

1 Iron overload promotes synaptic dysfunction and mediated excitotoxicity via

2 NMDAR–PSD-95–nNOS signaling in dopaminergic neurons

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ABSTRACT

Iron deposition in the substantia nigra (SN) is recognized as a critical factor in the pathogenesis of Parkinson's disease. However, the precise effects of iron on dopaminergic neuron damage, and the underlying molecular mechanisms remain largely unclear. This study aimed to investigate how excessive iron disrupts dopamine homeostasis and synaptic integrity, with a focus on NMDAR-mediated excitotoxicity. In iron dextran-treated rats, iron disrupted dopamine homeostasis and reduced the expression of NMDAR and AMPAR subunits, and several synaptic plasticity-related proteins in SN and striatum. Ferric ammonium citrate (FAC) inhibited spontaneous and evoked firing of dopaminergic neurons in the substantia nigra pars compacta. In the primary ventral midbrain (VM) neurons of rats, FAC elevated intracellular free iron and ROS, reduced mitochondrial membrane potential, and decreased total protein levels of NMDARs and AMPARs. Notably, in vitro study, FAC upregulated the expressions of PSD-95 and nNOS and increased membrane expression of GluN2B and GluN2A, as well as promoted GluN2B–PSD-95–nNOS complex assembly. Tat-NR2B9c, a peptide that disrupts the interaction between GluN2B and PSD-95, protected against iron-induced neurotoxicity in both cultured neurons and rats. This protection was associated with suppression of nNOS upregulation, NO overproduction, restoration of mitochondrial membrane potential and BDNF expression, which were achieved through inhibiting PSD-95–nNOS–GluN2B complex assembly. These results indicated that iron overload disrupted dopamine homeostasis, promoted synaptic deficits and NMDAR-mediated excitotoxicity via NMDAR–PSD-95–nNOS signaling, identifying the disruption of the PSD-95/NMDAR interaction as a novel protective strategy against iron-induced neuronal injury.

Key words: Parkinson's disease; dopamine; NMDAR; Iron; PSD-95

INTRODUCTION

Parkinson's disease (PD), a prevalent neurodegenerative disorder, is characterized by the progressive and selective loss of dopaminergic neurons within the substantia nigra pars compacta (SNpc). Although the precise mechanisms of PD were not fully understood, genetics, aging and environmental factors were reported to be involved in the etiology of PD. Metal exposure is considered as a potential risk factor for PD (Pyatha et al., 2022). Intriguingly, accumulating evidence from imaging and post-mortem studies has identified abnormal iron accumulation within the substantia nigra (SN) as a prominent and early pathological feature of PD (Bergsland et al., 2019; Dexter et al., 1987; Zecca et al., 2005), and iron accumulation has been reported to be strongly implicated in disease progression of PD (Devos et al., 2022; Gao et al., 2025). Although iron is indispensable for various physiological functions, excess iron can act as a potent catalyst for reactive oxygen species (ROS) generation through the Fenton reaction. This process induces oxidative damage to lipids, proteins, and DNA, and promotes the aggregation of α -synuclein, ultimately contributing to neuronal death. Moreover, it has been reported that elevated iron triggers the release of neuroinflammatory factors from activated microglia, which further aggravates the degeneration of dopaminergic neurons (Ryan et al., 2023; Wang et al., 2022). However, the precise molecular mechanisms by which elevated iron triggers degeneration of dopaminergic neurons and induces synaptic dysfunction, ultimately leading to motor dysfunction, remain incompletely elucidated. Synaptic dysfunction is one of the key mechanisms contributing to PD pathogenesis. Among the projection fibers received by dopaminergic neurons of SNpc, excitatory projection fibers are mainly glutamatergic fibers from subthalamic nucleus and cerebral cortex (Ammari et al., 2010; Fan et al., 2023; Hamani et al., 2004). Glutamate exerts its regulatory functions through a variety of receptors in the central nervous system (Fadahunsi et al., 2024; Hansen

et al., 2021; Xie et al., 2023). These receptors not only participate in excitatory neurotransmission, but also modulate synaptic plasticity. In addition, glutamate excitotoxicity, mediated largely through excessive activation of N-methyl-D-aspartate receptors (NMDARs), represents a critical mechanism of neuronal injury in various neurodegenerative diseases including PD (Kornhuber and Weller, 1997; Lin et al., 2025; Pershina et al., 2023). The scaffolding protein postsynaptic density protein 95 (PSD-95) plays a pivotal role in anchoring NMDARs at the postsynaptic density and modulating their function and trafficking, thereby critically influencing excitotoxic vulnerability (Shen et al., 2023; Yao et al., 2022; Yu et al., 2020). Our previous study revealed that activation of NMDAR reduced the expression of iron exporter ferroportin1 (FPN1) and increased the expression of iron importer divalent metal transporter 1 (DMT1), leading to iron accumulation within cells. NMDAR inhibitor can protect dopaminergic neurons and inhibit iron deposit in the SN of PD mice (Xu et al., 2017). However, whether and how iron overload interfaces with NMDAR signaling and synaptic organization, specifically within the vulnerable SNpc dopaminergic neurons, is a crucial unanswered question. Here, we investigated the mechanisms underlying iron-induced dopaminergic neurotoxicity using iron-induced rat models, primary cultured ventral midbrain neurons and PC12 cells with iron overload. Results showed that iron overload disrupted dopamine homeostasis and promoted synaptic deficits and NMDAR-mediated excitotoxicity and ultimately leading to the injury of dopaminergic neurons.

MATERIALS AND METHODS

Iron-induced PD animal models

Male Wistar rats used in this study were supplied by Qingdao Duren Fucheng Animal Husbandry Co., Ltd. (Qingdao, China). The animals were individually housed in standard cages with ad libitum access to food and water. The housing environment was maintained under controlled conditions: constant temperature and humidity, and a 12-hour light/dark cycle. Rats were acclimatized to the laboratory environment for one week prior to experimentation. Then rats received iron dextran (500 mg/kg) intraperitoneally (i.p.) for four hours to induce iron overload animal model as our previous studies (Jiang et al., 2006; Jiang et al., 2007b). Iron dextran, a complex where iron is tightly bound to dextran, is used to mimic iron overload and induce a Parkinsonian phenotype in laboratory animals. Our previous studies have also shown that iron content in the SN of these iron overload rats increased, which was associated with the degeneration of dopaminergic neurons and reduced dopamine release (Jiang et al., 2006; Jiang et al., 2007a). This further provide evidence that iron dextran could induce brain iron overload in the SN. All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Qingdao University (Ethics approval number 2025684).

Animal treatment

The rats were randomly divided into four groups: the control group received normal saline intraperitoneally (i.p.); the iron dextran group received iron dextran (500 mg/kg, i.p.); the Tat-NR2B9c + iron dextran group received Tat-NR2B9c (7.5 mg/kg, MedChemExpress, # HY-P0117) through tail vein (i.v.) 1 hour before receiving the same dose of iron dextran; and the iron dextran + Tat-NR2B9c group received Tat-NR2B9c (7.5 mg/kg) through tail vein (i.v.) 4 hours after receiving the same dose of iron dextran (behavioral experiments were

conducted after Tat-NR2N9c treatment for 1 hour).

The open field test

The open field test was used to evaluate the locomotor activity of rats. Each animal was gently placed in the center of a black open-field arena (100 cm×100 cm ×100 cm).

Locomotor behavior was recorded for 10 minutes via a video tracking system connected to a computer for automated analysis. The following parameters were measured: total distance moved, number of entries into the central zone, and the percentage of time spent in the center of the area.

The gait analysis

The gait analysis was used to evaluate the motor coordination and balance ability of rats. Gait Analysis System (CatWalkXT, Netherlands) is a system for quantitative analysis of gait and movement of rats, mice and other small mammals, which mainly consists of a main walkway unit and a high-speed image acquisition module (under the glass walkway). The main walkway unit includes tempered illuminated footprint glass plate, adjustable divider corridor, and dual-layer integrated LED lighting system. When animals step onto the glass walkway, the contact of their paws can alter the refractive index of the glass, triggering luminescence on the glass underside. High-speed cameras record these glowing paw imprints, and specialized software subsequently sorts, quantifies, and visualizes the collected gait metrics. Here, the parameters of the walking platform are set to 100 cm long and 9 cm wide, and the camera is 70 cm away from the glass bottom. We allowed rats to explore the specialized glass walkway three days before the experiment, and rats were trained once a day for 10 minutes. When the experiment was started, each animal was gently placed in the entrance of the glass walkway, let them go through the aisle independently, ensuring that they go straight all the way, without stopping, turning around, retreating or turning back. This process is completed in a dark room environment, and each mouse is tested at least 5 times. The following

parameters were measured: the run average speed, the stand time of the limbs to the walkway respectively.

Immunofluorescence and Imaging

Cell immunofluorescence staining was performed to confirm the purity of primary ventral midbrain (VM) neurons. Cells were fixed in 3.6% formaldehyde in phosphate buffered saline (PBS) for 10 minutes and permeabilized with 0.25% Triton X-100 for 20 minutes. Subsequently, cells were blocked with PBS containing 3% normal goat serum for 30 minutes, followed by the application of primary antibody MAP2 (Cell Signal Technology, #4542, 1:800) overnight at 4°C. After washing with PBS, fluorescent secondary antibody (Abcam, #ab150077, 1: 500) were added at room temperature for 2 hours, followed by incubation with DAPI (Beyotime Biotechnology, #C1006) for 10 minutes at room temperature. Then cells were sealed with 70% glycerin and observed under a laser scanning confocal microscope. Immunofluorescence staining was performed to assess the changes of TH-positive neurons in the SN of rats. Briefly, after the behavioral tests, animals were deeply anesthetized and transcardially perfused with 200 mL of PBS. The brains were then quickly removed. Coronal brain sections (20 µm thick) were prepared using a cryostat. For immunofluorescence staining, the sections were first permeabilized with 0.5% Triton X-100, then blocked with 10% donkey serum for 30 minutes. Subsequently, the sections were incubated with anti-TH primary antibody (Sigma Aldrich, #AB152, 1: 800) overnight at 4°C. The next day, after washing, the sections were incubated with a fluorescent secondary antibody (Abcam, #ab150077, 1: 500) for 2 hours at room temperature. After washing by PBS, the nuclei were stained with DAPI (Beyotime Biotechnology, #C1006) for 10 minutes. Finally, the stained sections were observed under a fluorescence microscope (Olympus, Japan).

Western blot

Cells or tissue samples were collected and lysed using RIPA lysis buffer containing protease

inhibitors (pepstatin 1 µg/mL, aprotinin 1 µg/mL, leupeptin 1 µg/mL) for 30 minutes on ice. The cell lysates were collected by centrifugation at 4°C, 12,000 rpm for 20 minutes. The protein concentration was quantified using BCA protein assay kit. Plasma membrane proteins were isolated using the Beyotime Biotechnology P0033 kit according to the manufacturer's instructions. The protein samples were boiled at 100°C for 5 minutes in loading buffer. The total 20 µg of protein was separated by 6%-8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% nonfat milk in Tris-buffered saline-tween-20 (TBST) buffer for 2 hours at room temperature and then incubated at 4°C overnight with primary antibody against TH (Sigma Aldrich, #AB152, 1:3000), VMAT-2 (Abcam, #ab259970, 1:1000), DAT (Abcam, #ab184451, 1:1000), GluN1 (Abcam, #ab109182, 1:1000), GluN2A (Abcam, #ab203197, 1:1000), GluN2B (Cell Signal Technology, #4207, 1:1000), phospho-GluN2B (Cell Signal Technology, #4208, 1:1000), PSD-95 (Thermo Fisher, #200-301-G33, 1:2000), GluA1 (Cell Signal Technology, #13185, 1:1000), phospho-GluA1 (Cell Signal Technology, #8084, 1:1000), activity-regulated cytoskeleton-associated protein (Arc) (Abcam, #ab309193, 1:1000), D1R (Abcam, #ab184247, 1:1000), V-GLUT2 (Abcam, #ab216463, 1:1000), nNOS (Cell Signal Technology, #4231, 1:1000), BDNF (Selleck, #E17G19, 1:1000), β-actin (Bioss, #bsm-63325R, 1:10000), GAPDH (Bioss, #bsm-52262R, 1:20000). The membranes were then washed and incubated with secondary HRP-conjugated antibodies for 1 hour at room temperature, and then followed by enhanced chemiluminescence (ECL) detection (BIO-RAD, USA). Image J software (NIH Image, Bethesda, MD, USA) was used to analyze the bands.

Brain slice preparation

Brain slices from the SNpc were prepared using C57BL/6 mice aged 15 to 20 days postnatally. The brains were removed rapidly and immersed in an ice-cold, oxygenated (95%

188 O₂/5% CO₂) cutting solution composed of (in mmol/L): 124 NaCl, 3 KCl, 0.5 CaCl₂·2H₂O, 6
189 MgCl₂·6H₂O, 1.3 NaH₂PO₄, 26 NaHCO₃, 10 glucose. Coronal slices (250 μm) containing the
190 SNpc were sectioned using a vibratome (Leica VT1000S). Subsequently, these slices were
191 incubated in oxygenated artificial cerebrospinal fluid (ACSF) with the following composition
192 (in mmol/L): 124 NaCl, 3 KCl, 2.4 CaCl₂·2H₂O, 1.3 MgCl₂·6H₂O, 1.3 NaH₂PO₄, 26
193 NaHCO₃, and 10 glucose. The slices were allowed to recover for one hour at a temperature of
194 36°C prior to experimentation.

195 **Whole-cell patch clamp recordings**

196 After equilibration, brain slices were transferred to a small-volume chamber (~0.5 mL)
197 mounted on the upright fixed stage of an Infrared Differential Interference Contrast
198 microscope (Nikon, Japan). The chamber was superfused with oxygenated artificial
199 cerebrospinal fluid (ACSF) at a flow rate of 1.5-2.0 mL/ min at room temperature (22–25°C).
200 Patch pipettes with resistances ranging from 3 to 5 MΩ were pulled from borosilicate glass
201 capillaries using a horizontal puller (P-97, Sutter Instrument, Novato, CA, USA). The internal
202 solution (290 mOsm) comprised the following components (in mmol/L): 130 K-gluconate, 10
203 HEPES, 0.6 EGTA, 5 KCl, 4 MgCl₂·6H₂O, 3 Na₂ATP, 0.3 Na₃GTP and 10 Na₂-
204 phosphocreatine; pH was adjusted to 7.3 with KOH. Whole-cell patch-clamp recordings of
205 SNpc dopaminergic neurons were conducted to record spontaneous and evoked spikes under
206 current-clamp mode utilizing a Multiclamp 700B amplifier (Molecular Devices, Union City,
207 CA). Electrophysiological data were acquired at 5 or 10 kHz through a Digital1440A
208 interface (Molecular Devices), in conjunction with a PC system. All data recordings were
209 performed at room temperature. Subsequent analysis was carried out using pCLAMP
210 software (Molecular Devices).

211 **Cell cultures**

212 The primary VM neuronal cultures were prepared from Wistar rat embryos at embryonic day

14-15. The ventral mesencephalon was microdissected in ice-cold DMEM/F12 solution under a stereomicroscope. Tissues were then digested with 0.25% trypsin-EDTA (Sigma-Aldrich) for 5 minutes at 37°C, followed by trituration using a fire-polished pasteur pipette in DMEM/F12 supplemented with 10% fetal bovine serum (FBS). Dissociated cells were resuspended in the DMEM/F12 solution that with 2% B27 (Gibco, #A3582801) and plated onto poly-D-lysine pre-coated coverslips at a density of approximately 1×10^5 cells/mL and maintained at 37°C in 5% CO₂. 5 µmol/L cytosine-D-Aminobiofuranoside was added to neurons after 3 days in vitro culture to kill glial cells. The cultures were established following previously described methods and were used for experiments at 7 days in vitro (DIV 7)(Bollimpelli and Kondapi, 2015). In this study, 100 µM ferric ammonium citrate (FAC), which could be rapidly taken up by cells, was used to induce intracellular iron overload as previously described (Oh et al., 2014; Shen et al., 2024).

Rat adrenal medullary pheochromocytoma PC12 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution. Cells between passages 6 and 10 were used for experiments. For treatment, cells were seeded at a density of 5×10^6 cells/mL in 6-well plates and allowed to adhere overnight. Subsequently, the culture medium was replaced with fresh FBS-free DMEM containing either 100 µmol/L FAC or/and 8 µmol/L Tat-NR2B9c and 0.5 µmol/L Saracatinib. The cells were then incubated at 37°C under 5% CO₂ for 24 hours(Thermo Fisher Scientific, USA)(Zheng et al., 2024).

Detection of reactive oxygen species (ROS) level

Intracellular ROS was detected by a molecular probe 2',7'-Dichlorofluorescein diacetate (DCFH2-DA) (Sigma-Aldrich, # D6883) (Yin et al., 2024). Briefly, cells were incubated with the reagent (5 µmol/L) for 30 minutes at 37°C followed by three washes with HBS. Cells

were resuspended in 1 mL HBS and fluorescence was analyzed using the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) at 488 nm (excitation) and 523 nm (emission). Data was collected from 10000 cells and results were analyzed by CELLQuest Pro system. Results are presented as FL1-H (Fluorescence 1-Histogram), setting the gated regions M1 and M2 as markers to observe the changing levels of fluorescence intensity.

Detection of mitochondrial function

The mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using the fluorescent dye Rhodamine123 (Solarbio, #IR1800). Cells were incubated with 1 μ M Rhodamine123 at 37°C for 30 minutes in the dark, followed by three washes with Hanks' Balanced Salt Solution (HBS). After washing, the cells were resuspended in 1 mL HBS and analyzed immediately on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using 488 nm excitation and 523 nm emission wavelengths. Data from 10,000 cells were collected for each sample, and fluorescence intensity was evaluated using the CELLQuest Pro software. Results are presented as FL1-H (Fluorescence 1-Histogram), with gated regions M1 and M2 used to quantify changes in fluorescence intensity.

Tetramethylrhodamine ethyl ester (TMRE), a cell-permeant cationic fluorescent probe, was used to monitor changes in mitochondrial membrane polarization. TMRE accumulates in active mitochondria with high membrane potential; loss of potential results in probe redistribution and reduced fluorescence. Following treatment, cells were incubated with 1 $\times$ TMRE staining solution (Beyotime Biotechnology, #C2001S) for 40 minutes at 37°C in the dark. Cells were then washed twice with pre-warmed culture medium. After adding pre-warmed medium, imaging was performed within 1 hour using a laser scanning confocal microscope (Olympus, Japan).

Detection of intracellular calcium ion (Ca^{2+})

Calcium imaging was measured using Fluo-4 AM (Beyotime Biotechnology, #S1060), a

Ca²⁺ sensitive fluorescent dye that can penetrate the cell membrane. Simply, cells were incubated with Fluo-4 AM staining solution (2 μmol/L) for 30 minutes at 37°C in the dark. Subsequently, the cells were washed with pre-warmed PBS three times to remove unloaded probe. Calcium imaging was performed with the laser scanning confocal microscope within 30 minutes (excitation at 488 nm, emission at 550±20 nm) (Olympus, Japan).

Detection of intracellular nitric oxide (NO)

NO-sensitive fluorescent probe 3-Amino,4-aminomethyl-2',7'-difluorescein, diacetate (DAF-FM DA, Beyotime Biotechnology, #S0019S) can penetrate the cell membrane and emit strong fluorescence upon reacting with NO. Briefly, cells were incubated with DAF-FM DA staining solution (5 μmol/L) in cell incubator at 37°C for 20 minutes. Then after washing with PBS for three times, the cells were observed under a laser confocal microscope within 30 minutes (excitation at 495 nm, emission at 515 nm) (Olympus, Japan).

Co-immunoprecipitation (Co-IP)

Immunoprecipitation of protein complexes (Co-IP), is a powerful technique to analyze protein-protein interactions. Here, the Co-IP Kit (MedChemExpress, #HY-K0202K) was used to detect the interaction between PSD-95 and GluN2B and nNOS. Firstly, suspending the magnetic beads by binding/washing buffer, discarding the supernatant after magnetic separation. And then, adding anti-PSD-95 (Abcam, #ab238135, 1:50) or Rabbit-IgG (Beyotime Biotechnology, #A7016, 1:50) to the magnetic beads, incubating for 4 h on a tumbling mixer in 4°C to bind the magnetic beads to the antibody thoroughly, then collecting the magnetic bead-antibody complex. Secondly, adding IP lysis buffer (containing PMSF and protease inhibitor) into the treated cells, incubating on ice for 10 minutes, centrifuge at 14000 g for 10 minutes at 4°C, and collect the supernatant as the protein samples for protein concentration determination. After concentration determination, each sample was divided into In-put group (10%), IgG group (45%) and IP group (45%). Thirdly, the samples of IgG

group and IP group were mixed with the corresponding magnetic bead-antibody complexes, and incubated overnight rotating at 4°C to form a magnetic bead-antibody-protein conjugate. Lastly, protein loading buffer (1×) was added to the conjugate, and heated in metal at 95°C for 5 minutes. After magnetic separation, the supernatant was taken for SDS-PAGE electrophoresis to detect the proteins that interacting with PSD-95.

Labile iron pool (LIP) measurement

After 7 days in culture, VM neurons were treated with FAC for 24 hours. Following treatment, the cells were gently washed with pre-warmed HEPES-buffered saline (HBS). To assess the LIP, cells were incubated with 0.5 µmol/L calcein-AM, prepared in HBS (pH 7.4), for 30 minutes at 37°C in the dark. After incubation, the cells were rinsed twice with HBS (pH 7.4) to remove excess probe. Subsequently, 100 µL of HBS buffer was added to each well. Fluorescence intensity was measured using a microplate reader with an excitation wavelength of 488 nm and an emission wavelength of 523 nm. All measurements were performed in triplicate to ensure reproducibility.

Enzyme-linked immunosorbent assay (ELISA)

The cell culture medium was collected from primary ventral midbrain neuronal cultures and centrifuged at 3000 r/min (approximately 1000×g) for 20 minutes at 4°C to remove cellular debris. The resulting supernatant was carefully collected and stored at –80°C until analysis. The concentration of glutamate in the supernatant was quantified using a commercial ELISA kit (Enzyme-linked Biotechnology, #ml063087), strictly following the manufacturer's protocol. Briefly, standards and samples were added to wells and incubated. After washing, a detection solution and substrate solution were applied. The reaction was then stopped by adding stop solution and the optical density (OD) of each well was measured at 450 nm using a microplate reader (Molecular Devices, UAS). All samples were assayed in duplicate, and glutamate concentrations were determined by interpolation from a standard curve.

Statistical analysis

The results were analyzed by GraphPad Prism 6.0 statistical software and data was expressed as mean±SEM. Significant difference between two groups was evaluated by unpaired student's t-test. One-way analysis of variance (ANOVA) followed by Turkey's test was used to compare the differences among multiple experimental groups. $P<0.05$ was considered to be statistically significant.

RESULTS

Iron-treated rats exhibited motor dysfunction and reduced nigral TH expression

Iron deposition in the SN is considered as a risk factor for PD (Casanova et al., 2024). To investigate the effects of iron overload, an acute iron overload rat model was established by intraperitoneal injection of iron dextran for 4 hours. It has been confirmed that intraperitoneal injection of iron dextran can induce brain iron overload in rat (Piloni et al., 2013). The motor ability of rats was evaluated using the open field test (OFT). The total distance and velocity of rats in the open field, as well as the frequency of entries into the central area, and the percentage of time spent in the center were examined. Results showed a significant reduction in the total distance, the velocity, frequency and the percentage of time in the central area of rats treated with iron dextran compared to the control group as shown in Figure 1A-1F.

Tyrosine hydroxylase (TH) and dopamine transporter (DAT) are markers of dopaminergic neurons. And DA depletion in the SN is an important manifestation in PD patients.

Therefore, we examined the protein expression of TH and DAT in the SN to estimate the damage of the dopaminergic neurons induced by iron dextran. Our results showed that the expression of TH and DAT in iron dextran group was significantly lower than the control group (Figure 1G, H). The experimental results showed that iron overload could lead to motor dysfunction and decreased expressions of TH and DAT in rats, suggesting iron dextran induced pathological changes of PD in rats.

The expression of glutamate receptors and synapse-associated proteins in the SN and striatum decreased in iron dextran-treated rats

Previous studies have shown that changes in synaptic function are involved in the pathogenesis of PD (Nguyen et al., 2019; Sharma and Burré, 2023). Glutamate is the most important excitatory neurotransmitter in the mammalian central nervous system and plays an important role in memory, synaptic plasticity and neuronal development (Meldrum, 2000; Ribeiro et al., 2017; Verma et al., 2022). In order to explore the effect of iron overload on the glutamatergic neurotransmission and synaptic function in PD, we assessed the expression of glutamate receptors (GluN1, GluN2A, GluN2B and GluA1) as well as synaptic-related proteins Arc and PSD-95 in the SN and striatum (Str) of iron dextran-treated rats by western blot. The results revealed a decrease in glutamate receptor expression including GluN1, GluN2A, GluN2B and GluA1 as well as synaptic proteins Arc and PSD-95 in the SN (Figure 1J-1R) and Str (Figure 2A-2I) of rats treated with iron dextran. These findings suggest that iron overload induced synaptic dysfunction within the SN and Str.

In addition, we detected the expression of nNOS within both SN and Str by western blot. Our results showed an increase in nNOS expression specifically within the SN of rats with iron overload compared to the control group (Figure 1S, T), while no significant change was observed within the Str that maybe associated with the susceptibility of different brain regions to oxidative stress (Figure 2J, K). It is known that activation of nNOS could produce nitric oxide, thus leading to excitatory toxicity of cells (Nicoll and Schulman, 2023; Wang et al., 2021). This suggests that iron overload may induce cell death by increasing nNOS and inducing excitatory toxicity specifically in the SN.

FAC inhibits SNpc dopaminergic neurons spontaneous and evoked firing activity

It has been reported that the spontaneous firing activity of dopaminergic neurons in the SNpc of PD animal model is disrupted (Chen et al., 2023). To explore whether iron overload could

362 affect spontaneous firing activity of dopaminergic neurons, we then investigated the effects
363 of FAC on the spontaneous firing activity of dopaminergic neurons in mouse brain slices.
364 Dopaminergic neurons in the SNpc were identified based on their morphology and
365 electrophysiological properties, as detailed in previous reports (Chen et al., 2015; Lu et al.,
366 2024). As illustrated in Figure 3A and B, treatment with FAC (100 $\mu\text{mol/L}$) resulted in a
367 decrease in neuronal firing rate from 1.40 ± 0.35 Hz to 0.62 ± 0.49 Hz after 3 minutes of
368 exposure to ACSF, further declining to 0.36 ± 0.49 Hz after 5 minutes, and nearly stopped
369 spontaneous firing by the end of a prolonged treatment period of 10 minutes. Additionally,
370 we assessed the dose-dependent effects of FAC using two lower concentrations (10 $\mu\text{mol/L}$
371 and 1 $\mu\text{mol/L}$). The application of these lower doses also significantly inhibited the firing rate
372 of dopaminergic neurons over time. To achieve an approximate 50% reduction in firing rate,
373 varying durations were necessary for each concentration. The highest dose (100 $\mu\text{mol/L}$)
374 required an average of 3.7 minutes, while the mid-range dose (10 $\mu\text{mol/L}$) took
375 approximately 4.6 minutes, and the lowest concentration (1 $\mu\text{mol/L}$) necessitated up to 8.0
376 minutes. Consequently, FAC inhibits spontaneous firing activity in SNpc dopaminergic
377 neurons in a manner that is both concentration- and time-dependent (Figure 3C). Notably,
378 administration of FAC at a concentration of 100 $\mu\text{mol/L}$ resulted in a rapid and significant
379 decrease in neuronal firing rates.

380 We subsequently selected the concentration of 100 $\mu\text{mol/L}$ to further investigate the effects of
381 FAC on the electrical activities of nigral dopaminergic neurons. As illustrated in Figure 3D,
382 for the six non-firing neurons examined, the resting membrane potential was hyperpolarized
383 from -53.10 ± 3.88 mV to -58.78 ± 2.92 mV following a 10-minute application of 100 $\mu\text{mol/L}$
384 FAC. A series of depolarizing currents (0-40 pA) were injected into the neurons to record
385 evoked firing activity. The application of FAC resulted in a significant reduction in action
386 potential numbers (Figure 3E, F). In conclusion, FAC inhibits both spontaneous and evoked

firing activities in dopaminergic neurons within brain slices.

FAC increased intracellular iron and ROS levels and decreased mitochondrial membrane potential

In order to further explore the mechanisms underlying iron-induced injury of dopaminergic neurons, primary cultured VM neurons were selected to do the following experiments. VM neurons were cultured to maturity at 7 days, which is a suitable time period to study dopaminergic neurons in VM neuron-enriched culture as previously reported (Bollimpelli and Kondapi, 2015). And immunofluorescence was used to assess the purity of the primary cultured VM neurons by detecting the ratio of MAP2-positive cells to the total number of cells. Our results showed that the purity of VM neurons exceeded 89% (Figure 3G, H). To further investigate the effect of FAC on intracellular iron level in VM neurons, we examined intracellular LIP in this study using Calcein-AM. After entering the cells, Calcein-AM is hydrolyzed by endogenous esterase to produce Calcein, which is retained in the cell and emits strong green fluorescence. It has a high affinity for Fe^{2+} and binding with Fe^{2+} leads to fluorescence quenching. Decreased fluorescence intensity signifies an increase in cytosolic chelatable iron. The results showed that the fluorescence intensity was decreased in VM neurons treated with FAC (100 $\mu\text{mol/L}$ for 24 hours), indicating an increase of intracellular LIP level (Figure 3I). Subsequently, we examined the levels of ROS and the changes of $\Delta\Psi\text{m}$ of VM neurons treated with FAC. The results revealed a significant increase in ROS level (Figure 3J, K) and marked reduction in $\Delta\Psi\text{m}$ (Figure 3L, M) in FAC-treated VM neurons compared to the control group. These findings indicate that iron overload induces oxidative stress and mitochondrial dysfunction in VM neurons.

Iron overload decreased the expression of glutamate receptors in primary cultured VM neurons

We have demonstrated that iron overload decreased the expression of glutamate receptors in

the SN and Str of rats, and also led to oxidative stress and mitochondrial dysfunction in primary cultured VM neurons. To further investigate the effect of iron overload on the expression of glutamate receptors in neurons, VM neurons were treated with 100 $\mu\text{mol/L}$ of FAC for 24 hours. Then we examined the expression of three subunits of NMDAR (GluN1, GluN2A, GluN2B) and AMPA receptor (AMPA) subunit GluA1 in VM neurons by western blot. As shown in Figure 4, the protein levels of GluN1, GluN2A, GluN2B and GluA1 decreased significantly in VM neurons treated with FAC, compared to the control group. Given that the function of NMDAR and AMPAR can be regulated through phosphorylation. Therefore, we examined the phosphorylation levels of GluN2B and GluA1. Results showed that phospho-GluA1 protein expression decreased, whereas the phosphorylation level of GluN2B remained unchanged. Given that phosphorylation is usually a transient event, we detected the changes of phosphorylation level of GluN2B after FAC treatment for 0.5 h, 1 h, 3 h, 6 h, 12 h and 24 h respectively. The results revealed that GluN2B phosphorylation at Tyr1472 were significantly increased at 1 h following FAC treatment (Figure 4J, K). Together, these findings suggest that iron overload alters both the expression and phosphorylation patterns of key glutamate receptor subunits in primary cultured VM neurons.

Iron overload disturbed dopamine homeostasis and affected the expression of synapse-associated proteins in VM neurons

Iron overload was shown to affect dopamine neurons in rat models. To determine whether iron overload affects key components of the dopaminergic system, we then examined the expression of VMAT-2, DAT and D1R in VM neurons after iron overload. The result is shown in the Figure 5A-5E. Compared with the control group, the expressions of D1R and VMAT-2 in FAC treated VM neurons were significantly decreased, while the expression of DAT had no significant change. These results suggest that iron overload causes the imbalance of dopamine homeostasis in VM neurons.

To further verify the effect of iron overload on synaptic function, we also examined the effect of iron overload on the expression of synaptic associated-related proteins in VM neurons. The result is shown in the Figure 5D, 5F-5I. The expression of Arc in FAC treated VM neurons decreased, while the expression of PSD-95 increased, compared to the control. It is known that activation of nNOS could produce nitric oxide, thus leading to excitotoxicity of neurons (Nicoll and Schulman, 2023; Sanjay et al., 2024). We further examined the expression of nNOS after FAC treatment. Results showed that FAC treatment can increase the expression of nNOS in VM neurons (Figure 5J, K). This is consistent with the results in iron overload rats, demonstrating an increase in nNOS expression within the SN of rats with iron overload compared to the control group (Figure 1S, T). This suggests that iron overload may induce cell death by increasing nNOS and inducing excitotoxicity in the SN. Furthermore, the expression of VGLUT-2 and the content of glutamate secreted by VM neurons after FAC treatment were examined to further explore the effect of iron overload on glutamatergic transmission. Results showed that the expression of VGLUT-2 decreased (Figure 5G, I) and the content of glutamate increased (Figure 5L), compared to the control. These results suggest that iron overload may affect the homeostasis of dopamine and glutamatergic transmission of VM neurons.

FAC increased the surface protein expression of GluN2A and GluN2B in VM neurons

Studies have demonstrated that the numbers of NMDARs and their subunit composition are not static, but change dynamically during development in a cell-specific and synapse-specific manner (Bessi eres et al., 2024; Paoletti et al., 2013). The distribution of glutamate receptors on the postsynaptic membrane plays a crucial role in determining the reactivity of postsynaptic cells to glutamate released by presynaptic neurons (Bod zeta et al., 2021; Scheefhals and Macgillavry, 2018). Therefore, regulating surface expression of receptor is particularly important. In this study, we extracted the membrane fraction of VM neurons to

examine the expression of glutamate receptors on the membrane. The results are illustrated in Figure 6, FAC treatment significantly increased the expressions of GluN1, GluN2A, GluN2B and phosphorylated GluN2B on the membrane of VM neurons, compared to the control group. Although phosphorylated GluA1 decreased significantly, no significant changes were observed in GluA1 expression between iron treatment and the control group. These results indicate that iron overload might affect glutamatergic neurotransmission of VM neurons by increasing the expression of GluN2A, GluN2B and phosphorylated GluN2B in the membrane of VM neurons.

Src family kinase plays a role in GluN2B phosphorylation and its membrane expression

The Src family kinase is one of the best-characterized tyrosine phosphorylation systems in neurons, which phosphorylates and stabilizes NMDARs and AMPARs at the cell surface. Specifically, Fyn phosphorylates the GluN2B subunit of the NMDAR at tyrosine residue 1472, thereby increasing NMDAR permeability and facilitating NMDA-mediated calcium influx (Lu et al., 2015). Additionally, RACK1 has been shown to inhibit the interaction between Fyn and GluN2B. To explore the potential mechanisms underlying iron-induced alterations in GluN2B phosphorylation, we examined the expression and phosphorylation status of Fyn. As shown in Supplementary Figure 1A-C, no significant differences were observed in either total Fyn protein levels or its phosphorylation following FAC treatment compared to the control group. Furthermore, we assessed RACK1 expression in both cytoplasmic and nuclear fractions of VM neurons. Similarly, RACK1 protein levels remained unchanged after FAC administration (Supplementary Figure 1D-F). In order to further clarify whether phosphorylation of GluN2B promotes its expression on the neuronal membrane, we treated neurons with Saracatinib (a potent Src family inhibitor) after FAC treatment. The result showed that Saracatinib could significantly inhibit the phosphorylation of GluN2B, and also decreased the level of GluN2B in membrane induced by FAC (Supplementary Figure

1G-I). These findings suggest that Src family kinase might play a role in GluN2B phosphorylation and its membrane expression.

PSD-95 inhibitor Tat-NR2B9c attenuates iron overload-induced toxicity in dopaminergic neurons

Our study indicates that iron overload might damage neurons via NMDAR–PSD-95–nNOS signaling. Therefore, disrupting the NMDAR-PSD-95-nNOS signaling axis has become one of the most promising neuroprotective strategies. Tat-NR2B9c is a PSD-95 inhibitor that specifically disrupts protein interactions between PSD-95 and NMDAR, as well as inhibits nNOS/PSD-95 interaction (Xu et al., 2024; Zhang et al., 2024). Our Co-IP results revealed that FAC enhanced the protein–protein interactions between PSD-95 and GluN2B, as well as between PSD-95 and nNOS in PC12 cells, suggesting an assembly of the GluN2B/PSD-95/nNOS complex under iron overload conditions. Importantly, Tat-NR2B9c attenuated these enhanced interactions, indicating its ability to disrupt the iron-induced aberrant protein complex formation (Figure 8D). In Supplementary Figure 1G-II, Tat-NR2B9c significantly reduced iron-induced GluN2B levels on the VM neuronal surface, suggesting its possible role in regulation GluN2B surface expression.

To explore the involvement of NMDAR–PSD-95–nNOS signaling in iron-induced damage, Tat-NR2B9c was used to investigate whether inhibiting NMDAR–PSD-95–nNOS signaling could protect against iron overload. Our results demonstrated that treatment with Tat-NR2B9c effectively alleviated iron-induced mitochondrial dysfunction in PC12 cells, as evidenced by improved mitochondrial membrane potential (Figure 7A, B). Additionally, Tat-NR2B9c could significantly inhibit iron-induced NO production and calcium overload in PC12 cells (Figure 7A, B). Furthermore, nNOS inhibitor 7-nitroindazole and GluN2B inhibitor ifenprodil can also alleviated iron-induced mitochondrial dysfunction, NO production and calcium overload (Figure 7A, B)

Moreover, Tat-NR2B9c significantly reversed the iron-induced upregulation of nNOS expression in PC12 cells, indicating a suppression of nitrosative stress (Figure 8A, B). Additionally, Tat-NR2B9c treatment counteracted the iron-induced downregulation of brain-derived neurotrophic factor (BDNF) protein expression in PC12 cells, suggesting a restorative effect on neurotrophic support (Figure 8A, C). Furthermore, in order to fully recapitulate the physiological environment of primary midbrain dopaminergic neurons, we further evaluated the protective effect of Tat-NR2B9c in primary VM neurons. The results demonstrated that Tat-NR2B9c effectively alleviated iron-induced calcium overload and NO production (Figure 8E-8G). In addition, Tat-NR2B9c treatment significantly reversed the iron-induced upregulation of nNOS expression, as well as the downregulation of BDNF and TH expression in VM neurons. (Figure 8H-8K). These findings collectively indicate that Tat-NR2B9c exerts neuroprotective effects in dopaminergic cells under iron overload conditions, likely through decoupling NMDAR–PSD-95–nNOS signaling and enhancing BDNF-mediated survival pathways. This supports the potential therapeutic effect of targeting PSD-95-related complexes in iron-related neurodegenerative models.

Tat-NR2B9c protects against iron-induced toxicity in iron-treated rats.

To evaluate the protective effect of Tat-NR2B9c against iron-induced behavioral and neuropathological changes, rats were treated according to the experimental design outlined in Figure 9A. The OFT revealed that iron dextran treatment significantly decreased the total distance moved, the residence time in the central area, and the movement speed, indicating impaired locomotor activity and anxiety-like behavior. Notably, pretreatment with Tat-NR2B9c effectively ameliorated these behavioral deficits, but no obvious therapeutic effect of Tat-NR2B9c was observed, which may be related to the severe injury of TH neurons induced by iron overload (Figure 9B–E). Additionally, gait analysis was performed for further behavioral assessment (Figure 9F–K). Results showed that iron dextran treatment

significantly decreased the average speed of rats (Figure 9G), and increased the stand time of limb (Figure 9H–K), indicated the abnormal gait and bradykinesia. Pretreatment with Tat-NR2B9c effectively ameliorated these gait deficits, but no obvious therapeutic effect was observed.

Immunofluorescence staining result showed that iron dextran markedly reduced the number of TH-positive neurons in the SN, and this reduction was attenuated by Tat-NR2B9c pretreatment (Figure 9L, M). Consistently, western blot analysis demonstrated that iron dextran decreased TH protein expression in both the SN and Str, and this reduction was significantly inhibited by Tat-NR2B9c treatment (Figure 9N–Q). Collectively, these results indicate that Tat-NR2B9c protects against iron-induced motor dysfunction and dopaminergic neuron loss in rats.

DISCUSSION

Accumulating evidence has highlighted iron accumulation in the SN as a key contributor to the pathogenesis of PD. In the present study, we have demonstrated that iron overload disrupts dopaminergic neurons through multiple mechanisms, including dopamine dyshomeostasis, synaptic dysfunction, disturbed spontaneous firing activity of dopaminergic neurons and NMDAR-mediated excitotoxicity. Moreover, Tat-NR2B9c exerts neuroprotective effects in dopaminergic cells against iron overload, likely through decoupling NMDAR-PSD-95-nNOS signaling and enhancing BDNF-mediated survival pathway. Substantial evidence has confirmed that iron deposition in the SN represents a key etiological factor in PD pathogenesis (Jonkman et al., 2019; Thomas et al., 2020). Although iron accounts for less than 2% of total body iron content, it plays essential roles in brain development, neurotransmitter synthesis, and metabolic processes. However, excess iron becomes detrimental by facilitating the interaction between dopamine and molecular oxygen, resulting in the production of cytotoxic quinones and ROS, an effect particularly prominent in

the SNpc (Hare and Double, 2016; Nakamura et al., 2019). In this study, we established an iron-induced PD rat model via intraperitoneal injection of iron dextran. We observed significant motor impairments in these iron dextran-treated animals. In addition, TH and DAT are well-established markers of dopaminergic neurons. Consistent with findings in PD patients (Niznik et al., 1991), our results indicated a pronounced decrease in TH and DAT protein levels in the SN of iron overload-induced PD rats, confirming the successful induction of a PD animal model.

Synaptic dysfunction is increasingly recognized as a critical component of PD pathology. Glutamate is the major excitatory neurotransmitter in the central nervous. Both NMDARs and AMPARs are integral to synaptic plasticity, which underlies complex behaviors. Most NMDARs consist of GluN1 and GluN2 subunits (Ladagu et al., 2023), with GluN2B and GluN2D being highly expressed in the SN (Jones and Gibb, 2005). Dopaminergic neurons in the SN receive substantial excitatory input, primarily glutamatergic projections from the subthalamic nucleus and cerebral cortex. To investigate whether iron overload could affect excitatory neurotransmission and synaptic function, we assessed the expression of glutamate receptors in the SN and Str of iron overload rats. We observed decreased expression of key receptor subunits—GluN1, GluN2A, GluN2B, and GluA1—in both SN and Str following iron dextran administration. Additionally, the synapse-associated proteins Arc and PSD-95 were also downregulated in iron overload rats. These findings suggest that iron overload not only induces oxidative stress but also significantly disrupts synaptic function both in SN and Str. The downregulation of NMDA and AMPA receptor subunits, along with postsynaptic density proteins, points to a likely mechanism through which iron contributes to synaptic dysfunction and subsequent motor deficits in PD.

In our study, we have also demonstrated that FAC treatment inhibited both spontaneous and evoked firing activities, and induced hyperpolarized resting membrane potential in

dopaminergic neurons within brain slices. Mitochondrial dysfunction has been reported to induce disturbance of membrane potential, subsequently decreases firing activity of dopaminergic neurons in the SN (Kriete, 2012), leading to reduced dopamine release in the striatum and locomotion deficit in PD (González-Cabrera et al., 2017). Our in vitro results showed that iron overload increased intracellular iron and ROS levels and decreased mitochondrial membrane potential. This suggested that FAC-induced oxidative stress and mitochondrial dysfunction might be responsible for the reduction of spontaneous firing rate in dopaminergic neurons of SN and decreased dopamine release in the striatum. In addition, our study has demonstrated that iron overload in VM neurons induces a profound dysfunction of dopaminergic neurons, characterized by a concomitant reduction in the expression of VMAT-2 and D1R. The coordinated downregulation of these dopamine-related proteins, coupled with diminished firing frequency and action potential generation, provides compelling evidence that the disruption of dopamine homeostasis was induced by iron overload.

As mentioned above, our results have revealed the impaired synaptic function and decreased expression of NMDAR and AMPAR in the SN and Str in iron-treated rats. Our in vitro results further confirmed the decreased expressions of Arc, GluN1, GluN2A, GluN2B and GluA1 in iron-treated VM neurons, which was consistent with the results in the rat model of iron overload. In addition, we also examined the expression of phosphorylated GluN2B and phosphorylated GluA1 in this study. Result showed decreased expression of phosphorylated GluA1 after FAC treated for 24 hours, and increased expression of phosphorylated GluN2B after FAC treated for 1 hour. Despite the loss of this increase at 24 hours, we cannot exclude a functional role of phosphorylation in GluN2B membrane expression. Phosphorylation of GluA1 have been shown to control AMPAR trafficking, thereby regulating synaptic plasticity. Our results suggest the possible dysfunction of synaptic plasticity in iron overload neurons. However, the total protein measurement is inadequate to clarify synaptic-specific

protein changes, follow-up experiments measuring NMDAR and AMPAR expression in purified synaptosomal fractions would offer more direct evidence regarding iron-induced synaptic dysfunction. Additionally, our in vitro results showed increased expression of PSD95 in VM neurons after iron overload. This observation is different from our in vivo findings, where PSD-95 expression was decreased in the SN of iron-overloaded rats. We propose that this discrepancy might arise from fundamental differences between the two experimental systems. PSD-95 contains multiple protein-interaction domains that enable binding to various membrane proteins including NMDARs. Through these interactions, PSD-95 could affect the surface expression of NMDARs (Kim and Sheng, 2004; Prybylowski et al., 2002; Roche et al., 2001). Therefore, we then examined the surface protein expression of NMDARs in VM neurons after iron overload. Results showed that iron overload increased the expressions of GluN1, GluN2A, GluN2B and phosphorylated GluN2B on the membrane of VM neurons. These results indicated that iron might promote the membrane expression of NMDA receptors in VM neurons. Phosphorylation is known to enhance receptor function, ion permeability, and membrane localization (Ardito et al., 2017). Accordingly, the altered phosphorylation of GluN2B observed in our study may promote its membrane redistribution. The GluN2B-PSD-95-nNOS complex has been reported to facilitate GluN2B phosphorylation at Tyr1472, triggering downstream neurotoxic cascades (Zhu et al., 2020), and PSD-95 may also contribute to membrane retention of GluN2B and GluN2A (Horak et al., 2014; Lussier et al., 2015). Thus, we propose that in VM neurons, iron overload may facilitate the assembly of the GluN2B–PSD-95–nNOS complex and upregulate PSD-95, thereby driving its redistribution to the membrane. In addition, our result showed that a Src family inhibitor Saracatinib could significantly inhibit the phosphorylation of GluN2B, and also decreased the level of GluN2B in membrane induced by FAC. These results suggest that Src-family kinases might mediate GluN2B phosphorylation, which in turn promotes the

637 expressiion of GluN2B in the membrane of VM neurons under iron overload conditions.
 638 NMDAR-mediated excitotoxicity is one of the important mechanisms contributing to the
 639 degeneration of dopaminergic neurons (Hallett and Standaert, 2004; Shimazu et al., 2000).
 640 The increased surface expression of GluN2A, GluN2B and phosphorylated GluN2B might
 641 increase the function of NMDARs, thereby leading to more Ca^{2+} influx into neurons, which
 642 could further activate NOS, leading to excessive nitric oxide (NO) production and induce
 643 NMDAR-mediated excitotoxicity. In models of ischemic injury, the GluN2B–PSD-95–nNOS
 644 complex translocates to the membrane, further amplifying NO-mediated neuronal injury (Fan
 645 et al., 2025; Shi et al., 2017; Zhou et al., 2010). In addition, NMDAR overactivation results in
 646 a significant down-regulation in *Bdnf* transcription, which could be rescued by blocking
 647 either GluN2A or GluN2B (Zhou et al., 2013). Here, our results also suggest that iron
 648 overload might be involved in the regulation of BDNF prosurvival signaling by upregulation
 649 of GluN2A and GluN2B, thereby inducing excitotoxicity and cell death.
 650 In our study, we have observed that iron promoted the assembly of GluN2B–PSD-95–nNOS
 651 complex. However, how excess iron triggers this abnormal assembly of GluN2B–PSD-95–
 652 nNOS complex is not explored. Evidence has shown that in the rotenone model, dopamine-
 653 derived ROS relieved the Mg^{2+} block of NMDARs, enhancing Ca^{2+} influx and excitotoxicity
 654 (Chen et al., 2012). Since Fe^{2+} produces ROS and synergizes with dopamine to exacerbate
 655 oxidative stress in dopaminergic neurons (Wu and Johnson, 2011), we propose that iron
 656 potentiates NMDAR activity might via a similar ROS-dependent pathway.
 657 Importantly, in this study, we also found that Tat-NR2B9c, a selective PSD-95 inhibitor,
 658 attenuated iron-induced neurotoxicity in dopaminergic cells. As we have mentioned above,
 659 Tat-NR2B9c is a PSD-95 inhibitor that specifically disrupts protein interactions between
 660 PSD-95 and NMDAR, as well as inhibits nNOS/PSD-95 interaction. Our Co-IP

661 results revealed FAC enhanced the protein–protein interactions between PSD-95 and
662 GluN2B, as well as between PSD-95 and nNOS, suggesting an assembly of the PSD-
663 95/nNOS/GluN2B complex under iron overload conditions. Importantly, Tat-NR2B9c
664 attenuated these enhanced interactions, indicating its ability to disrupt the iron-induced
665 aberrant protein complex formation. Collectively, these findings suggest that Tat-NR2B9c
666 exerts neuroprotective effects against iron-induced toxicity, at least in part, by restoring
667 normal expression patterns of key synaptic proteins and preventing the pathological assembly
668 of the PSD-95–nNOS–GluN2B complex, thereby mitigating downstream excitotoxic
669 signaling.

670 The present findings also demonstrate that Tat-NR2B9c significantly suppresses iron-induced
671 NO overproduction and calcium overload, two key mediators of excitotoxic neuronal injury.
672 Moreover, the protective effects were mimicked by both the nNOS inhibitor 7-nitroindazole
673 and the GluN2B inhibitor ifenprodil, indicating that iron toxicity is critically dependent on
674 the activation of the GluN2B-containing NMDA receptor and its downstream nNOS
675 signaling. Given that Tat-NR2B9c is known to disrupt the physical interaction between
676 NMDA receptors and postsynaptic scaffolding proteins (e.g., PSD-95), these results
677 collectively suggest that Tat-NR2B9c exerts its neuroprotection against iron-induced damage
678 by uncoupling the pathological GluN2B-PSD-95-nNOS complex, thereby attenuating NO
679 production and calcium dysregulation. This mechanism positions Tat-NR2B9c as a promising
680 therapeutic strategy for neurodegenerative conditions associated with iron overload, such as
681 Parkinson’s disease or other movement disorders, by targeting a specific protein–protein
682 interaction rather than broadly blocking glutamate receptors.

683 In addition, our results showed that Tat-NR2B9c reduced iron-induced increase of nNOS
684 expression and restored iron-induced decrease in BDNF expression. These findings further
685 support the notion that Tat-NR2B9c exerts neuroprotection in dopaminergic cells under iron

overload conditions via decoupling NMDAR–PSD-95–nNOS signaling and enhancing BDNF-mediated survival pathways. Collectively, these results highlight the significance of PSD-95/NMDAR/nNOS complex in iron-induced excitotoxicity and identify PSD-95 as a potential therapeutic target for neuroprotection. By interfering with this scaffolding protein, Tat-NR2B9c offers a strategy to mitigate excitotoxic injury without directly blocking NMDA receptors, thereby preserving physiological glutamatergic transmission.

In conclusion, our study provides comprehensive evidence that iron overload contributes to the degeneration of dopaminergic neurons through synergistic mechanisms involving oxidative stress, dopamine dyshomeostasis, synaptic dysfunction, and enhanced NMDAR–PSD-95–nNOS signaling. Targeting the PSD-95–NMDAR interaction may represent a promising neuroprotective strategy for delaying or preventing iron-related neurodegeneration including PD.

DATA AVAILABILITY

The data that support the findings of this study are available on request from the corresponding author, upon reasonable request

SUPPLEMENTARY MATERIAL

Supplemental Data

DECLARATION OF COMPETING INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

X.D.G.: Writing – original draft, Visualization, Methodology, Formal analysis; Z.X.X. (Zhixin Xiao): Writing–original draft, Investigation, Validation, Methodology; L.Q.: Software, Methodology, Investigation; R.Y.X.: Validation, Methodology, Investigation;

Z.X.X. (Zhaoxia Xiang): Validation, Methodology. L.M.S.: Validation, Supervision, Resources, Conceptualization; J.X.X.: Supervision, Project administration; H.M.X.: Writing – review & editing, Resources, Formal analysis, Funding acquisition, Data curation, Project administration. All authors read and approved the final version of the manuscript.

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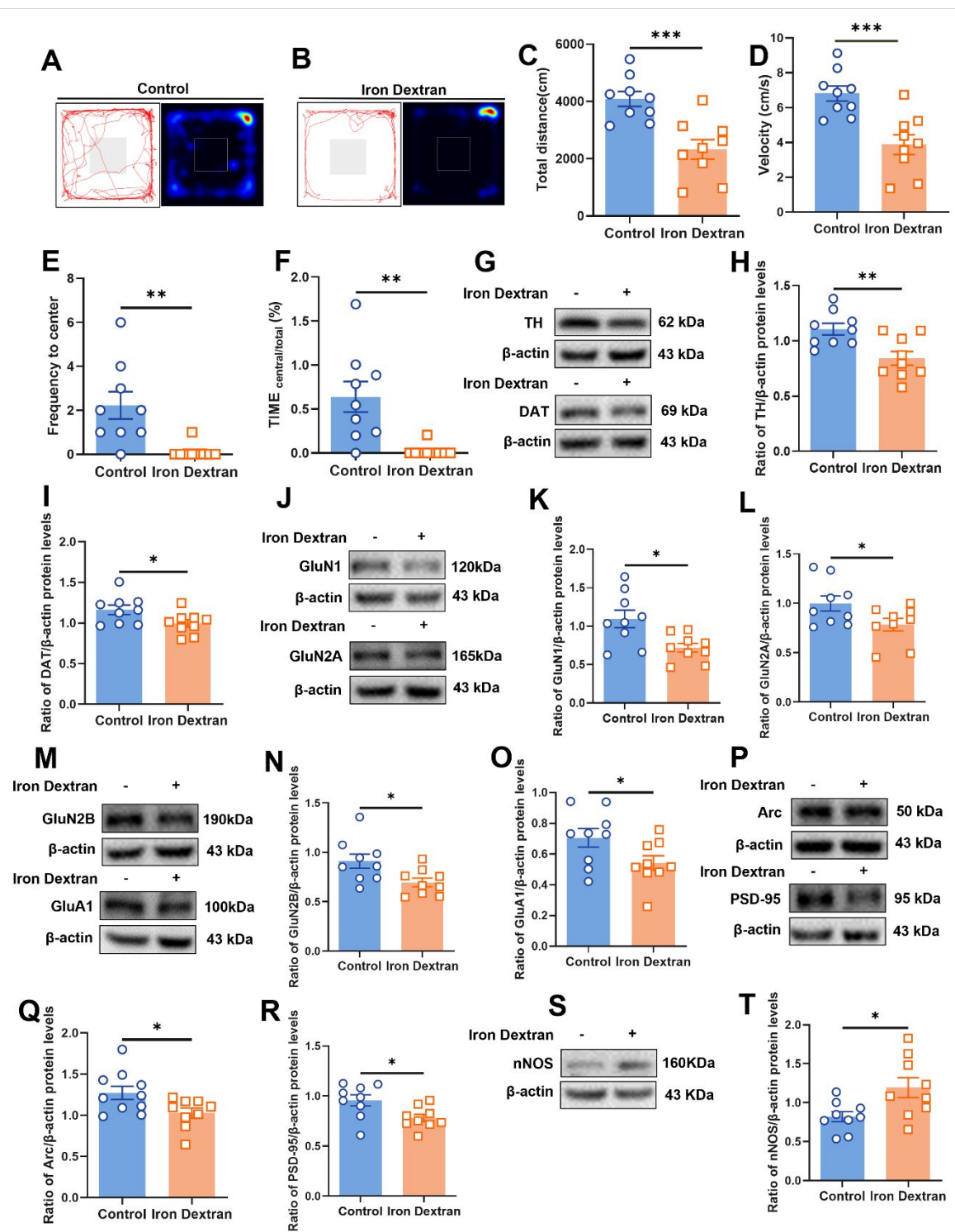


Fig. 1 Iron dextran induced motor deficits of rats and decreased protein expression of TH, DAT, and synapse-associated proteins in the SN.

A: Trajectory of rats in open field test in control group. B: Trajectory of rats in open field test in iron-dextran group. C-F: The total distance, velocity, and frequency in the central area and percent time spent in the center of rats in open field test. G, H: The expression of TH protein in the SN of rats. G, I: The expression of DAT protein in the SN of rats. J-T: The expressions of GluN1, GluN2A, GluN2B, GluA1, Arc, PSD-95 and nNOS protein in SN of rats. Western blot was applied to examine the protein levels. GluN1 (J, K), GluN2A (J, L),

GluN2B (M, N), GluA1 (M, O), Arc (P, Q), PSD-95 (P, R), nNOS (S, T). Data are presented as mean $\pm$ SEM, *t*-test, n=9, *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; compared with the control group.

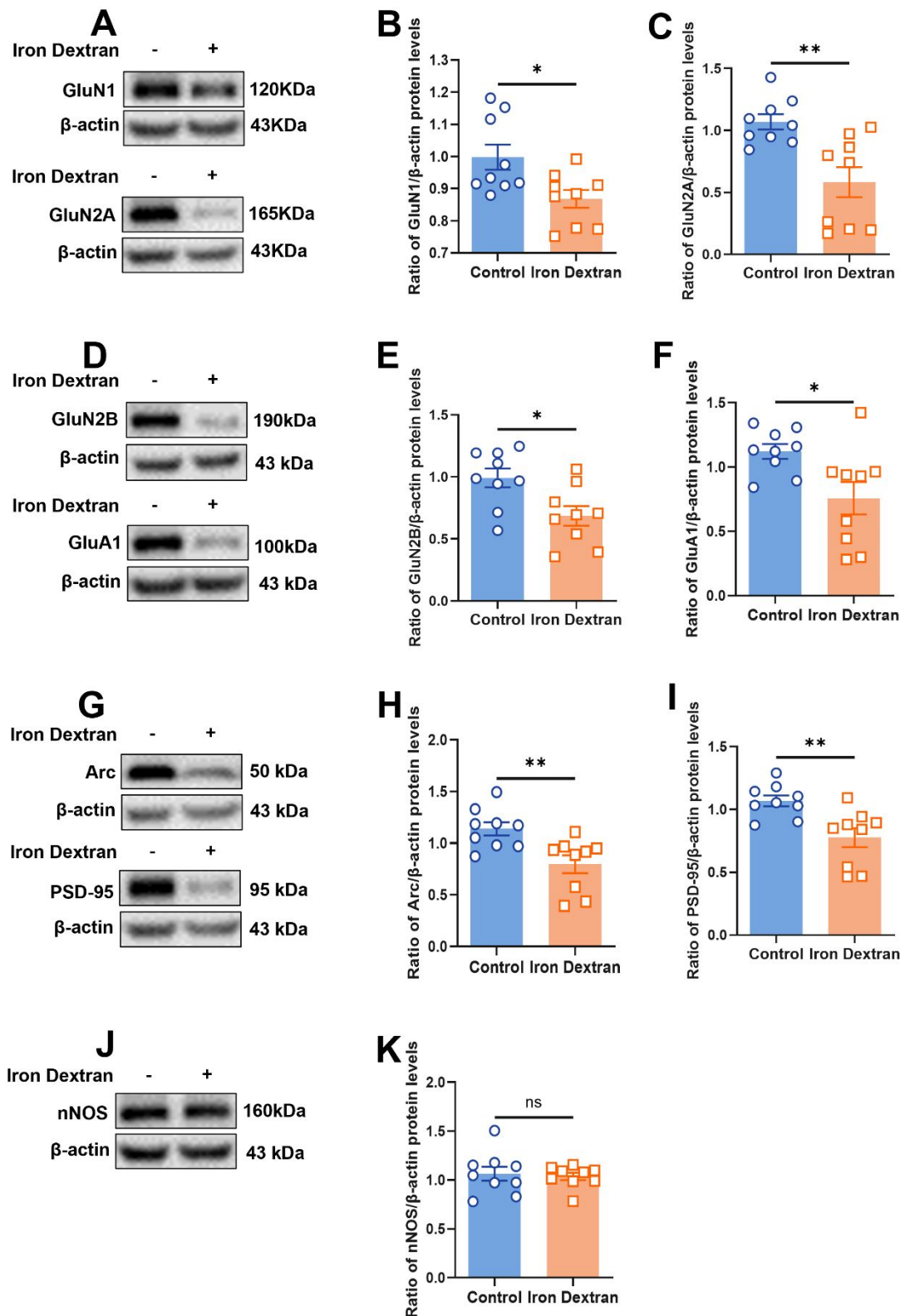


Fig. 2 The expression of synapse-associated proteins decreased in Str of iron dextran treated rats.

A-K: The expressions of GluN1, GluN2A, GluN2B, GluA1, Arc, PSD-95 and nNOS protein in Str of rats.

Western blot was applied to examine the protein levels. GluN1 (A, B), GluN2A (A, C), GluN2B (D, E)

GluA1 (D, F), Arc (G, H), PSD-95 (G, I), nNOS (J, K). Data are presented as mean $\pm$ SEM; *t*-test, *n*=9; ns:

945 Not significant; *: $P < 0.05$; **: $P < 0.01$; compared with the control group.

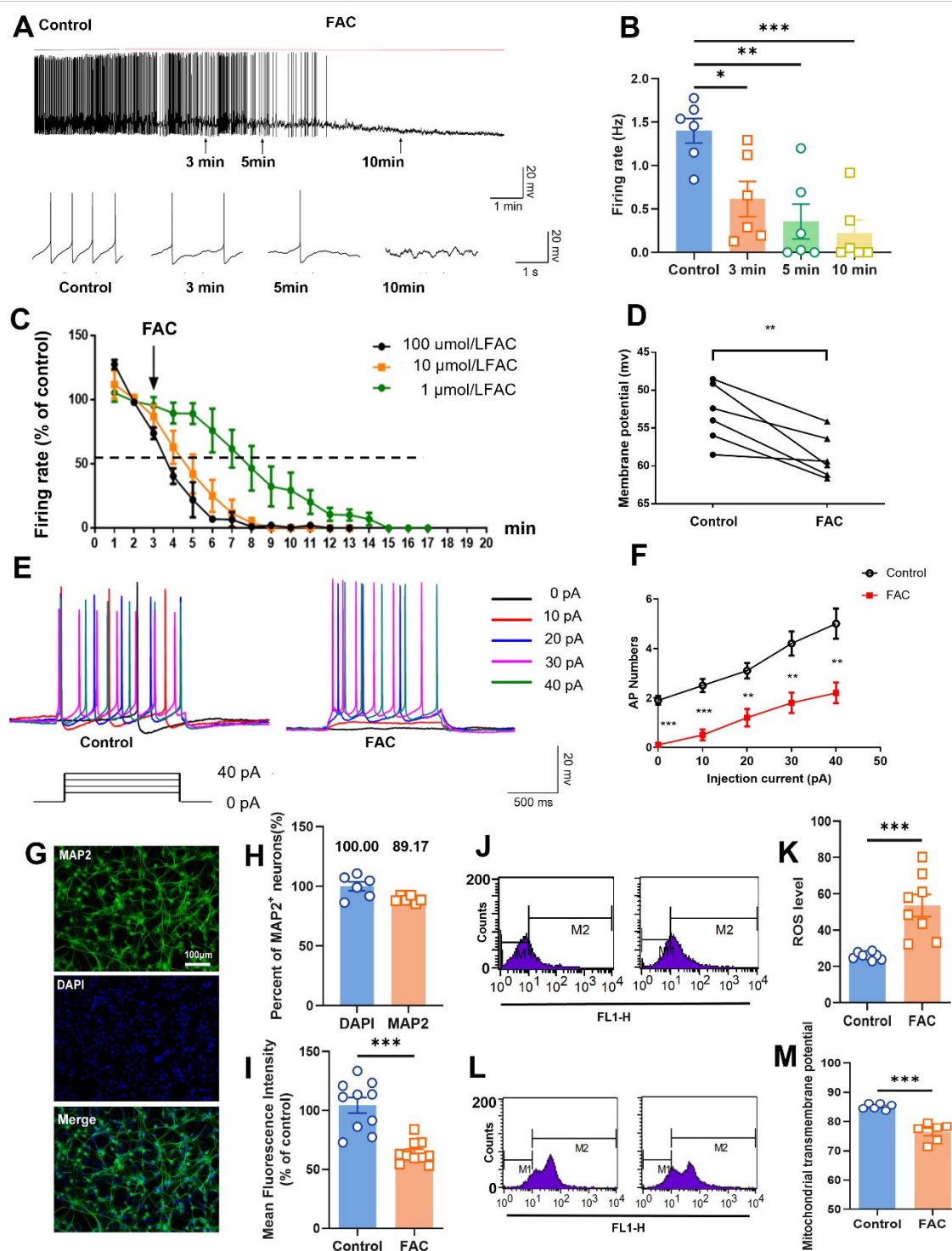


Fig. 3 FAC decreased the firing activity of dopaminergic neurons in the SNpc, and increased intracellular iron and ROS levels and decreased mitochondrial membrane potential ($\Delta \Psi_m$).

A, B: Representative recordings and summarized data illustrating that 100 $\mu\text{mol/L}$ FAC significantly inhibited the spontaneous firing frequency of dopaminergic neurons. One-way ANOVA analysis, $n=6$. C: The inhibitory effect of FAC (1, 10, and 100 $\mu\text{mol/L}$) on the spontaneous firing activity of SNc dopaminergic neurons in mice was both time- and concentration-dependent. All quantified data are normalized to control

group (pre-drug baseline). One-way ANOVA analysis, n=6 for each concentration. D: The membrane potential of dopaminergic neurons was hyperpolarized following application of 100 $\mu\text{mol/L}$ FAC. Paired-samples *t*-test, n=6. E, F: Representative recordings and summarized data illustrating that 100 $\mu\text{mol/L}$ FAC reduced the number of evoked action potentials (AP) when dopaminergic neurons were subjected to current injections ranging from 0 to 40 pA. Data are presented as mean $\pm$ SEM, Two-way repeated-measures ANOVA analysis, n=10; The change of intracellular $\Delta\Psi\text{m}$ in VM neurons was observed after treatment with FAC. G, H: Detection of the purity of the cultured ventral midbrain neurons, that was indicated by the co-location ratio of neuron markers MAP2 to DAPI. n=6. I: The fluorescence intensity of Calcein-AM in the VM neurons. Each bar represented the mean $\pm$ SEM of 3 independent experiments. *t*-test, n=10. J, K: The intracellular ROS production in VM neurons. Each bar represented the mean $\pm$ SEM of 3 independent experiments. *t*-test, n=8. L, M: The detected of $\Delta\Psi\text{m}$ in VM neurons. Each bar represented the mean $\pm$ SEM of 3 independent experiments. n=6; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; compared with the control group.

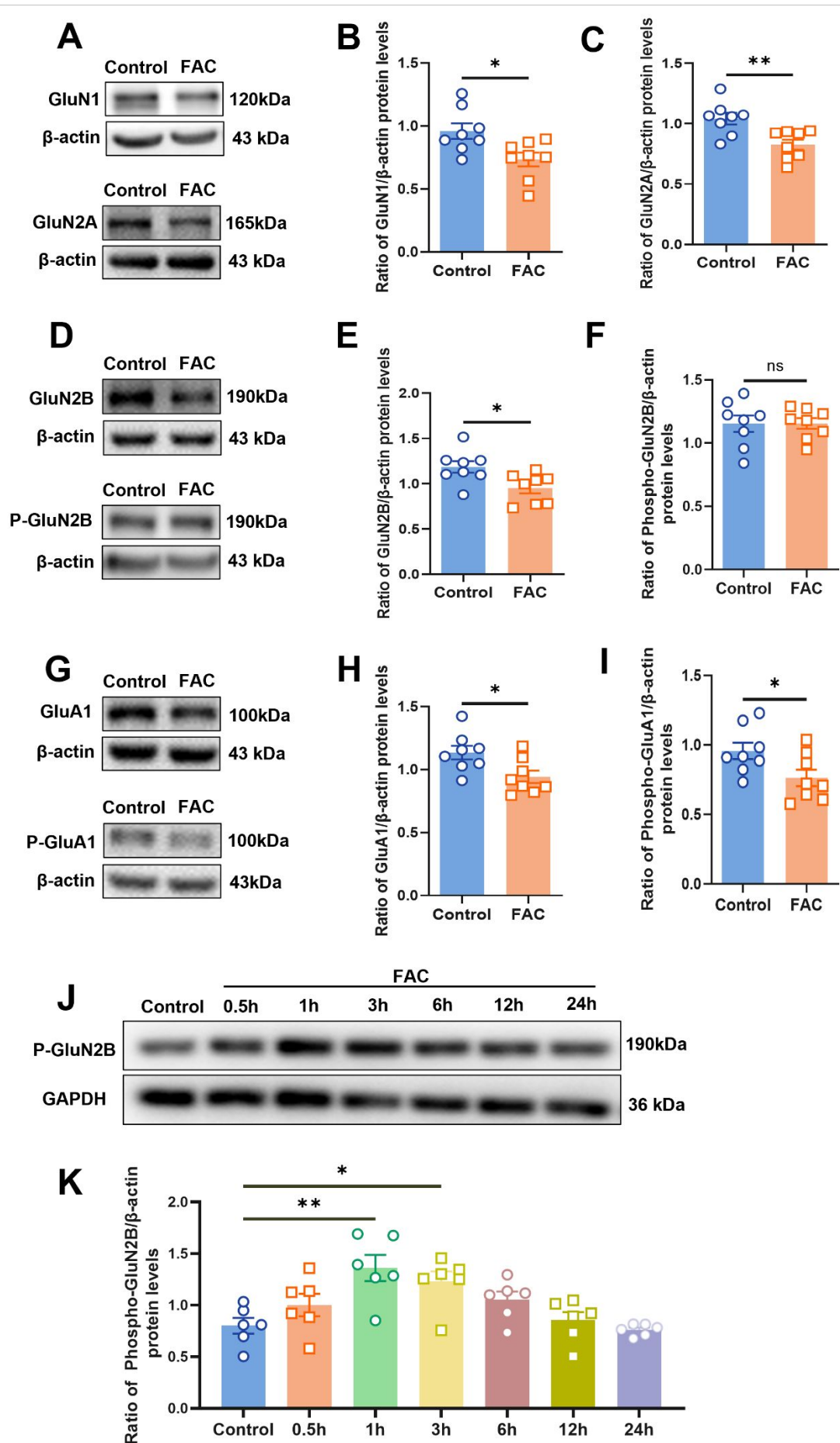


Fig. 4 The total protein expression of glutamate receptors in VM neurons after iron treatment.

A-I: The expressions of GluN1, GluN2A, GluN2B, p-GluN2B, GluA1, p-GluA1 protein in primary VM neurons. Western blot was applied to examine the protein levels. GluN1 (A, B), GluN2A (A, C), GluN2B (D, E), p- GluN2B (D, F), GluA1 (G, H), p-GluA1 (G, I), *t*-test, *n*=8; time-dependent changes of p-GluN2B following FAC treatment (J, K), One-way ANOVA analysis, *n*=6. Data are presented as mean $\pm$ SEM; ns: Not significant; *: *P*<0.05; **: *P*<0.01; compared with the control group.

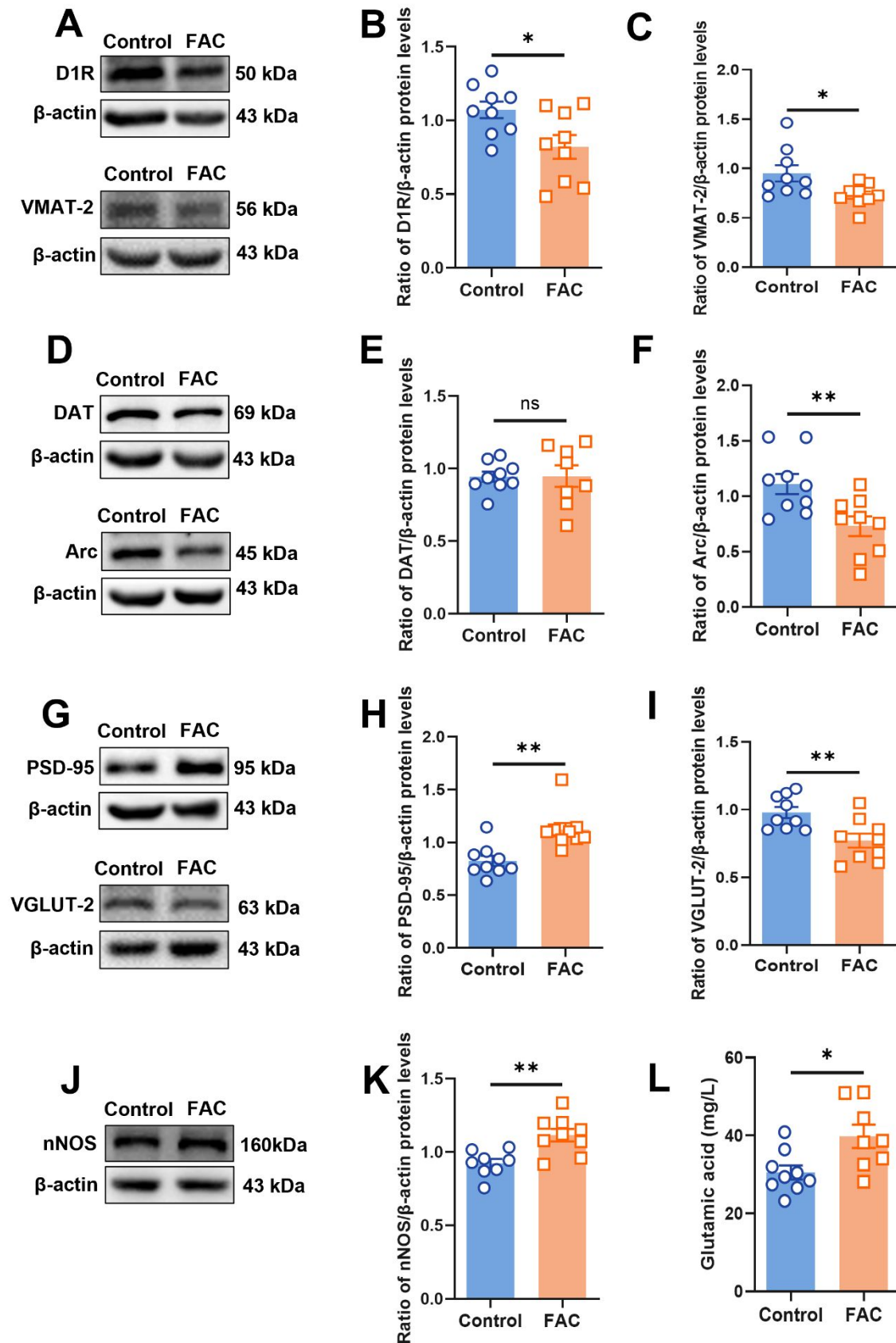


Fig. 5 Iron overload disrupts DA homeostasis and glutamate release and affects the expression of synapse-associated proteins in VM neurons

A-K: The expressions of D1R, VMAT-2, DAT, Arc, PSD-95, VGLUT-2, nNOS protein in primary VM neurons. Western blot was applied to examine the protein levels. D1R (A, B), VMAT-2 (A, C), DAT (D, E), Arc (D, F), PSD-95 (G, H), VGLUT-2 (G, I), nNOS (J, K). L: The glutamate level in the supernatant of VM neurons was detected by ELISA. Each bar represented the mean $\pm$ SEM of 3 independent experiments. *t*-test, n=9; ns: Not significant; *: *P*<0.05; **: *P*<0.01.

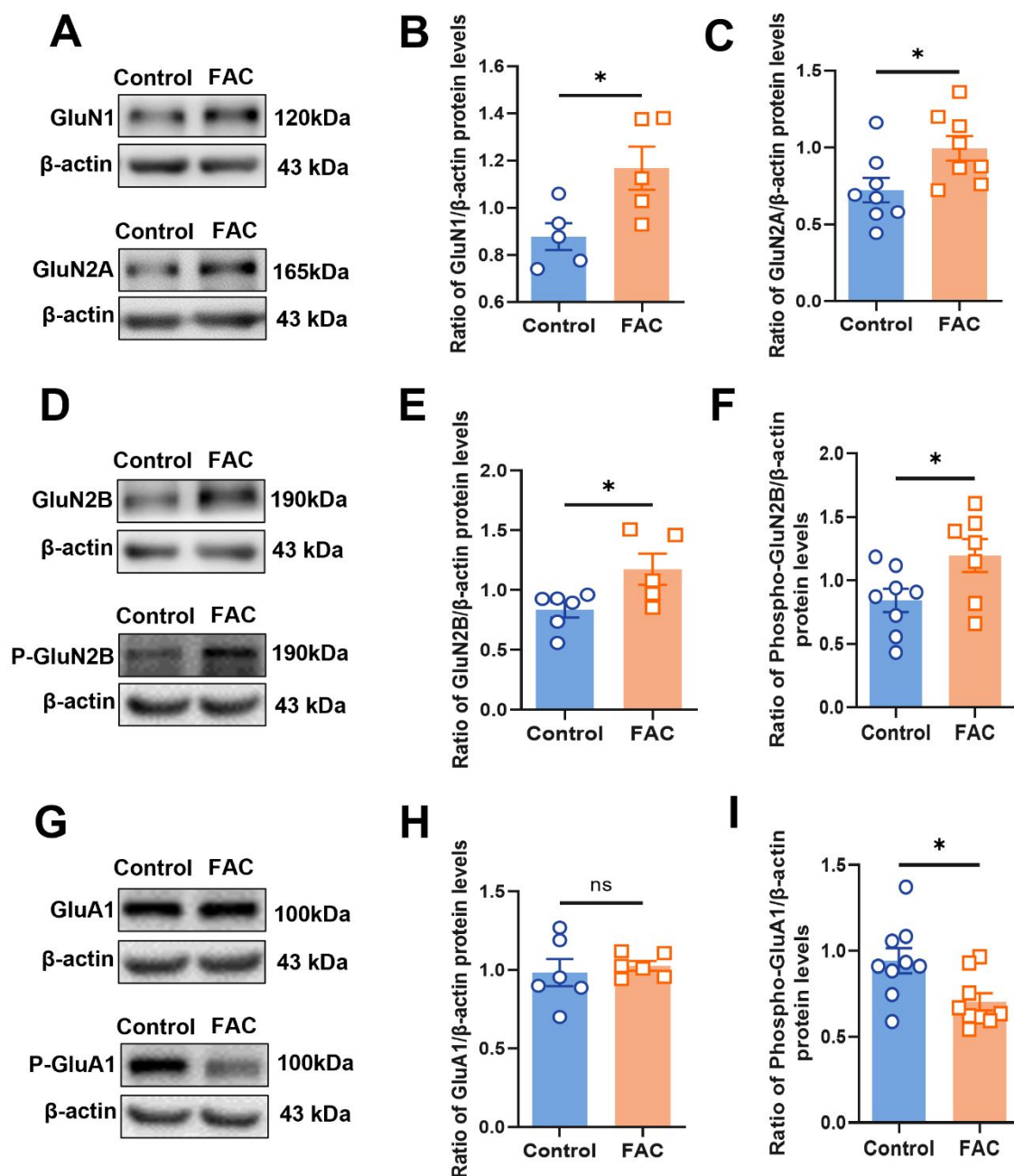


Fig. 6 The protein expression of glutamate receptors in the membrane of VM neurons after iron treatment.

A-I: The expressions of GluN1, GluN2A, GluN2B, p-GluN2B, GluA1, p-GluA1 protein in the membrane of primary VM neurons. Western blot was applied to examine the protein levels. GluN1 (A, B), GluN2A (A, C), GluN2B (D, E), p- GluN2B (D, F), GluA1 (G, H), p-GluA1 (G, I). Data are presented as mean $\pm$ SEM, *t*-test, $n \geq 5$; ns: Not significant; *: $P < 0.05$; compared with the control group.

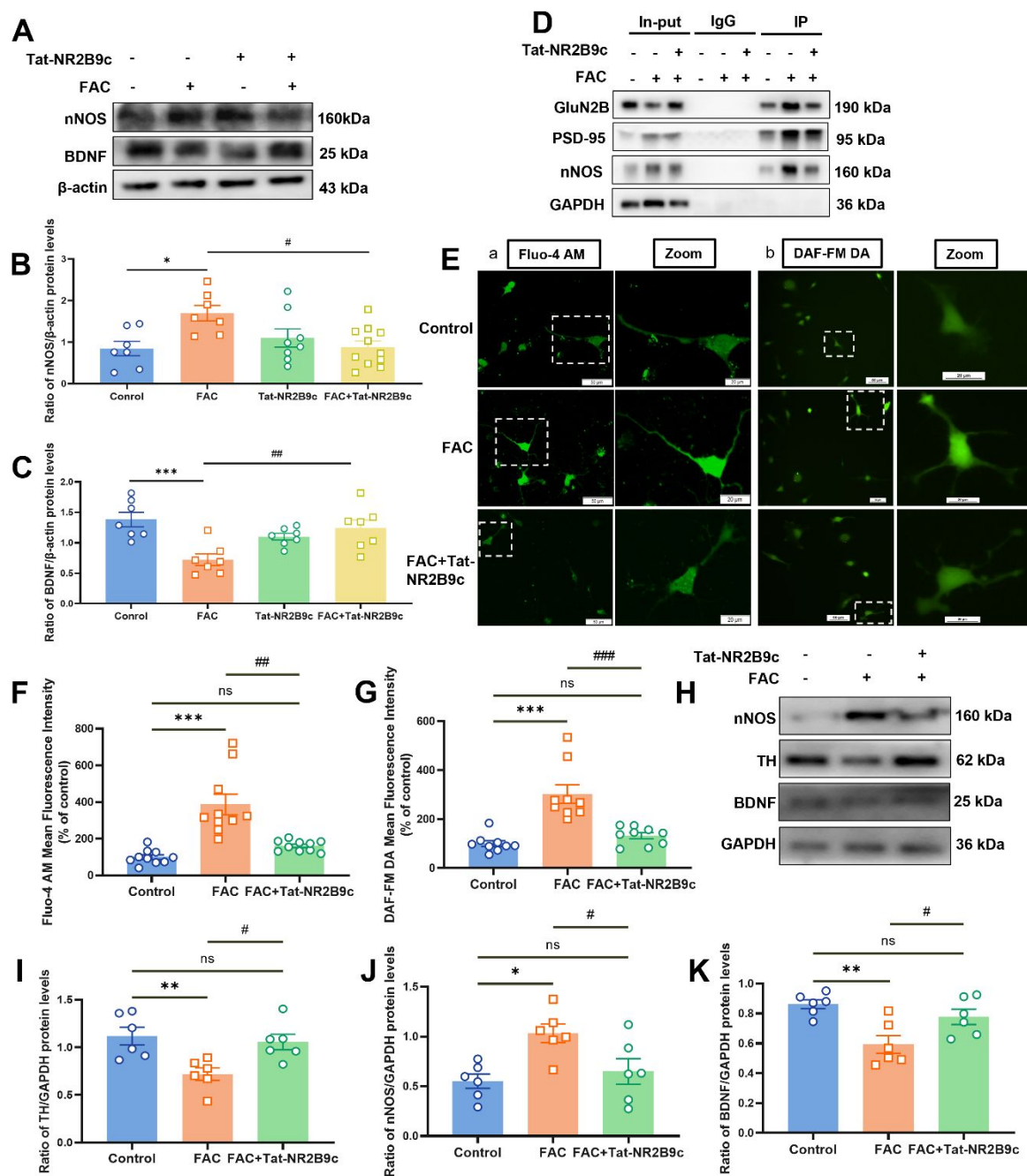


Fig. 8 Tat-NR2B9c attenuates iron-induced toxicity in dopaminergic neurons

A-C: The expression of nNOS and BDNF in PC12 cells. $n \geq 7$; D: Co-IP result showed the interactions between GluN2B, PSD-95 and nNOS in PC12 cells; E-a, F: The detected of Fluo-4 AM fluorescence intensity in primary VM neurons. $n=10$; E-b, G: The detected of DAF-FM DA fluorescence intensity in primary VM neurons. $n=9$; H-K: The expression of nNOS, BDNF and TH expression in primary VM neurons. $n=6$, One-way ANOVA analysis. Data are presented as mean $\pm$ SEM, ns: Not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; #: $P < 0.05$; ##: $P < 0.01$; ###: $P < 0.001$.

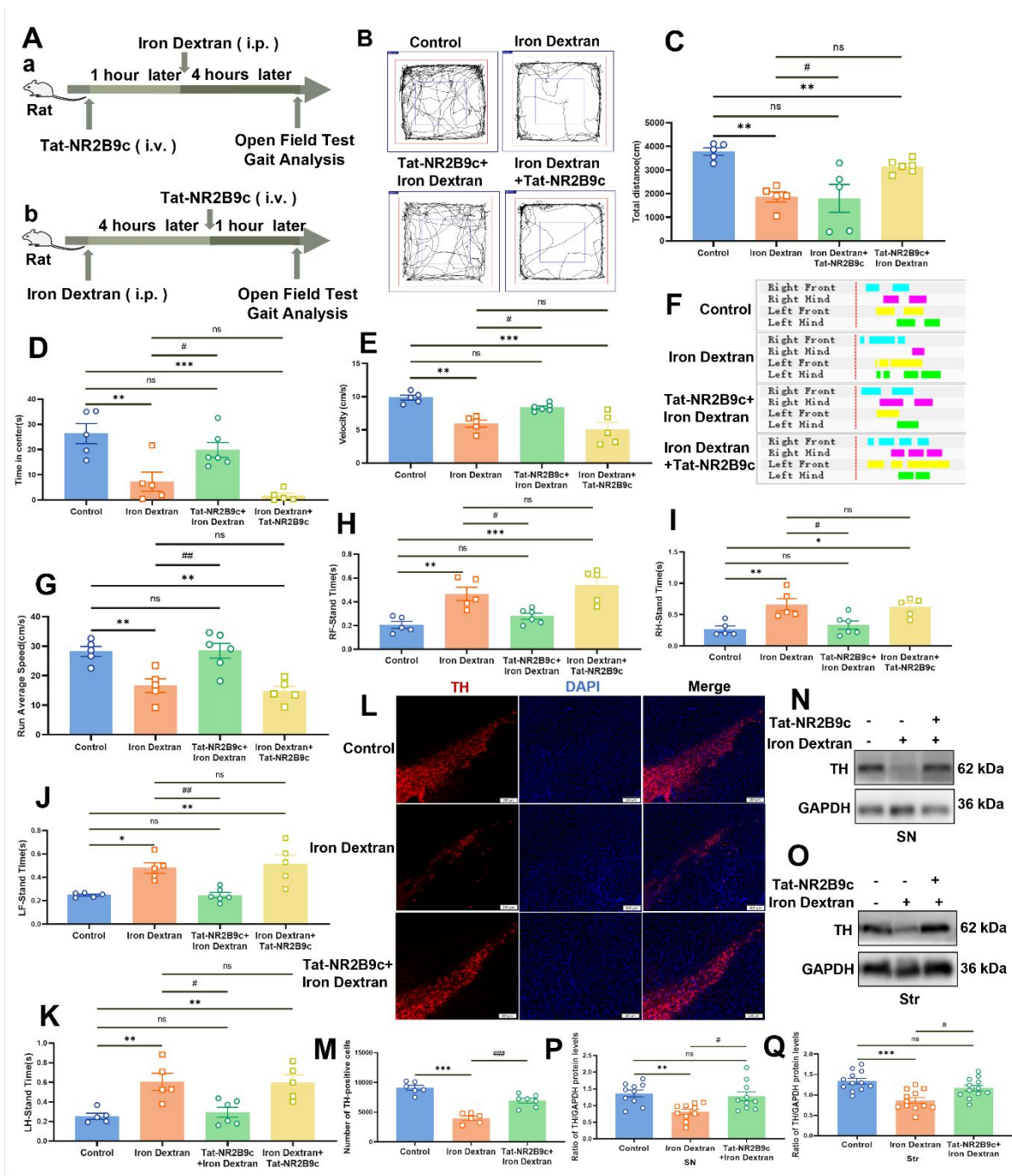
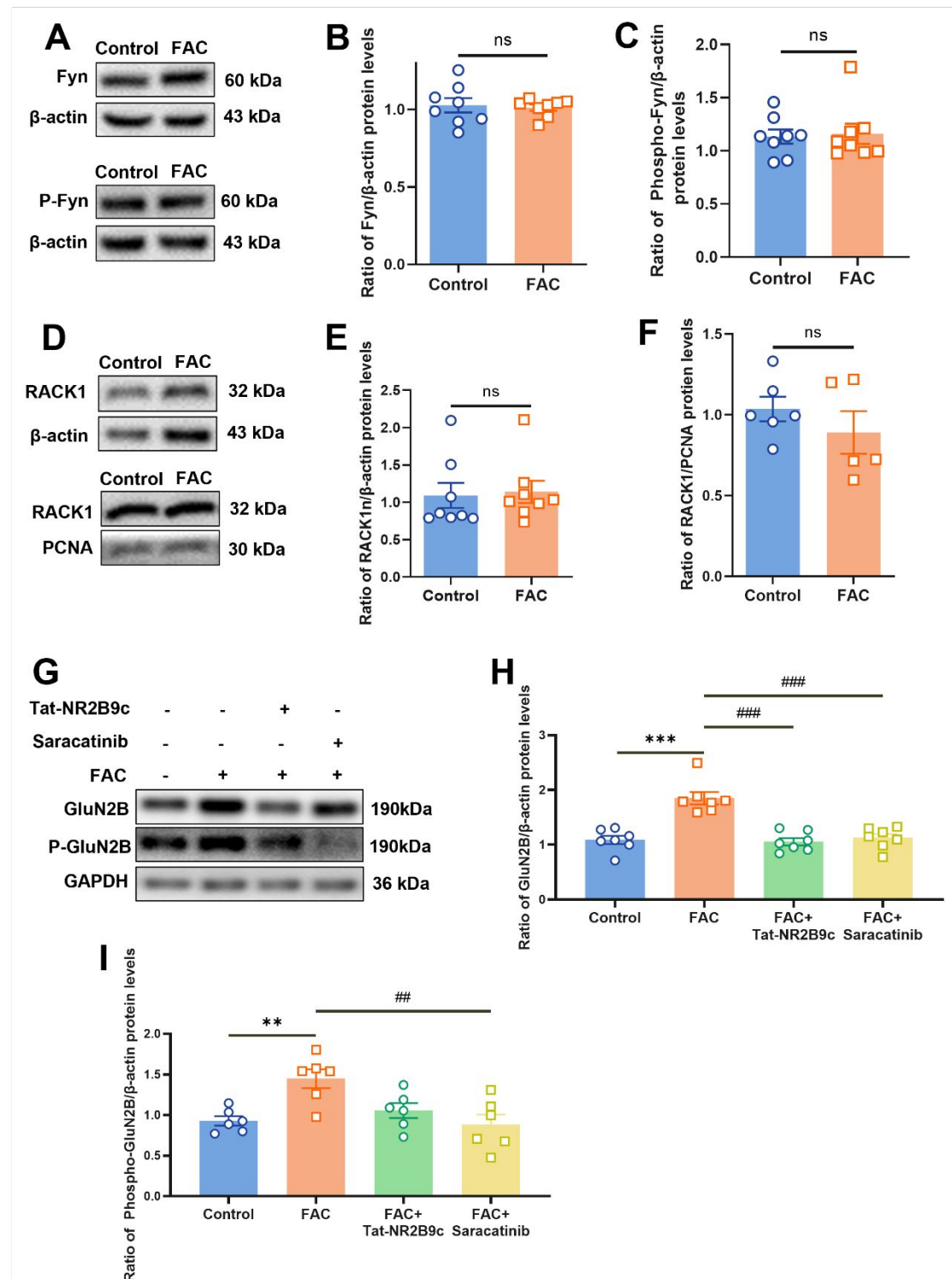


Fig. 9 Tat-NR2B9c protects against iron-induced toxicity in iron-induced rats

A: Experimental design. A-a, the experimental design of Tat-NR2B9c+iron dextran group; A-b, the experimental design of iron dextran+Tat-NR2B9c group. Abbreviations: i.v., tail vein intravenous injection; i.p., intraperitoneal injection. B-E: The results of open field test. The moved total distance, the residence time in the central area and the movement speed were detected in rats. F-K: The results of gait analysis. The run average speed and the stand time of limb were detected in rats. Abbreviations: RF, right front; RH, right hind; LF, left front; LH, left hind. L, M: Immunofluorescence staining of frozen sections showed the number of TH neurons in SN of rats. N-Q: The expression of TH protein in SN and Str of rats. Data are presented as

mean $\pm$ SEM; One-way ANOVA analysis, $n \geq 5$; ns: Not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; #, $P < 0.05$; ##: $P < 0.01$; ###: $P < 0.001$.



Supplementary Fig. 1 Involvement of Src-family kinases in GluN2B membrane expression.

A-I: The expressions of Fyn, p-Fyn, RACK1, PCNA, GluN2B, p-GluN2B protein in the primary VM neurons. Western blot was applied to examine the protein levels. Fyn (A, B), p-Fyn (A, C), cytoplasmic

RACK1 (D, E), nuclear RACK1 (D, F), *t*-test, *n*=8; GluN2B (G, H), p-GluN2B(G, I), One-way ANOVA analysis, *n*=6. Data are presented as mean ± SEM; ns: Not significant, **: *P*<0.01; ***: *P*<0.001; #: *P*<0.01; ###: *P*<0.001.

铁超载通过 NMDAR-PSD-95-nNOS 信号介导兴奋性毒性并促进多巴胺能神经元突触功能障碍

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摘要

铁在黑质 (substantia nigra, SN) 的沉积被认为是帕金森病发病的关键因素。然而, 铁对多巴胺能神经元损伤的确切影响以及潜在的分子机制尚不清楚。这项研究旨在探究过量铁如何破坏多巴胺稳态和突触完整性, 重点关注了 NMDAR 所介导的兴奋性毒性。结果表明在右旋糖酐铁处理的大鼠中, 铁破坏多巴胺稳态, 并降低了 SN 和纹状体中 NMDAR 和 AMPAR 亚单位及几种突触可塑性相关蛋白的表达。此外, 柠檬酸铁铵 (ferric ammonium citrate, FAC) 抑制黑质致密部多巴胺能神经元的自发和诱发放电。在大鼠原代腹侧中脑 (ventral midbrain, VM) 神经元中, FAC 能增加细胞内游离铁和 ROS 水平, 降低线粒体膜电位, 并下调总 NMDARs 和 AMPARs 的蛋白水平。在体外实验中, FAC 能上调 PSD-95 和 nNOS 蛋白的表达, 增加 GluN2B 和 GluN2A 在神经元膜表面的水平, 并促进 GluN2B-PSD-95-nNOS 复合物的形成。Tat-NR2B9c 是一种能够阻断 GluN2B 和 PSD-95 相互作用的肽, 我们研究表明, 在原代 VM 神经元和大鼠模型中, Tat-NR2B9c 均可抑制铁诱导的神经毒性。该保护作用表现为抑制 nNOS 上调、减少 NO 过量产生、恢复线粒体膜电位和上调 BDNF 蛋白表达, 其机制是通过抑制 PSD-95-nNOS-GluN2B 复合体形成实现的。以上结果表明, 铁超载通过 NMDAR-PSD-95-nNOS 信号通路破坏多巴胺稳态, 诱导突触功能障碍及 NMDAR 介导的兴奋性毒性。而干扰 PSD-95/NMDAR 相互作用有望成为对抗铁诱导神经元损伤的一种新型保护策略。

关键词: 帕金森病; 多巴胺; NMDA 受体; 铁; 突触后致密蛋白 95



uncorrected proof

