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Downregualtion of dynamin-related protein 1 attenuates glutamate-induced excitotoxicity via regulating mitochondrial function in a calcium dependent manner in HT22 cells

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ABSTRACT

Glutamate-mediated excitotoxicity is involved in many acute and chronic brain diseases. Dynamin related protein 1 (Drp-1), one of the GTPase family of proteins that regulate mitochondrial fission and fusion balance, is associated with apoptotic cell death in cancer and neurodegenerative diseases. Here we investigated the effect of downregulating Drp-1 on glutamate excitotoxicity-induced neuronal injury in HT22 cells. We found that downregulation of Drp-1 with specific small interfering RNA (siRNA) increased cell viability and inhibited lactate dehydrogenase (LDH) release after glutamate treatment. Downregulation of Drp-1 also inhibited an increase in the Bax/Bcl-2 ratio and cleavage of caspase-9 and caspase-3. Drp-1 siRNA transfection preserved the mitochondrial membrane potential (MMP), reduced cytochrome c release, enhanced ATP production, and partly prevented mitochondrial swelling. In addition, Drp-1 knockdown attenuated glutamate-induced increases of cytoplasmic and mitochondrial Ca^{2+}, and preserved the mitochondrial Ca^{2+} buffering capacity after excitotoxicity. Taken together, these results suggest that downregulation of Drp-1 protects HT22 cells against glutamate-induced excitatory damage, and this neuroprotection may be dependent at least in part on the preservation of mitochondrial function through regulating intracellular calcium homeostasis.

1. Introduction

Glutamate is the most abundant excitatory neurotransmitter in the central nervous system (CNS). In the case of brain trauma, stroke, epilepsy, and chronic neurodegenerative disorders, increased concentrations of extracellular glutamate caused by excessive vesicular release, disruption of cellular uptake, or liberation of glutamate following necrotic cell lysis may induce neuronal damage through receptor-mediated excitotoxicity [1]. The excitotoxic mechanisms of glutamate include hyper-activation of glutamate receptors, massive influx of calcium ions, over-activation of Ca^{2+}-dependent lethal second messengers and enzymes, and followed mitochondrial dysfunction that eventually causes death of neurons [2]. Inhibition of glutamate-induced activation of downstream detrimental cascades is a critical step for identification of potential therapeutic targets for future pharmacological and genetic intervention strategies.

Mitochondria are unique among cell organelles because of their involvement in cellular energy metabolism and intracellular calcium mobilization [3]. The important role of mitochondria in glutamate-induced excitotoxicity has been demonstrated by the deterministic influence of mitochondrial dysfunction in apoptotic and/or necrotic cell death and by protective effects of several pharmacological agents conferred by preservation of mitochondrial function [4]. Several hallmarks of mitochondrial dysfunction can be found after glutamate-induced excitotoxicity, including loss of the mitochondrial membrane potential (MMP), opening of the permeability transition pore and structural collapse [5]. An imbalance in mitochondrial division and mitochondrial fusion is one of the most important structural abnormality found in a large number of mitochondrial dysfunction related neurological diseases, and the GTPase gene dynamin-related protein 1 (Drp-1) is considered to be a key molecular in regulating mitochondrial fission [6]. Previous studies have been suggested that the outer membrane proteins, such as Fis1 and Mff, and the pro-apoptotic Bcl-2 family protein Bax function directly or indirectly as Drp-1 receptors to promote mitochondrial fission [6,7]. Drp-1 activation leads to abnormalities in mitochondrial structure and function, inhibits ATP generation, and activates pro-apoptotic signaling cascades [8]. In the present study, we have examined the involvement of Drp-1 in glutamate induced excitotoxicity in HT22 cells, an immortalized mouse hippocampal cell line. Knocking down of

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Drp-1 expression was performed by using small interfering RNA (siRNA)-induced gene silencing, and mitochondrial function was measured in injured HT22 cells and isolated mitochondria. The changes of intracellular calcium homeostasis were also determined to investigate the potential mechanism with focus on regulation of intracellular calcium homeostasis.

2. Materials and methods

2.1. Cell culture

HT22 mouse hippocampal cells were obtained from the Institute of Biochemistry and Cell Biology, SIBS, CAS. The cells were grown in DMEM plus 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin) in a humidified incubator with 5% CO_2 and 95% air. Growth medium was removed and replaced by medium containing glutamate (5 mM) for induction of apoptosis.

2.2. RNA interference (RNAi)

Drp-1 siRNA (sc-45953, Santa Cruz, CA, USA) and control siRNA (sc-37007, Santa Cruz, CA, USA) were dissolved separately in Optimem I (Invitrogen, CA, USA). After 10 min of equilibration at room temperature, each RNA solution was combined with the respective volume of the Lipofectamine 2000 solution, mixed gently and allowed to form siRNA liposomes for 20 min. The HT22 cells were transfected with the transfection mixture in antibiotic-free cell culture medium for 72 h before glutamate treatment, and subjected to various measurements.

2.3. Immunocytochemistry (ICC)

After being fixed with 4% paraformaldehyde for 15 min at room temperature, HT22 cells were washed with NaCl/Pi permeabilized with 0.2% Triton X-100, and incubated with primary antibodies (anti-Drp-1, 1:200 and anti-Cleaved-Caspase-3, 1:100) overnight at 4 °C. Cells were then incubated with Alexa 488-conjugated secondary antibodies for 2 h at 37 °C, and Hoechst 33342 (10 μg/ml) was used to stain nucleus. Images were captured with an Olympus FV10i Confocal Microscope (Tokyo, Japan). At least six images of each group were taken by an evaluator blinded to the experimental conditions.

2.4. Cell viability assay

Cell viability assay was performed by using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) that can be reduced to purple-coloured formazan by intact cells. After various treatments, cell viability was assessed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI) according to the manufacturer's instructions. The absorbance was measured with an automatic microplate reader at a wavelength of 492 nm. Results are presented as a percentage of the control.

2.5. LDH release assay

Cytotoxicity was determined by the release of LDH with a diagnostic kit (Jiancheng Bioengineering, Nanjing, China) according to the manufacturer's instructions. Briefly, 50 μl of supernatant from each well was collected to assay LDH release. The samples were incubated with NADH and pyruvate for 15 min at 37 °C, and the reaction was stopped by adding 0.4 M NaOH. The activity of LDH was calculated from the absorbance at 440 nm. The results were normalized to the maximal LDH release, which was determined by treating control wells for 60 min with 1% Triton X-100 to lyse all cells.

2.6. Measurement of MMP

MMP was measured using the fluorescent dye Rh123 as reported previously [9]. Rh123 was added to HT22 cells to achieve a final concentration of 10 μM for 30 min at 37 °C after the cells had been treated and washed with phosphate buffered saline (PBS). The fluorescence was observed by using an Olympus BX60 microscope with the appropriate fluorescence filters (excitation wavelength of 480 nm and emission wavelength of 530 nm).

2.7. Quantification of cytochrome c release

Cytochrome c release into the cytoplasm was assessed after subcellular fraction preparation. HT22 cells were washed with ice-cold PBS for three times and lysed with a lysis buffer containing protease inhibitors. The cell lysate was centrifuged for 10 min at 750*g* at 4 °C, and the pellets containing the nuclei and unbroken cells were discarded. The supernatant was then centrifuged at 15,000*g* for 15 min. The resulting supernatant was removed and used as the cytosolic fraction. The pellet fraction containing mitochondria was further incubated with PBS containing 0.5% Trition X-100 for 10 min at 4 °C. After centrifugation at 16,000*g* for 10 min, the supernatant was collected as mitochondrial fraction. The levels of cytochrome c in cytosolic and mitochondrial fractions were measured using the Quantikine M Cytochrome C Immunoassay kit obtained from R&D Systems (Minneapolis, MN, USA). Data were expressed as ng/mg protein.

2.8. Measurement of ATP synthesis

Isolated mitochondria were utilized to measure ATP synthesis with a luciferase/luciferin-based system as described elsewhere [10]. Thirty μg of mitochondria-enriched pellets were resuspended in 100 μl of buffer A (150 mM KCl, 25 mM Tris–HCl, 2 mM potassium phosphate, 0.1 mM $MgCl_2$, pH 7.4) with 0.1% BSA, 1 mM malate, 1 mM glutamate and buffer B (containing 0.8 mM luciferin and 20 mg/ml luciferase in 0.5 M Tris-acetate pH 7.75). The reaction was initiated by addition of 0.1 mM ADP and monitored for 5 min using a microplate reader.

2.9. Measurement of mitochondria swelling

Mitochondria swelling was measured following a previously published protocol [11]. Briefly, isolated mitochondria were suspended in fresh swelling buffer (0.2 M sucrose, 10 mM Tris-MOPS, pH 7.4, 5 mM succinate, 1 mM phosphate, 2 μM rotenone, and 1 μM EGTA-Tris, pH 7.4) at 0.5 mg/ml, and the swelling of mitochondria was monitored by a decrease in absorbance at 540 nm in the presence of $CaCl_2$ (200 μM).

2.10. Calcium imaging

Intracellular calcium concentrations were measured by Fura-2-AM [12]. HT22 cells grown on glass slides were loaded with 5 μM Fura-2-AM for 45 min before glutamate treatment at room temperature. Cells were then placed in an open-bath imaging chamber containing Dulbecco's NaCl/P_i supplemented with 20 mM glucose. With a Nikon inverted fluorescence microscope, cells were excited at 345 and 385 nm, and the emission fluorescence at 510 nm was recorded. Images were collected and analyzed with METAFLUOR image-processing software. The Ca^{2+} concentration values were then calculated, and Ca^{2+}-insensitive fluorescence was subtracted from each wavelength before calculations were performed.

2.11. Measurement of mitochondrial calcium

Cell permeable Rhod-2 AM was used as a mitochondria selective Ca^{2+} indicator. The cells were loaded with 2 mM Rhod-2 AM for 30 min before various treatments, and then washed three times. The fluorescence intensities were immediately analyzed with a Nikon inverted fluorescence microscope. Results are presented as a fold of the control.

2.12. Measurement of mitochondrial calcium buffering capacity

Mitochondrial calcium buffering capacity was estimated with the Ca^{2+} sensitive Calcium Green 5 N fluorescent dye. Incubation medium was composed of 125 mM KCl, 20 mM HEPES (pH 7.2), 2 mM KH_2PO_4, 2 mM $MgCl_2$, 5 mM succinate, 1 μM rotenone and 0.2 mM ADP, with 1 μg/mL oligomycin and 1 μM Calcium Green 5 N. Bolus additions of $CaCl_2$ were made to the 60 μg of mitochondria in suspension in 30 nM increments and changes in Calcium Green 5 N fluorescence were recorded at an emission of 532 nm.

2.13. Western blot analysis

Equivalent amounts of protein were loaded and separated by 10% SDS–PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat milk solution in tris-buffered saline with 0.1% Triton X-100 (TBST) for 1 h, and then incubated overnight at 4 °C with the primary Drp-1 antibody (1:1000), Bax (1:800), Bcl-2 (1:800), Cleaved Caspase-9 (1:500) or Caspase-9 (1:600) antibody dilutions in TBST. After that the membranes were washed and incubated with secondary antibody for 1 h at room temperature. Immunoreactivity was detected with Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). An analysis software named Image J (Scion Corporation) was used to quantify the optical density of each band.

2.14. Statistical analysis

Statistical analysis was performed using SPSS 16.0, a statistical software package. Statistical evaluation of the data was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Downregulation of Drp-1 reduces glutamate-induced excitotoxicity

To investigate the biological functions of Drp-1 in glutamate-induced excitotoxicity, HT22 cells were transfected with Drp-1 small interfering RNA (Si-Drp-1) or control siRNA (Si-Control). Western blotting analysis and morphological staining indicated that Drp-1 expression was significantly reduced in cells after their transfection with Drp-1 siRNA (Fig. 1A and B). After treatment with 5 mM glutamate for 24 h, the viability of the cells transfected with Drp-1 siRNA was higher than that of cells transfected with control siRNA (Fig. 1C). In contrast, knockdown of Drp-1 partly reversed the increase of LDH release induced by glutamate in HT22 cells (Fig. 1D).

3.2. Downregulation of Drp-1 attenuates glutamate-induced apoptosis

To investigate the effects of Drp-1 on excitotoxicity-associated apoptosis, the main cell death type of glutamate-induced neuronal injury, HT22 cells were transfected with Drp-1 siRNA or control siRNA for 72 h before glutamate treatment. As shown in Fig. 2A, the expression of apoptosis-related molecules, including Bax, Bcl-2, and caspase-9, was detected by western blotting analysis. Glutamate treatment caused increases in the Bax/Bcl-2 ratio and the activation of caspase-9, which were partly prevented by Drp-1 siRNA transfection, but not control siRNA (Fig. 2B and C).

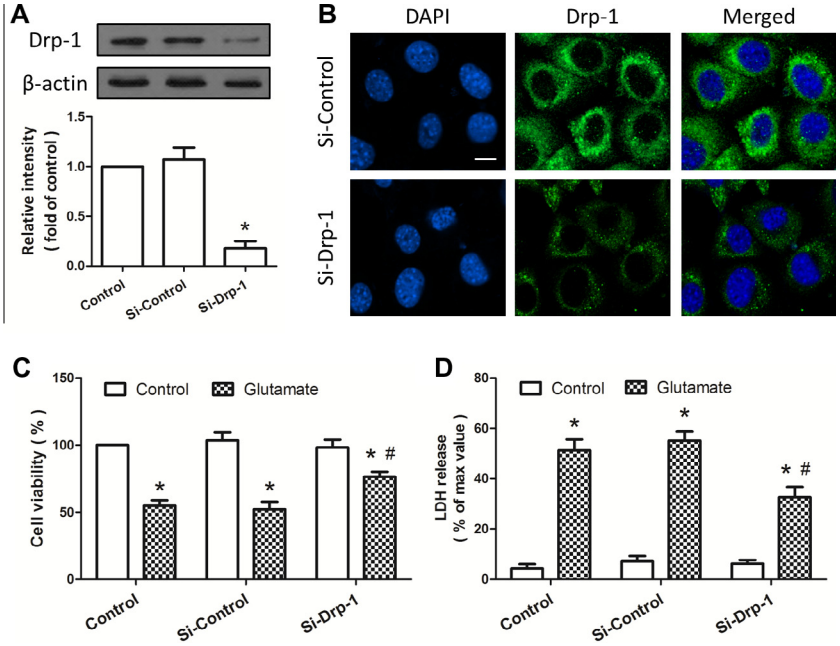

Fig. 1. Downregulation of Drp-1 reduces glutamate-induced excitotoxicity. HT22 cells were transfected with Drp-1 specific siRNA (Si-Drp-1) or control siRNA (Si-Control) for 72 h. After transfection, expression of Drp-1 was examined by western blot analysis (A) and immunostaining (B). Scale bar: 10 μm. After treatment with 5 mM glutamate, cell viability was measured with the MST assay (C), and cytotoxicity was measured with the LDH assay (D). The data were represented as means ± SD from five experiments. $^{*}p < 0.05$ vs. control. $^{\#}p < 0.05$ vs. control siRNA.

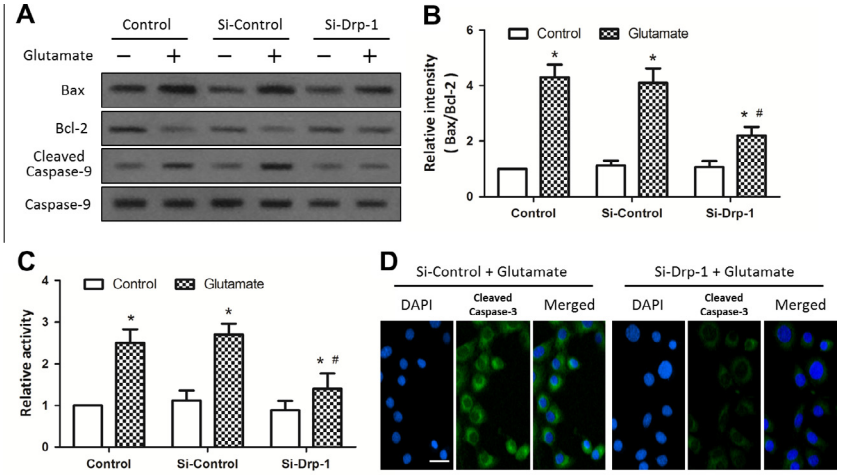

Fig. 2. Downregulation of Drp-1 attenuates glutamate-induced apoptosis. HT22 cells were transfected with Drp-1 specific siRNA (Si-Drp-1) or control siRNA (Si-Control) for 72 h before treatment with 5 mM glutamate. The expression levels of Bax, Bcl-2, cleaved caspase-9 and caspase-9 were determined by western blot analysis (A), and the Bax/Bcl-2 ratio (B) and activity of caspase-9 (C) were calculated. The cleavage of caspase-3 was measured by immunostaining (D). Scale bar: 40 μm. The data were represented as means ± SD from five experiments. $^{*}p < 0.05$ vs. control. $^{\#}p < 0.05$ vs. control siRNA.

Immunoblot analysis demonstrated an increase in the cleavage of caspase-3 after glutamate treatment in control siRNA transfected cells, whereas downregulation of Drp-1 by transfection with Drp-1 siRNA significantly attenuated the cleavage of caspase-3 (Fig. 2D), indicating the anti-apoptotic activity of Drp-1 knockdown.

3.3. Downregulation of Drp-1 inhibits glutamate-induced mitochondrial dysfunction

To characterize the effects of Drp-1 on mitochondrial dysfunction induced by glutamate treatment, the change of MMP was monitored with Rh123 probe (Fig. 3A), while release of cytochrome c into the cytoplasm was determined by an immunoassay kit after subcellular fraction preparation (Fig. 3B and C). The results showed that glutamate-induced loss of MMP and increase of cytochrome c release were partly reversed by Drp-1 siRNA transfection, but not affected by control siRNA transfection. After transfection and glutamate treatment, mitochondria were isolated and purified from the cells in each group to further confirm the effects of Drp-1 knockdown on mitochondrial function. Downregulation of Drp-1 partly rescued the decrease in mitochondrial ATP production in isolated mitochondria from Drp-1 siRNA transfected cells when compared to control siRNA (Fig. 3D). Moreover, we also tested the effects of Drp-1 knockdown on mitochondria swelling, which was induced by adding 200 μM Ca^{2+} into isolated mitochondria (Fig. 3E). We found that glutamate-induced mitochondria swelling was significantly decreased in Drp-1 siRNA transfected cells compared to control siRNA transfected cells.

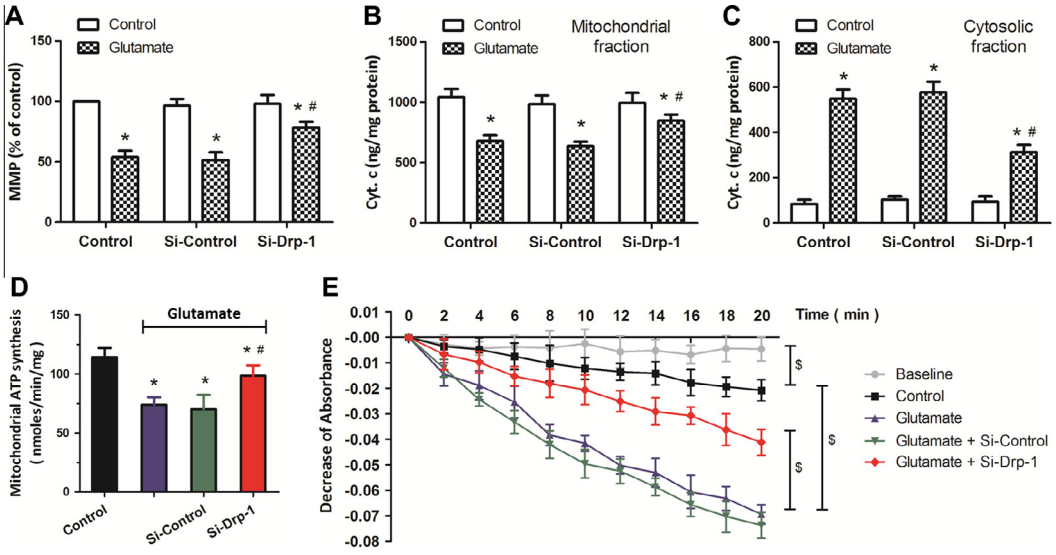

Fig. 3. Downregulation of Drp-1 inhibits glutamate-induced mitochondrial dysfunction. HT22 cells were transfected with Drp-1 specific siRNA (Si-Drp-1) or control siRNA (Si-Control) for 72 h before treatment with 5 mM glutamate. The MMP levels (A), mitochondrial Cytochrome c (B), and cytosolic Cytochrome c (C) were measured. After transfection and glutamate treatment, mitochondria in each group was isolated and purified. Levels of ATP synthesis was determined (D). Mitochondrial swelling was examined by monitoring the absorbance at 540 nm induced by 200 μM Ca^{2+} (E), and the baseline absorbance was measured without Ca^{2+}. The data were represented as means ± SD from five experiments. $^{*}p < 0.05$ vs. control. $^{\#}p < 0.05$ vs. control siRNA. $^{\$}p < 0.05$.

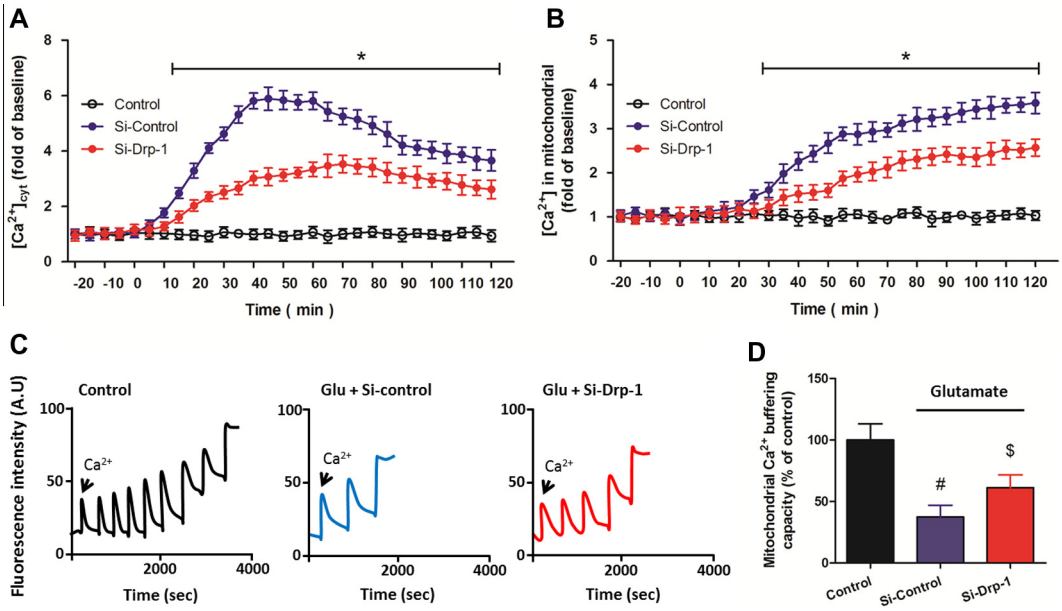

Fig. 4. Downregulation of Drp-1 preserves intracellular calcium homeostasis. HT22 cells were transfected with Drp-1 specific siRNA (Si-Drp-1) or control siRNA (Si-Control) for 72 h before treatment with 5 mM glutamate. The cytoplasmic Ca^{2+} concentration (A) and mitochondrial Ca^{2+} concentration (B) were measured up to 120 min after glutamate treatment. After transfection and glutamate treatment, mitochondria in each group was isolated and purified. Relative Ca^{2+} uptake capacity of isolated mitochondria were determined (C) and calculated (D). The data were represented as means ± SD from five experiments. $^{*}p < 0.05$. $^{\#}p < 0.05$ vs. control. $^{\$}p < 0.05$ vs. control siRNA.

3.4. Downregulation of Drp-1 preserves intracellular calcium homeostasis

To determine whether downregulation of Drp-1 attenuates glutamate-induced excitotoxicity by preserving intracellular calcium homeostasis, we used calcium imaging to detect the changes of cytoplasmic calcium concentration after glutamate treatment (Fig. 4A). Downregulation of Drp-1 resulted in decreased Ca^{2+} overloading and improved Ca^{2+} recovery after glutamate treatment compared to control siRNA. Fig. 4B shows dynamic changes of mitochondrial Ca^{2+} as monitored by Rhod-2 AM probe and expressed as fold of the baseline for up to 120 min following glutamate treatment. Knockdown of Drp-1 attenuated the increase of mitochondrial Ca^{2+} induced by glutamate treatment from 25 to 120 min after excitotoxicity. Furthermore, we also examined the calcium buffering capacity in isolated mitochondria following transfection and glutamate treatment. As shown in Fig. 4C, the peaks corresponded to sequential bolus additions of 30 nM of Ca^{2+}, and the downward deflections reflected mitochondrial Ca^{2+} uptake. Glutamate treatment resulted in a ~60% reduction in Ca^{2+} buffering capacity in isolated mitochondria, whereas downregulation of Drp-1 significantly preserved the Ca^{2+} buffering capacity compared to control siRNA (Fig. 4D).

4. Discussion

Dynamic structural changes of the mitochondrial network, including continuous remodeling by fusion and fission events, is one of the most important means recently characterized that might adapt mitochondrial function to various pathological conditions [13]. Impaired regulation of mitochondrial dynamics and defects in mitochondrial fission and fusion proteins may limit mitochondrial motility, increase oxidative stress, reduce energy generation, and result in accumulating of mitochondrial DNA defects, thereby promoting neuronal dysfunction and cell death [14]. Multiple mitochondrial membrane GTPases that regulate mitochondrial networking have been identified, and mitochondrial fission is thought to be mainly mediated by Drp-1 [15]. Increased expression of Drp-1 and enhanced mitochondrial fission can be observed in cancer cells and injured neuronal cells in neurodegenerative diseases [16], and these observations expose Drp-1 as a potential therapeutic target. Drp-1 was shown to interact with Bax to form complexes at mitochondrial fission sites, mediating the outer mitochondrial membrane permeabilization and cytochrome c release [17]. Moreover, Drp-1 overexpression amplified Bax-mediated release of cytochrome c induced by staurosporine [18]. A more recent study showed that tBid-induced loss of mitochondrial outer membrane permeabilization in a Drp-1-dependent manner [19]. In the present study, the results of western blotting analysis showed that downregulation of Drp-1 decreased the Bax/Bcl-2 ratio and the activation of caspase-9 induced by glutamate treatment. These data was confirmed by immunoblot analysis showing that Drp-1 knockdown significantly attenuated the cleavage of caspase-3, the central molecular in the execution-phase of caspase-dependent apoptosis. Altogether, these findings indicated that downregulation of Drp-1 may preserve the balance of proapoptotic and antiapoptotic Bcl-2 family proteins thereby preventing caspase-dependent apoptosis after glutamate exposure.

Increasing evidence has shown that mitochondria suffer severe damage, including changes in morphology, function and protein expression, in neurons under excitotoxicity conditions [20,21]. The mitochondria are the primary organelles that produce high energy phosphate, and the loss of MMP reflects the impaired performance of electron transport chain [22]. Both in vitro and in vivo studies have conclude that mitochondrial energy metabolism is extremely sensitive to ROS impairment, and the ROS that are synthesized in excess during stress may aggravate mitochondrial dysfunction and lead to mitochondrial swelling, the most commonly observed ultrastructural changes following neuronal injury [21]. We showed that downregulation of Drp-1 increased mitochondrial ATP generation, attenuated mitochondrial swelling and prevented loss of MMP, suggesting a preservation of mitochondrial function after Drp-1 knockdown. We also found that

downregulation of Drp-1 significantly inhibited glutamate-induced cytochrome c release, the typical response of neurons to death-inducing stimuli [23]. It has been well demonstrated that opening of mitochondria permeability transition pore (mPTP) is the key factor in mitochondrial swelling and the loss of MMP after neuronal injury, and cytochrome c can be released from mitochondria via mPTP opening-dependent pathways [24]. It is possible that Drp-1 participates in mPTP pathophysiological responses to glutamate-induced excitotoxicity in HT22 cells, and whether Drp-1 knockdown protect against mitochondrial dysfunction via regulating mPTP related proteins, such as voltage-dependent anion channel (VDAC), needs to be further investigated.

One of the key events that causes mitochondrial injury is an abnormal increase in intracellular Ca^{2+} and followed mitochondrial Ca^{2+} uptake [24]. Intramitochondrial Ca^{2+} seems to play a two-faced role in the regulation of mitochondrial function. On the one hand, Ca^{2+}-dependent stimulation of NAD(P)H and followed ATP generation, through activation of Ca^{2+} sensitive dehydrogenases and metabolite carriers, serves to adapt energy and substrate production to increased cellular needs [25]. On the other hand, mitochondria serve as an intracellular calcium buffer shaping Ca^{2+} signaling, and mitochondrial Ca^{2+} loading has been shown to paly crucial role in apoptotic cell death [26]. Recent evidence have validated mitochondrial Ca^{2+} overloading as a key effector in the apoptotic response induced by stimuli like oxygen and glucose deprivation (OGD), H_2O_2, or arachidonic acid [27,28]. C_2 ceramide was shown to sensitize mitochondria to Ca^{2+}impulses from $InsP_3$ or ryanodine receptor ($InsP_3R$, RyR)-mediated Ca^{2+} release, leading to MPT and depolarization, activating the effector caspases and, finally, triggering apoptotic cell death [29]. Inhibition of Ca^{2+} transport into mitochondria through targeting the mitochondrial calcium uniporters (mCU), such as uncoupling proteins (UCPs), has been demonstrated to protect neurons from cell death mediated by glutamate [30]. In the present study, we found that downregulation of Drp-1 attenuated intracellular Ca^{2+} overload and inhibited mitochondrial Ca^{2+} uptake after glutamate exposure. Moreover, the ~60% reduction in mitochondrial buffering capacity observed after glutamate treatment in control siRNA transfected cells was partly reversed by downregulation of Drp-1, adding further evidence to the involvement of intracellular calcium homeostasis regulation in Drp-1 knockdown-induced protection against excitotoxicity.

In conclusion, our present study showed that downregulation of Drp-1 using specific siRNA protects HT22 cells against glutamate-mediated excitotoxicity via antiapoptotic activity. Moreover, these neuroprotective effects were partly dependent on attenuation of mitochondrial dysfunction and preservation of intracellular calcium homeostasis.

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