Protective role of mRNA demethylase FTO on axon guiding molecules of nigro-striatal projection system in manganese-induced Parkinsonism

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Abstract

Background

Parkinson's disease (PD) is a neurodegenerative disease caused by environmental and genetic factors. Manganese (Mn) exposure is a major environmental cause of PD. Cellular and molecular mechanism of Parkinsonism caused by Mn has not been explored clearly. In addition, patients with Mn-induced Parkinsonism show poor therapeutic response to levodopa. Therefore, there is need to explore the mechanisms underlying neurotoxicity of Mn exposure.

Methods

In short, we used SH-SY5Y cells and C57BL/6 mice to characterize Mn-induced Parkinsonism. We measured the behavioral, histological, ultrastructural and nigro-striatal projection system changes, cell viability, axon growth, and other target indicator levels, which led to the discovery of a novel mechanism of Mn-induced neurotoxicity.

Results

The findings of the current study showed that inhibition of fat mass and obesity-associated protein (FTO)-mediated demethylation of N⁶-methyladenosine (m⁶A) mRNA aggravates Mn-induced motor dysfunction. Notably, FTO level is low in Mn exposure model mice and enhances occurrence of dyskinesia in mice. Over-expression of FTO reduces m⁶A methylation in the key axon guiding molecules of nigro-striatal projection system, including ephrin-A5 and ephrin-B2. It increases ephrin-B2 mRNA decay through the m⁶A reader YT521-B homology domain family proteins 2 (YTHDF2).

Conclusions

The findings of this study show that FTO, a m⁶A demethylase, performs an indispensable function in Mn-induced Parkinsonism. Notably, re-expression of FTO and ephrin-B2 improved motor dysfunction after Mn exposure.

Background

Parkinson's disease (PD) is a neurodegenerative disease caused by environmental and genetic factors [1]. Approximately 3 million PD cases are reported in China, and WHO predicts that this number will increase to 5 million by 2030 [2]. Most PD cases are sporadic cases caused by environmental factors, and only less than 10% of PD cases are caused by genetic factors [3]. Manganese (Mn) exposure is one of the major environmental risk factors that cause PD [4]. Mn is a quintessential component that is important

for human health. It is an enzyme cofactor involved in neurotransmitter synthesis and in normal neuron and glial function [5]. Exposure to high levels of Mn can cause headache, lower limb weakness, cognitive impairment, muscle tone increase, tremor, gait difficulties and other symptoms similar to those of PD. The condition is known as manganism [6], or Mn-induced Parkinsonism [7, 8]. The *2019 Report on the State of the Ecology and Environment in China* reported that Mn is one of the heavy metals that exceed sanitary requirements of ingesting water [9]. China has adopted active measures, such as clean production, use of gasoline with limited Mn and elimination of Mn from drinking water. However, environmental Mn pollution is still at an excessive level due to challenges in corporate governance, poor government supervision, and insufficient maintenance of equipment. In the central nervous system (CNS), excessive Mn mainly accumulates in the striatum [10, 11]. Study reports that manganism is associated with the modifications of striatal dopamine (DA) pathway, which is mainly manifested by decrease in DA content [12]. However, studies on the molecular mechanisms for Mn induced dysregulation of DA levels are limited. In addition, specific mechanisms involved in motor dysfunction associated with simultaneous exposure to Mn have not been explored.

Accurate establishment of neural circuits involves the axon navigating the neurons to their target [13], which is essential for DA neurons of the substantia nigra to release DA after projecting to the striatum. Studies report that axon guidance regulation of dopaminergic neurons and spinal motoneurons plays important role in brain development [14, 15]. The divergent member of the erythropoietin-producing hepatocyte receptor-interacting proteins (ephrins) mediate cell-cell communication thus regulating axon guidance and neural projection. Ephrins promotes developmental axon pathfinding by modulating actin cytoskeleton dynamics [16]. Researchers report that ephrins are abundantly expressed in the striatum [17]. Sieber *et al*/[18] reported that ephrin signaling is important in normal connectivity and function of midbrain DA neurons. The present study postulated that Mn regulation of the motor function may be associated with the function of ephrins signaling. However, the specific molecular mechanisms were not elucidated.

Hundreds of RNA modifications have been reported, and N⁶-methyladenosine (m⁶A) is one of the most ubiquitous internal changes that regulate gene expression at the post-transcriptional level [19]. m⁶A modification regulates several phases of RNA metabolism, such as RNA translation, decay and splicing [20, 21]. The latest reported m⁶A demethylase FTO shows that m⁶A modifications can be dynamically regulated [22]. However, the targets and features of m⁶A modification in CNS have not been explored. m⁶A modification can regulate neuronal function such as motor function [23], axon regeneration [24], and synapse function [25]. In addition, Hess *et al*/reported that inhibition of FTO gene impairs neuronal activity and motor function [26]. However, the function of m⁶A eraser FTO on axon guiding molecules of nigro-striatal projection system in Mn-induced Parkinsonism pathogenesis has not been fully elucidated.

The findings of the current study show that FTO is implicated in regulation of motor function and mediation of axon guiding molecules against Mn-induced Parkinsonism *in vitro* and *in vivo*. These findings show the specific mechanism of Mn-induced motor dysfunction mediated by FTO and ephrins.

Moreover, these findings show that re-expression of FTO and ephrin-B2 improve motor dysfunction after Mn exposure.

Materials And Methods

Reagents and chemicals

Manganese chloride ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$) and actinomycin D, and other chemical compounds were bought from Sigma Chemical Co. (Saint Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was supplied with Transgen Institute of Biotechnology (Beijing, China). FD Rapid GolgiStain™ kit was supplied by FD NeuroTechnologies (Ellicott City, MD, USA). Enzyme-linked immunosorbent assay (ELISA) kits of mice DA was acquired from Baolai Biotechnology Institute (Jiangsu, China). Ethyl ester derivative of MA (MA2, the FTO inhibitor) was a present from Professor Caiguang Yang of the Chinese Academy of Sciences. Fluoro-Gold (FG) was purchased from Kaiji Biotechnology Co., Ltd. (Jiangsu, China). The adeno-associated virus 5 (AAV5)-FTO and AAV5-ephrin-B2 were purchased from Genecreate Biological Co., Ltd. (Wuhan, China). Lentiviral particles for over-expression of FTO (FTO-OE) were purchased from Hesheng Gene Technology Co., Ltd. (Beijing, China). Anti-m⁶A antibody was bought from Synaptic Systems (USA, 202003). Protein-A bead was bought from Thermo Fisher (USA, 10002D). RNasin was provided by Promega Biotechnology Co., Ltd. (Beijing, China). Rabbit antibody of FTO, methyltransferase like 3 (METTL3), α -ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5), YTH domain-containing family proteins 1/2 (YTHDF1/2) were bought from Proteintech Group, Inc (Wuhan, China). Rabbit antibody of tyrosine hydroxylase (TH) was bought from Cell Signaling Technology, Inc. Rabbit antibody of METTL14, ephrin-A1/3, ephrin-B1/3 were sold from ABclonal Biotechnology Co., Ltd. (Wuhan, China). Rat antibody of dopamine transporter (DAT), mouse antibody of ephrin-A5, ephrin-B2, Dopamine- and cAMP-regulated neuronal phosphoprotein 32 kDa (DARPP32) and GADPH were offered from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Mouse antibody of ephrin-A2/4 was bought from Boasosen Biotechnology Co., Ltd. (Beijing, China). Horseradish peroxidase (HRP) conjugated anti-rabbit, mouse and rat secondary antibody were provided by means of Boasosen Biotechnology Co., Ltd. (Beijing, China). Real-time quantitative PCR (RT-qPCR) kit and Trizol reagent were supplied by TaKaRa Biotechnology Co, Ltd (Dalian, China). Other chemical substances were supplied through neighborhood chemical suppliers. All chemical substances reagents have been analytical grade or the pharmaceutical grade.

Animals

The research was carried out on adult C57BL/6 mice (20 ± 2 g; N = 350; Among them, 30 pregnant mice are needed for primary neuron culture) which have been bought from the Laboratory Animal Centre of China Medical University, Shenyang, China (SPF grade, certificate no. SCXK2013-0001). The mice were housed in plastic cages with 12-h light/dark cycle (temperature, $25^\circ\text{C} \pm 1^\circ\text{C}$, humidity, $55\% \pm 5\%$). Water and Food were accessible to the mice. All experiments were performed following the care and use guidelines of laboratory animals by Institutional Animal Care and Use Committee of China Medical University. The study protocol conformed to the National Institutes of Health Guide for Care and Use of

Laboratory Animals (NIH Publications No. 8023, revised 1978). Attempts were made to minimize the number of animals used and to reduce animal suffering.

Primary neuron and SH-SY5Y culture

Neonatal C57 BL/6 mice were decapitated with scientific scissors without use of an anesthetic. The cranium was opened with ophthalmic forceps after sterilization with 75% alcohol. D-Hank's buffer solution was added to the dissected striatal tissue, and routinely separated with surgical devices under a light microscope. The tissue was digested with 0.125% trypsin solution for 20–30 min, and diluted with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) containing 20% fetal bovine serum (FBS). Isolated neurons were seeded into 60-cm² plastic tissue culture flasks precoated with poly-L-lysine after purification by filtration through a 200-mesh sieve. Cultures were maintained in 5% CO₂/95% air at 37°C. After cell attachment, DMEM medium was replaced with Neurobasal™-A Medium supplemented with B27 (Life Technologies). Neurons were further purified by addition of cytosine arabinoside and cultured for 24 h (5 µM, Sigma-Aldrich). Primary cultures yielded more than 95% neurons, which was determined through NeuN antibody immunostaining after culturing for approximately 7 days.

SH-SY5Y cells were purchased from the National Centre for Cell Sciences of China. Cells were maintained in 5% CO₂/95% air at 37°C and cultured with DMEM supplemented with 10% FBS, streptomycin, and penicillin.

Animal experiments

One hundred forty mice were randomly assigned to sixteen groups based on their weight, with 12 mice in each group. The first part included the control group, 12.5, 25, and 50 mg/kg MnCl₂ groups. The second part included the AAV5 negative control (NC) group, AAV5-FTO control group, AAV5-NC + 50 mg/kg MnCl₂ group, and AAV5-FTO + 50 mg/kg MnCl₂ group. The third part included the control group, 20 mg/kg MA2 control group, 50 mg/kg MnCl₂ group, and 20 mg/kg MA2 + 50 mg/kg MnCl₂ group. The fourth part included the AAV5-NC group, AAV5-NC + 50 mg/kg MnCl₂ group, AAV5-NC + 20 mg/kg MA2 + 50 mg/kg MnCl₂ group, and AAV5-ephrin-B2 + 20 mg/kg MA2 + 50 mg/kg MnCl₂ group. Mice in the control group received a subcutaneous (s.c.) injection of 0.9% NaCl and intraperitoneal (i.p.) injection of 0.9% NaCl after 2 h. Mice in the MnCl₂ groups received s.c. injections of 0.9% NaCl and i.p. injections of 12.5, 25, and 50 mg/kg MnCl₂ after 2 h. Mice in the MA2 control group were administered with s.c. injections with 20 mg/kg MA2, and i.p. injections of 0.9% NaCl after 2 h. Mice in the 20 mg/kg MA2 + 50 mg/kg MnCl₂ group received s.c. injections with 20 mg/kg MA2, and i.p. injections of 50 mg/kg MnCl₂ after 2 h. The concentration of treatments were 5 mL/kg. Treatments were administered daily two weeks.

For AAV5 viral injection, mice were anaesthetized with 5% chloral hydrate and positioned on a stereotaxic apparatus. Bilateral holes were drilled into the cranium at 0.5 mm foreside and 2.8 mm outside the bregma for administration of treatments into the striatum regions. An AAV5 expressing either FTO or ephrin-B2 (AAV5-FTO or AAV5-ephrin-B2) or hrGFP (AAV5-NC) was obtained through stereotactic injection.

A glass cannula stuffed with a virus was placed on the striatum (-3.5 mm), and 0.2 μL virus was administered at a rate of 0.1 $\mu\text{L}/\text{min}$. The scalp was then sealed. Follow-up exposure was carried out 10 days after virus injection, and then behavioral experiments or other tests were carried out.

After the behavioral experiment, all the mice were sacrificed through decapitation under anesthesia. Striatum of 20 mice in each group was separated in an ice bath. 4 out of the 20 mice were used for histological and ultrastructural analysis of the striatum. Among the other 16 mice from each group, 4 were used for the detection of Mn and DA levels. 2 were used for immunofluorescence detection. 2 were used for determination of ephrin-B2 m⁶A mRNA expression, 2 for electrophysiological recording, and 2 mice were used for fluorogold retrograde tracing. Striatum for the other mice was used for determination of target mRNA level, protein, and other indicator levels.

Lentiviral generation and infection.

Lentiviral particles for over-expression of NC and FTO were purchased from Hesheng Gene Technology Co., Ltd. (Beijing, China). For over-expression of ephrin-B2, METTL3, METTL14, YTHDF1 or YTHDF2, cells were transfected with plasmids using lipofectamine 2000 (Sigma, USA) following the manufacturer's instructions. After 48–72 h of transfection, the virus-containing supernatant was obtained and used to infect target cells, followed by screening with puromycin (Solarbio, 2.5 $\mu\text{g}/\text{mL}$) for 7 days.

Cells treatments

Cells were cultured in media for 24 h with 0, 125, 250, and 500 μM MnCl_2 . In the 0 and 500 μM MnCl_2 groups, cells were used for over-expression of FTO, ephrin-B2, YTHDF2, FTO + METTL3 + METTL14, FTO + ephrin-B2, and FTO + YTHDF1 or YTHDF2. In addition, For the MA2 intervention groups, cells were cultured in media for 24 h with 0 μM MnCl_2 , 200 μM MA2, 500 μM MnCl_2 , and 200 μM MA2 (pretreated for 6 h) + 500 μM MnCl_2 . The relevant experiments were then carried out.

Cell viability analysis

The cells were seeded into 96-well plate at $5 \times 10^3/\text{well}$ and cultured in 10% FBS containing DMEM for 24h. Then, cells were treated in 100 μL of DMEM according to the above groups. After incubating for 24 h, add 10 μL of CCK-8 reagent to every well, and incubate for extra 4h. Absorbance of every well was measured at 450 nm. Each well has two duplicate wells to confirm the reproducibility.

Automated observation of axon growth with the live cell imager

Cells and axon growth trends were observed using Lionheart FX Automated Live Cell Imager (BioTeK, USA). The relevant treatment was given to every group following the study design. The control group was used to adjust the photo parameters. The duration of observation was 24 h, and data were recorded every hour.

Rotarod test

Mice were positioned on a Rotary rod instrument (UGO 47650, Italy) and skilled for 5 min at the 10 rpm speed for 3 days. For testing, mice were positioned on a rod that turned around accelerating velocity beginning with 5 rpm and accelerating up to 30 rpm in 60 s. Experiments were performed three times.

Forced running wheel test

Mice were positioned on a wheel fatigue meter (YLS-10B, Yiyao, China) to explore their staying power capabilities. Mice were skilled at 30 rpm for 10 min, and the fatigue running distance was determined. The wheel fatigue meter was stopped if mice fail to run within 10 sec after the electric shock (1 mA) and the wheel fatigue meter was started after mice rest for 30 sec. The experiments were performed in triplicates.

Open field test

The open field experimental analysis system for mice was provided by China Medical University. The mice were placed in the laboratory to adapt to the environment for 10 minutes. This experiment is performed in a quiet, low-light environment. The indoor temperature was maintained at about 26°C. The mice were positioned in the open field and the mice activity was observed within 5 min. Observation indicators include total horizontal movement distance and horizontal movement speed. Clean up the labyrinth after the experiment of each mouse, and try to avoid external interference that will cause unnecessary adverse effects on the experimental results.

Gait analysis

In order to enable all mice to walk to the end of the runway at a uniform speed without external stimulation, the mice were trained for 3 days. Data was collected on the 4th day and recorded 3 times for each mouse. The collected data was analyzed with Cat Walk XT Version10.6, and the average value of each index was taken. The related indexes are run duration, average speed, swing speed, and maximum variation.

Determination of Mn levels

The inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x, USA) was used for analysis of Mn levels in striatum of mice. Striatum was predigested, and 0.1, 1, 5, 10, 50, and 100µg/L series of Mn standard solutions were prepared for future use. The content of Mn in the striatum was then determined with internal standard method.

Measurement of DA levels

Total DA levels in the striatum were quantified using ELISA kits of mice DA (Baolai, Jiangsu, China) following the manufacturer's directions. The sample value within the detectable range was used for analysis. Experiments were performed in triplicates.

Immunofluorescence of FTO, ephrin-B2, TH, DARPP32, and DAT

Expression and localization of FTO and ephrin-B2 were determined in striatum and neurons, respectively, through immunofluorescence. In addition, immunofluorescence triple staining of TH, DARPP32 and DAT in striatum was used to explore whether nigro-striatal projection neurons were damaged. Samples were dehydrated with xylene and alcohol, and tissue sections were steamed with sodium citrate for antigen retrieval. Samples were blocked in 10% Goat serum for 30 min at 25°C. Samples were then treated with rabbit antibody of FTO (1:100), mouse antibody of ephrin-B2 (1:100), rabbit antibody of TH (1:100), mouse antibody of DARPP32 (1:50), and rat antibody of DAT (1:50) overnight at 4°C. On the second day, after 1 h of rewarming and washing 3 times with phosphate buffered solution (PBS), samples were treated with goat anti-mouse IgG (1:100), goat anti-rabbit IgG (1:100), and goat anti-rat IgG (1:100) for 1 h at 25°C. For immunofluorescence of FTO and ephrin-B2, samples were counterstained using 2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride (DAPI) for 5 min after washing 3 times with PBS. Then, samples were mounted with mounting medium for further study. Laser scanning confocal microscope (A1R, Nikon, Japan) was used to capture images.

Hematoxylin and eosin (HE) staining

After the mice were perfused with 4% paraformaldehyde, the striatum tissue was removed. The striatum of mice was serially dehydrated in xylene and ethanol. The striatum was sectioned at 5 µm thickness after paraffin embedded. Then, HE staining was done for morphological observation with high resolution panoramic imaging system (CS2, Leica, Germany).

Golgi-cox staining

Brains of mice were removed quickly from the skull after euthanizing. After washing, the brains were stained with the FD Rapid GolgiStain™ kit (MD, USA). Firstly, the brains were incubated in the impregnation solution (A and B) for 14–15 days. Afterwards, the brains were treated with Solution C for the 48–72 h. Microtome Cryostat (1950, Leica, Germany) was used to cut 200 µm slices. Slices were mounted on a gelatin-coated slides, then dehydrated, stained, and coverslipped. Then, dendritic spines was observed with high resolution panoramic imaging system (CS2, Leica, Germany).

Nissl staining

Tissue sections were sequentially stained with series alcohol dehydration. The sections were then treated with 1% thionine for 1 h, then the samples were washed with distilled water. 70% alcohol color separation was performed for several sec to several minutes. The sections were dehydrated with 70%, 80%, 95%, and 100% alcohol, re-spectively, with 2 min for each. Next, the sections were washed with anhydrous ethanol twice (with 5 min for each time), followed by washing with xylene twice (with 10 min for each time). Finally, the sections were observed with high resolution panoramic imaging system (CS2, Leica, Germany).

Fluorogold retrograde tracing

Two mice in each group were injected with 2% FG solution into the striatum via brain stereotaxic injection. After 5 days of survival, the mice were anesthetized and perfused with 4% paraformaldehyde solution. The brain was taken out and fixed with 4% paraformaldehyde solution for 4 hours. Then the brain tissue was placed in a 20% sucrose solution at 4°C overnight. The next day, freeze sectioning was performed, and the section thickness was 30 µm. The sections were washed with 0.01 mM PBS and fixed in 4% paraformaldehyde for 10 min. After washing 3 times with PBS, samples were mounted with mounting medium for further study. Laser scanning confocal microscope (A1R, Nikon, Japan) was used to capture images.

Electron microscopy studies

After the mice were perfused with 4% paraformaldehyde, the striatum was fixed with 2.5% glutaraldehyde for 1h. A 1-mm-thick slice was made and fixed overnight. Postfixation of slice was carried out in 1% OsO₄ for electron microscopy observation. Then, tissues were embedded in spur resin after dehydrated in series of acetone. Blocks were photographed in a transmission electron microscope (JEM-1400-FLASH, Japan) after stained with uranyl acetate and lead citrate.

Electrophysiological recording of striatum slices

Spontaneous discharge frequency in the striatum was recorded in 300 µm slices. Slices were cut using vibratome in a modified artificial cerebrospinal fluid (mACSF) saturated with 5% CO₂/95% O₂. Slices were then placed on an incubation chamber with normal ACSF and incubated at 25°C for at least 1 h before recording. Analysis was performed with a MED64 flat microelectrode array system (Alpha of Japan).

Western Blotting analysis

Total protein of striatum was extracted with RIPA buffer and protease inhibitors. BCA reagent was used for protein quantification. Proteins samples were transferred onto polyvinylidene difluoride (PVDF) membranes after gel electrophoresis. PVDF membranes were then blocked using 5% bovine serum albumin fraction V for 1h, then the membranes were incubated with primary antibody against FTO (1:500), METTL3 (1:1000), METTL14 (1:500), ALKBH5 (1:1000), YTHDF1 (1:2000), YTHDF2 (1:1000), ephrin-A1 (1:1000), ephrin-A2 (1:500), ephrin-A3 (1:1000), ephrin-A4 (1:500), ephrin-A5 (1:500), ephrin-B1 (1:1000), ephrin-B2 (1:1000), ephrin-B3 (1:500), and GAPDH (1:5000) overnight at 4°C. In the next day, membranes were incubated with corresponding secondary antibodies for 1 h at 25°C after washing 3 times with Tris-buffered saline with 0.1% Tween 20 (TBST). Analysis was performed with the multifunctional imaging system (AZURE C500, USA). GAPDH was used as an internal standard.

RT-qPCR assay

Total RNA was dissolved in RNase-free water (TaKaRa) after extraction using the Trizol reagent (TransGen, Beijing, China). First strand cDNA was obtained from total RNA using reverse transcriptase Kit (TaKaRa) following manufacturer's instructions. RT-qPCR was performed using QuantStudio 6 Flex

fluorescence quantitative PCR instrument (Thermo Fisher, USA). Primer sequences of FTO, METTL3, METTL14, ALKBH5, YTHDF1, YTHDF2, ephrin-A1, ephrin-A2, ephrin-A3, ephrin-A4, ephrin-A5, ephrin-B1, ephrin-B2, ephrin-B3, Grb4, GIT1, HPRT1, and β -actin for RT-qPCR analysis are presented in Supplementary Table 1. HPRT1 or β -actin genes were used as internal controls. Comparative CT method ($\Delta\Delta$ CT) was used to quantify the target genes. All experiments were performed in triplicates.

Quantification of m⁶A in total RNA

The ELISA-based m⁶A Quantitative Kit (Epigentek, P-9005-96) was used to quantify m⁶A levels of total RNA (200 ng). In accordance to the manufacturer's instructions, quantification was performed in triplicates.

Actinomycin D assay

Samples were treated with 2 μ M actinomycin D (Act-D, Sigma, USA) for 0, 3, and 6 h. Cells were collected and RNA was isolated for RT-qPCR. HPRT1 gene was used as an internal control as HPRT1 mRNA does not bind to YTHDF2, is rarely affected by Act-D, and does not undergo m⁶A modifications.

m⁶A-RT-qPCR

Total RNA was used for m⁶A-immunoprecipitation and 500 ng of RNA was used as an input sample. 100 μ g of RNA was diluted with 40 μ L IP buffer (0.05 M Tris-HCl, 0.75 M NaCl, 0.5% Igepal, and RNase-free water) and treated with m⁶A antibody (Synaptic Systems, Germany) for 3.5 h at 4°C. Recombinant protein-A bead (Waltham, MA) containing BSA was added to the RNA solution and shaken at 4°C for 4 h. The m⁶A IP portion was eluted with elution buffer (300 μ L 20mM CaCl₂, 2 μ L RNasin, 2 μ L RVC, and 0.75 μ L 20 mg/mL Proteinase K) after washing three times with IP buffer. The collected total eluate was concentrated in an RNA concentrator to a final volume of 20 μ L. The concentration of RNA was determined and reverse transcribed to cDNA. RT-qPCR was used to determine m⁶A levels of mRNA. Data were calculated using the %input = $2^{(-\Delta C_{t\text{normalized RIP}})}$ method.

Statistical analysis

Results were presented as means $\pm$ standard deviations after measured at least three times. The SPSS 22 software was used to analysis all statistical. The one-way analysis of variance (ANOVA) followed by Bonferroni test were used to determined the differences between the means for multiple comparison. *P*-value of < 0.05 or *P*-value of < 0.01 were considered statistically significant.

Results

Effect of FTO on Mn-induced parkinsonism

To explore the role of FTO in Mn-induced striatal motor dysfunction, the level of Mn in normal striatum and Mn-exposed striatum was determined by ICP-MS. The findings showed that levels of Mn in striatum increased significantly after Mn exposure compared with the control group (Fig. 1A). Further, mRNA and protein levels of FTO and other methylases in striatum and neurons were determined. Analysis showed that mRNA and protein levels of FTO were significantly downregulated after Mn exposure compared with the control group (Fig. 1B, C, F, and G). Notably, other methylases levels including METTL3/14, ALKBH5, and YTHDF1/2 were not significantly different after Mn exposure compared with the control group (Supplementary Fig. 1A-T). Immunofluorescence was used to determine specific FTO protein levels in striatum and neurons. Analysis showed that FTO level (Green) was significantly decreased after Mn exposure compared with the normal group (Fig. 1D, H). In addition, m⁶A ELISA was used to determine changes in total RNA m⁶A level in striatum and neurons after Mn exposure. The findings showed that the level of total RNA m⁶A in striatum and neurons increased significantly after Mn exposure compared with the control group (Fig. 1E, I). These findings indicate that FTO is downregulated in striatum and neurons after Mn exposure, resulting in significant increase in the level of total RNA m⁶A in striatum. This implies that FTO plays an inhibitory role in Mn-induced motor dysfunction.

Role of FTO in Mn-induced motor dysfunction and morphological analysis

Effect of over-expression FTO or inhibition of FTO activity in the striatum on regulating the symptoms was explored to verify that Mn-induced motor disorders were caused by down-regulation of FTO in the striatum. AAV5 were used to express FTO (AAV-FTO) or hrGFP (AAV-control) in the striatum through stereotactic injection (Supplementary Fig. 2A, B), resulting in selective re-expression in bilateral striatum (Fig. 2A and B, Supplementary Fig. 2C). In addition, activity of FTO was inhibited by intraperitoneal administration of MA2, a selective inhibitor of FTO. The results showed that treatment of MA2 did not affect FTO expression (Supplementary Fig. 2D and E). Re-expression of FTO in mice exposed to Mn significantly decreased total RNA m⁶A levels, with no significant effect on levels of Mn in the striatum (Fig. 2C and Supplementary Fig. 3A). On the contrary, inhibition of FTO activity in mice exposed to Mn significantly increased total RNA m⁶A levels, with no significant effect on levels of Mn in the striatum (Supplementary Fig. 2F and Supplementary Fig. 3K). Re-expression of FTO in striatum exposed to Mn significantly increased their endurance times and fatigue running distance (Fig. 2D and E). Furthermore, it improved the distance covered and speed in the open field (Fig. 2F and Supplementary Fig. 3B-D). In addition, it significantly improved mice gait, mean intensity, run duration, average speed, swing speed and maximum variation performances in gait tests (Fig. 2G and Supplementary Fig. 3E-J). On the contrary, motor function was damaged more severely after inhibition of FTO activity (Supplementary Fig. 2G-J and Supplementary Fig. 3L-T). These findings indicate that inhibition of FTO significantly reduces motor performances, implying that FTO plays a protective role in Mn-induced motor dysfunction.

Effects of Mn on striatum morphology were determined by HE staining. In the control group, the nucleus of striatum was stained clearly. The nucleus was round and giant with evident nucleoli (Fig. 2H). The 50

mg/kg MnCl_2 group (Fig. 2H) showed significant interstitial vacuolation and cell swelling compared with the control group. Re-expression of FTO significantly improved neuropathological injury compared with the 50 mg/kg MnCl_2 group (Fig. 2H). Nissl staining in the control group showed that Nissl bodies were clearly stained, arranged neatly and the number was normal (Fig. 2I). Analysis showed significant degeneration of Nissl bodies, deletion and lighter staining in the 50 mg/kg MnCl_2 group compared with the normal control group (Fig. 2I). Re-expression of FTO significantly alleviated deformation and deletion of Nissl bodies compared with the 50 mg/kg MnCl_2 group (Fig. 2I). Observation under an electron microscope showed that the nucleus of the control group was approximately round and the structure was normal (Fig. 2J). Neuronal nucleus pyknosis, organelle disappearance, swelling and degeneration were observed in the 50 mg/kg MnCl_2 group (Fig. 2J). Notably, re-expression of FTO significantly improved neuronal damage compared with the 50 mg/kg MnCl_2 group (Fig. 2J). Changes in dendritic density and/or morphology were investigated to explore the effects of Mn on axonal guidance regulates synaptic plasticity (Fig. 2K-L). Morphology of dendrites was significantly damaged and the density of total dendritic spines decreased significantly after exposure to 50 mg/kg MnCl_2 compared with normal control group (Fig. 2K-L). Re-expression of FTO significantly improved the morphology of dendrites and the density of total dendritic spines compared with the 50 mg/kg MnCl_2 group (Fig. 2K-L).

Spontaneous discharge activity reflects the function of striatal neurons. Acute brain slices from mice were used to record electrical signals through substrate embedded electrodes to explore the discharge activity of the striatum. A typical electrical signal obtained using a microelectrode is shown in Fig. 2M. Individual spontaneous potential was recorded when the individual spontaneous potential exceeds the threshold (± 0.02 mV). Neurons from the striatum of the control group showed normal spontaneous activity (Fig. 2M). The amplitude and frequency of spike waves decreased after treatment with 50 mg/kg MnCl_2 compared with the control group (Fig. 2M). Re-expression of FTO significantly improved the damaged spontaneous discharge frequency compared with the 50 mg/kg MnCl_2 group.

On the contrary, the morphology was severely damaged and electrophysiological signals of striatum were significantly reduced after inhibition of FTO activity compared with over-expression of FTO (Supplementary Fig. 2K-P). These findings indicate that FTO plays a promoting role in Mn-induced striatal injury.

Effect of Mn exposure on nigro-striatal projection system

To explore the role of Mn treatment on nigro-striatal projection system, FG labeling (Fig. 3A), the immunofluorescence triple staining of TH, DARPP32 and DAT (Fig. 3B), the DA levels (Fig. 3C) and neuronal axons growth (Fig. 3D-E) were determined to evaluate the survival rate of nigro-striatal neurons. In the control group, the boundary of neurons was clear and evident cell body were observed (Fig. 3A). In the 50 mg/kg MnCl_2 group, the boundary of neurons was unclear, the distribution of cells was uneven, the staining was lighter and disappeared significantly compared with the control group. (Fig. 3A). Re-expression of FTO significantly improved survival rate of nigro-striatal projection neurons compared with the 50 mg/kg MnCl_2 group (Fig. 3A).

TH, DARPP32 and DAT are specific markers of nigro-striatal projection neurons, and their expression can reflect damage of nigro-striatal projection neurons. After treatment with 50 mg/kg MnCl_2 , the nigro-striatal projection neurons marked with TH, DARPP32 and DAT were significantly reduced, and the projection system was severely damaged compared with the control group (Fig. 3B). Re-expression of FTO significantly restored the damage to the projection system compared with the 50 mg/kg MnCl_2 group (Fig. 3B). After treatment with 50 mg/kg MnCl_2 , DA levels decreased significantly compared with control group (Fig. 3C). DA levels increased significantly after re-expression of FTO compared with the 50 mg/kg MnCl_2 group. Neuronal axons grew slowly and shrank in the 50 mg/kg MnCl_2 group compared with control group (Fig. 3D and E). Neuronal axon length increased significantly after re-expression of FTO compared with the 50 mg/kg MnCl_2 group. On the contrary, the substantia-striatal projection system was significantly damaged after inhibition of FTO activity compared with that after over-expression of FTO (Fig. 3F-J).

Role of FTO function in neuron and SH-SY5Y cells

To explore the role of FTO in neuron and SH-SY5Y cells, the levels of FTO and total RNA m^6A and performed cytotoxicity were determined using neuron and SH-SY5Y cells (Fig. 4A-H). Over-expression of FTO (Fig. 4A-B, and Supplementary Fig. 4A) in Mn-induced FTO-low neuron and SH-SY5Y cells decreased the levels of total RNA m^6A , and cytotoxicity (Fig. 4C-D). Inhibition of FTO activity in neurons and SH-SY5Y cells significantly increased the levels of total RNA m^6A and cytotoxicity (Fig. 4E-H). These findings indicate that FTO alleviates Mn-induced cytotoxicity *in vitro*.

Role of m^6A in FTO functions in neuron and SH-SY5Y cells

To explore the effect of m^6A in neuron and SH-SY5Y cells function, the gain-of-function of the m^6A writers METTL3/14 were determined (Fig. 5A-D, and Supplementary Fig. 4B-E). Forced over-expression of METTL3/14 significantly increased total m^6A levels (Fig. 5E, and Supplementary Fig. 4F) and decreased cell viability (Fig. 5F, and Supplementary Fig. 4G). Furthermore, over-expression of METTL3/14 inhibited effects of over-expression of FTO on the total m^6A levels and cell viability (Fig. 5E-F, and Supplementary Fig. 4F-G). These findings show that m^6A modification of mRNA increases toxicity and viability of Mn exposure on cells, indicating that m^6A exacerbates Mn-induced parkinsonism.

Identification of key target genes for FTO function

Axon guiding molecules are expressed in the nigro-striatal projection system, whereas ephrins are mainly expressed in the striatum [17, 27]. Inactivation of the FTO gene impairs DA receptors, controls activity and behavior of neurons in a dose-dependent manner. Therefore, analysis was performed to determine if ephrins are key targets of FTO after Mn exposure. Analysis showed that Mn exposure significantly reduces levels of ephrin-A4/5 and ephrin-B2 compared with the control group. Notably, levels of other ephrins of Mn exposed group showed no significant difference with those in the control group

(Fig. 6A-B, and Supplementary Fig. 5A-D). Analysis showed that over-expression of FTO significantly increased ephrin-A5 and ephrin-B2 levels (Fig. 6C-H, and Supplementary Fig. 5E-F). In addition, over-expression of FTO significantly decreased mRNA m⁶A levels of ephrin-A5 and ephrin-B2, with no significant effect on expression of ephrin-A4 (Fig. 6I). On the other hand, downregulation of FTO activity significantly decreased ephrin-A5 and ephrin-B2 levels, and increased mRNA m⁶A levels of ephrin-A5 and ephrin-B2 (Supplementary Fig. 5E-G). Notably, over-expression of FTO showed no effect on expression of other methylases (Supplementary Fig. 5H-K) implying that Mn affects expression of ephrins only by regulating FTO. These findings indicate that FTO inhibits expression of ephrin-A5 and ephrin-B2 after Mn exposure.

Role of YTHDF2 on function of FTO in neurons

m⁶A regulates gene expression through YTH domain-containing family proteins [28]. In the present study, a gain function test was performed to explore mechanism of regulation of gene expression by Mn via determining m⁶A RNA methylation (Fig. 7A) of YTHDF1/2. Over-expression of YTHDF1 showed no effect on the mRNA levels of ephrin-A5 and ephrin-B2 in the control group and FTO over-expression cells (Fig. 7B-E). However, over-expression of YTHDF2 significantly decreased levels of ephrin-B2 in both control and FTO over-expression cells, with no significant effect on expression of ephrin-A5 (Fig. 7F-I). In addition, over-expression of FTO significantly increased the stability of ephrin-B2 mRNA (Fig. 7J). Over-expression of YTHDF2 decreased the stability of ephrin-B2 mRNA (Fig. 7K). Immunofluorescence double staining of FTO and ephrin-B2 showed that Mn did not change the position of FTO and ephrin-B2. However, it decreased expression level of FTO and ephrin-B2. Notably, over-expression of FTO antagonized the effect of Mn (Fig. 7L). Grb4 and GIT1 are downstream target genes of ephrins, and play key roles in regulating axonal growth. The findings of the present study showed that Mn treatment decreased mRNA expression of Grb4 and GIT1 (Fig. 7M). Over-expression of FTO and activation of ephrin-B2 significantly increased expression of Grb4 and GIT1 (Fig. 7N-P). These findings show that YTHDF2 modulates expression of FTO target genes by mediating RNA decay.

Role of FTO and ephrin-B2 in Mn-induced motor dysfunction

The relationship between ephrin-B2 and FTO-mediated motor dysfunction was explored. Forced activation of ephrin-B2 in FTO gene suppressor mice significantly increased performance of mice in rotating rod, fatigue meter, open field and gait tests (Fig. 8A-D and Supplementary Fig. 6A-I). Similarly, forced activation of ephrin-B2 in FTO suppressor mice significantly alleviated Mn-induced striatal morphological and functional damage (Fig. 8E-J). In addition, forced activation of ephrin-B2 in FTO suppressor neuronal cells significantly improved Mn-induced nigro-striatal projection system damage (Fig. 8K-O). These findings show that ephrin-B2 is a potential target of FTO in the striatum. In summary, the findings of the current study indicate that FTO plays a critical role in inhibiting effect of ephrin-B2 in Mn induced nigro-striatal projection system dysfunction, implying that activation of FTO can promote expression of ephrin-B2 and then antagonize Mn-induced motor dysfunction.

Discussion

PD is a neurodegenerative disease characterized by bradykinesia. In the top 10 most populated countries in the world, the number of people with PD is projected to be between 8.7 and 9.3 million by 2030 [29]. Several studies report that PD is associated with disruptions in metallic ion homeostasis-mainly Mn [30, 31]. High levels of Mn results in parkinsonian-like symptoms known as manganism. This is mainly because neurodegeneration in PD occurs mainly in the DA neurons of the substantia nigra whereas Mn toxicity affects striatum. Both types of damage can decrease DA levels [30, 31]. Ephrins are important axon guidance molecules which play important roles in regulating motor function [32]. However, mechanisms of Mn in regulating motor function through axon guidance molecular ephrins has not been explored clearly.

Posttranscriptional regulation is implicated in axon guidance, and several RNA-binding proteins are involved in these mechanisms [13, 33]. The findings of the current study show significant down-regulation of FTO in Mn treated group in a dose-dependent manner. FTO inhibits striatal nerve and motor dysfunction caused by manganism *in vivo*. In addition, FTO inhibits toxicity of manganism cells *in vitro*. Notably, at the molecular level, FTO over-expression decreases total RNA m⁶A level and upregulates key manganism inhibitory genes ephrin-A5 and ephrin-B2, and increases their mRNA stability. mRNA decay of ephrin-B2 gene in manganism cells is regulated by m⁶A reader YTHDF2. On the contrary, pretreatment with FTO selective inhibitor MA2 aggravates Mn-induced neurological dysfunction. FTO is the RNA demethylase of m⁶A [34], which has the opposite effect on the stability of mRNA. The findings of this study showed that regulation of ephrin-B2 is partly regulated through the m⁶A/YTHDF2 mechanism due to the following reasons: (1) FTO over-expression increased mRNA stability and level of ephrin-B2 gene, and decreased the abundance of m⁶A; (2) YTHDF2 and METTL3/14 over-expression decreased mRNA stability and level of ephrin-B2 gene, and reversed the effect of FTO over-expression. These findings imply that in Mn-induced parkinsonism, increase in m⁶A level leads to RNA degradation of ephrin-B2 gene through a YTHDF2-dependent mechanism. However, several other undiscovered additional effects of FTO-regulated demethylation should be explored.

Mn neurotoxicity is correlated with impaired DA, glutamatergic and GABAergic transmission and neuroinflammation. Mn toxicity causes extrapyramidal motor dysfunction due to preferential accumulation of Mn in the striatum [35]. Previous studies report that ephrin signaling is important for normal connectivity and function of midbrain DA neurons [36, 37]. To verify that the observed defects resulted from the loss of FTO and ephrin-B2 mainly in the striatum, the role of ephrin-B2 activation in the striatum of mice in improving motor dysfunction was explored. The findings showed that increase in expression level of ephrin-B2 significantly improved their motor function. On the contrary, pretreatment with MA2 damaged motor function. These findings imply that deregulation of FTO and ephrin-B2 are significant mechanisms underlying Mn-induced neurotoxicity in mice. Future studies should be conducted to further explore anti-Mn effect of ephrin-B2.

Striatum is associated with several neurodegenerative diseases such as PD [38] and Huntington's Disease [39]. Effect of Mn linked on motor function in the striatum should be explored further. The findings of the current study show that Mn exposure causes pathological effects on the striatum characterized by cell loss, unusual shape and decrease in the number of neurons. These findings are consistent with previous findings by Song *et al*/ [40]. The findings of the current study showed that increase in ephrin-B2 significantly alleviates neuropathological damage and ultrastructural changes caused by manganism. Notably, the neuropathological injury was severe in the MA2 pretreatment groups. These findings show the effects of FTO and ephrin-B2 on ultrastructural changes of Mn-induced neurotoxicity. Studies report that axon guide molecules perform an important role in regulating synaptic formation. Notably, axon guide molecules determine the location of synapses and regulate neuronal function [41–43]. In the current study, increased expression of ephrin-B2 antagonized Mn-induced dendritic spine density dysfunction. These findings show for the first time that ephrin-B2 plays an important role in Mn-induced neuronal damage.

Brain DA neurons play important role in motor function control and related disorders by transmitting electrophysiological signals [44–47]. In addition, spontaneous firing activity is responsible for placing baseline DA ranges in the striatum [48], which regulate cognitive and motor function [49, 50]. Therefore, determinants of the spontaneous firing activity *in vivo* play important roles in regulation of DA signaling and motor function. Notably, Mn-induced disappearance of spontaneous discharge was improved by ephrin-B2 activation. Spontaneous discharge frequency was damaged in MA2 pretreatment groups. These findings indicate a novel mechanism for Mn regulation of spontaneous firing rate of DA neurons in manganism.

Mn induced Parkinsonism is mainly caused by decrease in DA content in the striatum [12]. DA is derived from release of DA neurons from the substantia nigra after projecting to the striatum. This pathway is known as the nigro-striatal projection pathway of DA neurons [51]. To explore the effect of Mn exposure on nigro-striatal projection system, FG labeling, immunofluorescence triple staining of TH, DARPP32 and DAT, DA levels and neuronal axons growth were determined to evaluate the survival rate of nigro-striatal neurons. The findings showed that Mn treatment significantly damaged the nigro-striatal projection system, whereas re-expression of FTO and ephrin-B2 significantly increased survival rate of the projection neurons. Notably, substantia-striatal projection system was severely damaged after inhibition of FTO activity. These findings show that down-regulation of FTO and ephrin-B2 significantly affect morphology and function of the nigro-striatal projection system, implying that FTO mediated axon guidance promotes Mn-induced dysfunction of the nigro-striatal projection system, resulting in the failure of DA to be transmitted to the striatum, which may eventually cause striatal motor dysfunction.

Conclusions

In summary, the findings of the current study show that FTO over-expression inhibits Mn-induced parkinsonism and promotes expression of ephrin-B2 gene, partially through inhibits ephrin-B2 mRNA degradation, which is mediated by YTHDF2. In addition, promoting the expression of ephrin-B2

antagonizes Mn-induced parkinsonism in mice. These findings provide a basis to explore the specific mechanisms of Mn-induced motor dysfunction and provides a novel approach for treatment of Mn-induced parkinsonism.

Abbreviations

AAV5: adeno-associated virus 5; ALKBH5: α -ketoglutarate-dependent dioxygenase alkB homolog 5; CCK-8: Cell Counting Kit-8; CNS: central nervous system; DA: dopamine; DAPI: 2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride; DARPP32: Dopamine- and cAMP-regulated neuronal phosphoprotein 32 kDa; DAT: dopamine transporter; DMEM: Dulbecco's modified Eagle's medium; ELISA: Enzyme-linked immunosorbent assay; ephrins: erythropoietin-producing hepatocyte receptor-interacting proteins; FBS: fetal bovine serum; FG: Fluoro-Gold; FTO: fat mass and obesity-associated protein; HE: Hematoxylin and eosin; HRP: Horseradish peroxidase; ICP-MS: inductively coupled plasma mass spectrometry; i.p: intraperitoneal; m⁶A: N⁶-methyladenosine; MA2: Ethyl ester derivative of MA; mACSF: modified artificial cerebrospinal fluid; METTL3: methyltransferase like 3; MnCl₂: Manganese chloride; Mn: Manganese; NC: negative control; OE: over-expression; PBS: phosphate buffered solution; PD: Parkinson's disease; PVDF: polyvinylidene difluoride; RT-qPCR: Real-time quantitative PCR; s.c: subcutaneous; TH: tyrosine hydroxylase; YTHDF1/2: YT521-B homology domain family proteins 1/2

Declarations

Availability of data and material

All data generated or analyzed during this study are included in this published article.

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Authors' contributions

Each author notably contributed to the article. Zhipeng Qi: design and draft the article; Jiashuo Li, Ke Zhang, Yanan, Liu, Xinxin Yang, Minghui Li, Xianchao Du, Lei Zhang, Yi Wen, Bin Xu, Wei Liu, and Zhaofa Xu: revise the manuscript; Yu Deng: conception, design, and revise it severely for essential intellectual content, and final approval of the version to be published. All authors read and permitted the final version of the manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

FTO is upregulated in striatum after Mn exposure. A Mn levels in striatum. B, F mRNA expression level of FTO in striatum and neurons. C, G FTO protein levels in striatum and neurons. D, H Immunofluorescence staining of FTO in striatum and neurons. Nuclei are counterstained with DAPI in blue, FTO are counterstained in green. Scale bar, 100µm. E, I Total RNA m⁶A levels in striatum and neurons. Data are expressed as mean ± S.D. (n≥3). *P<0.05, **P<0.01 indicates statistical significance compared with control group.

Figure 2

Selective FTO re-expression or inhibition of FTO activity in the striatum rescues defects in motor function and morphological changes after Mn exposure. A mRNA expression level of FTO in striatum. B FTO protein levels in striatum. C Total RNA m⁶A levels in striatum. D Endurance times on the rotating rod. E Fatigue running distance in the fatigue meter. F Heat map of activity in the open field. G Mean intensity in the gait tests. H HE staining of striatum after Mn exposure and/or intervention by FTO expression. I Nissl

staining of striatum after Mn treatment and/or intervention by FTO expression. J Electron microphotograph of striatum after Mn treatment and/or intervention by FTO expression. Green arrow-heads represent the nuclear membrane, n representative nuclei, blue arrow-heads represent mitochondria, black arrows represent endoplasmic reticulum. K-L Golgi-Cox staining of striatum after Mn exposure and/or intervention by FTO expression. M Trace representative spontaneous firing recorded by MED64 after Mn exposure and/or intervention by FTO expression. Magnification of the sections stained with HE, Nissl, and Golgi-Cox was $\times 400$. Magnification of the electron microphotograph was $\times 4,000$. Data are expressed as mean $\pm$ S.D. ($n \geq 3$). * $P < 0.05$, ** $P < 0.01$ indicates statistical significance compared with the control (AAV5-NC) group; # $P < 0.05$ indicates statistical significance compared with the 50 mg/kg MnCl₂ (AAV5-NC) group.

Figure 3

Effect of Mn exposure on nigro-striatal projection system. A, F Retrograde labeling of nigro-striatal projection neurons with FG after Mn exposure and/or intervention by FTO expression. B, G Immunofluorescence triple staining of TH, DARPP32 and DAT. TH are counterstained in green, DARPP32 are counterstained in red, and DAT are counterstained in purple. C, H DA levels of striatum after Mn exposure and/or intervention by FTO expression. D-E, I-J Changes in neuronal axons growth with live cell station after Mn exposure and/or intervention by FTO expression. Magnification of sections stained with FG was $\times 400$. Data are expressed as mean $\pm$ S.D. ($n \geq 3$). * $P < 0.05$, ** $P < 0.01$ indicates statistical significance compared with the control (with or without AAV5-NC) group; # $P < 0.05$ indicates statistical significance compared with the 50 mg/kg MnCl₂ (with or without AAV5-NC) group.

Figure 4

Effect of FTO re-expression or inhibition of FTO activity in neurons and SH-SY5Y cells. A-B, E-F Expression levels of FTO in neuron and SH-SY5Y cells by real time PCR and immunoblot analysis after over-expression or inhibition. C, G Total RNA m⁶A levels in neuron and SH-SY5Y with FTO-NC, FTO-OE (FTO over-expression), or MA2 (inhibition of FTO activity). D, H Cell viability assay in neuron and SH-SY5Y cells with FTO-NC, FTO-OE (FTO over-expression), or MA2 (inhibition of FTO activity). Data are expressed

as mean $\pm$ S.D. ($n \geq 3$). * $P < 0.05$, ** $P < 0.01$ indicates statistical significance compared with the control (with or without FTO-NC) group; # $P < 0.05$, ## $P < 0.01$ indicates statistical significance compared with the 50 mg/kg MnCl₂ (with or without FTO-NC) group.

Figure 5

Function of m⁶A in neuron cells. A-D Expression levels of METTL3/14 or FTO neuron cells by real time PCR and immunoblot analysis after over-expression. E Total RNA m⁶A levels in neuron cells. F Cell viability assay using neuron cells. Data are expressed as mean $\pm$ S.D. ($n \geq 3$). * $P < 0.05$, ** $P < 0.01$ indicates statistical significance compared with the control (FTO-NC+METTL3/14-NC) group; # $P < 0.05$, ## $P < 0.01$ indicates statistical significance compared with the 50 mg/kg MnCl₂ (FTO-NC+METTL3/14-NC) group.

Figure 6

Ephrin-A5 and ephrin-B2 are key target genes for FTO in neuron cells. A-B Expression levels of ephrin-A4/5 and ephrin-B2 in neuron cells determined by real time PCR and immunoblot analysis. C-E Expression levels of ephrin-A4/5, and ephrin-B2 in neuron cells after exposure to 500 μ M MnCl₂ and/or FTO over-expression. F-H Expression levels of ephrin-A4/5 and ephrin-B2 in neuron cells after exposure to 500 μ M MnCl₂ and/or FTO over-expression determined by immunoblot analysis. I mRNA m6A levels of ephrin-A4/5 and ephrin-B2 in neuron cells after exposure to 500 μ M MnCl₂ and/or FTO over-expression. Data are expressed as mean $\pm$ S.D. ($n \geq 3$). * $P < 0.05$, ** $P < 0.01$ indicates statistical significance compared with the control (FTO-NC) group; # $P < 0.05$, ## $P < 0.01$ indicates statistical significance compared with the 50 mg/kg MnCl₂ (FTO-NC) group.

Figure 7

FTO regulates its target genes through m6A/YTHDF2-mediated mRNA decay pathway. A Expression levels of YTHDF1/2 in neuron cells as determined by immunoblot analysis. B-E Levels of ephrin-A5 and ephrin-B2 in cells after FTO and YTHDF1 over-expression. F-I Levels of ephrin-A5 and ephrin-B2 in cells after FTO and YTHDF2 over-expression. J-K RT-qPCR analysis of stability of ephrin-B2 mRNA with FTO or YTHDF2 over-expression. L Immunofluorescence double staining of FTO and ephrin-B2. Nuclei are

counterstained with DAPI in blue, FTO are counterstained in green, ephrin-B2 are counterstained in red. Scale bar, 100µm. M-P Expression levels of Grb4 and GIT1 in neuron cells as determined by real time PCR with or without FTO and ephrin-B2 over-expression. Data are expressed as mean ± S.D. (n≥3). *P<0.05, **P<0.01 indicates statistical significance compared with the control (FTO-NC with or without ephrin-B2-NC) group; #P<0.05, ##P<0.01 indicates statistical significance compared with the 50 mg/kg MnCl2 (FTO-NC with or without ephrin-B2-NC) group.

Figure 8

Selective ephrin-B2 activation in the striatum rescues defects in motor function. A Endurance times on the rotating rod with inhibition of FTO activity in combination with activation of ephrin-B2. B Fatigue running distance in the fatigue meter. C Heat map of activity in the open field. D Mean intensity in the gait tests. E HE staining of striatum. F Nissl staining of striatum. G Electron microphotograph of striatum. Green arrow-heads represent the nuclear membrane, n representative nuclei, blue arrow-heads represent mitochondria, black arrows represent endoplasmic reticulum.. H-I Golgi-Cox staining of striatum. J Trace representative spontaneous firing recorded by MED64. K Retrograde labeling of nigro-striatal projection neurons with FG. L DA levels of striatum. M The immunofluorescence triple staining of TH, DARPP32 and DAT. TH are counterstained in green, DARPP32 are counterstained in red, and DAT are counterstained in purple. N-O Observe the changes of neuronal axons growth with live cell station. The magnification of the sections stained with HE, Nissl, FG and Golgi-Cox was $\times 400$. The magnification of the electron microphotograph was $\times 4,000$. Data are represented as mean $\pm$ S.D. ($n\geq 3$). * $P<0.05$, ** $P<0.01$ indicates statistical significance in contrast with the control (ephrin-B2-NC) group; # $P<0.05$, ## $P<0.01$ indicates statistical significance in contrast with the 50 mg/kg MnCl₂ (ephrin-B2-NC) group. & $P<0.05$, && $P<0.01$ indicates statistical significance in contrast with the 20 mg/kg MA2+50 mg/kg MnCl₂ (ephrin-B2-NC) group.

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