh5 positively regulates Bcl2. We hypothesized that Alkbh5 erased the m^6^A modification on Bcl2 mRNA, resulting in increased stability of Bcl2 mRNA. As previously reported, in epithelial ovarian cancer Alkbh5 increases the stability of Bcl2 by modulating m^6^A modification. Bcl2 is known to play a critical role in cerebral ischemic stroke, and our previous results also demonstrate that Bcl2 protein expression is significantly reduced in MCAO-treated rats. We thought that this reduction was most likely due to an increase in Bcl2 methylation levels, causing a decrease in Fto, leading to an increase in Bcl2 degradation. Therefore, we overexpressed Fto in neurons, which significantly increased Bcl2 mRNA. However, a recent paper reported that Mettl3-mediated dynamic m^6^A modification in human acute myeloid leukemia promoted Bcl2 translation. Therefore, we do not rule out the presence of some mechanisms other than m^6^A modification maintaining mRNA stability in cerebral ischemia-reperfusion injury. In summary, these results indicate that the m^6^A demethylases Alkbh5/Fto are involved, at least in part, in the mechanism of cerebral ischemia-reperfusion injury through m^6^A modification.

In addition, in order to demonstrate that m^6^A modification does play a role in I/R, we used drugs that are reported to promote and inhibit m^6^A modification. Consistent with the expected results, promoting m^6^A modification with Betaine aggravated the damage caused by OGD/R treatment. In contrast, inhibition of m^6^A modification with CL alleviated the damage caused by OGD/R treatment in neurons. However, previous studies have shown that feeding rats betaine protects them from the effects of traumatic brain injury. This is discrepancy probably due to the use of different models and drug concentrations. Thus, we do not completely rule out the biological effects caused by mechanisms other than these two drugs.

In conclusion, we revealed the mechanism of m^6^A modification after cerebral ischemia-reperfusion injury (Figure 6P). I/R-induced neuronal apoptosis was regulated by the m^6^A erasers Alkbh5/Fto. Therefore, Alkbh5/Fto are potential therapeutic targets for cerebral ischemia-reperfusion injury.

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Author contributions
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Conflict of interest statement
The authors declare that there is no conflict of interest.

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ORCID iD
Junlu Wang https://orcid.org/0000-0002-7486-2207

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